ELUCIDATING THE ROLES OF THE DIGUANYLATE CYCLASES CELR AND AVMA IN ATTACHMENT AND CELLULOSE SYNTHESIS WITH THE RESPONSE REGULATOR DIVK IN AGROBACTERIUM TUMEFACIENS

BY

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DISSERTATION

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Abstract

*Agrobacterium tumefaciens*, the causative agent of crown gall disease in plants, physically interacts with plant cells to remain anchored within the nutrient-rich rhizosphere. Part of this interaction involves the production of cellulose fibrils by the bacteria, leading to the aggregation of the cells to each other as well as to the plant surface. While this interaction between the bacteria and plants has been partially characterized, little is known concerning the regulatory system that controls cellulose synthesis within *A. tumefaciens*. One possible mechanism is through the secondary messenger cyclic diguanosine monophosphate (c-di-GMP), a small molecule used in numerous complex physiological processes in bacteria, including regulation of exopolysaccharide production. In this study, we attempt to characterize the effects of two diguanylate cyclases (DGC), CelR and AvmA, and other components involved in regulating cellulose production and attachment.

CelR positively influences cellulose synthesis through the production of c-di-GMP. This effect occurs when the DGC is activated, likely by phosphorylation. Overexpressing *celR* also results in changes in cell morphology, attachment, unipolar polysaccharide (UPP) production and virulence, although deleting the gene does not affect any of these phenotypes. Overexpressing a DGC of similar structure to CelR, AvmA, also affects these processes. Deleting the gene impacts attachment, UPP deployment and virulence, suggesting that *avmA* is important for regulating the processes not under direct control of *celR*. The cellulose synthase subunit, CelA, contains a c-di-GMP-binding PilZ domain, which is necessary for the complex to produce the polymer in response to the signal molecule. Deleting *divK*, the first gene of a two-gene operon with
celR, also reduced cellulose production. This requirement for the response regulator was alleviated by expressing a constitutively active form of CelR, suggesting that the DGC must be activated and that DivK acts upstream of this event. A constitutively-active form of DivK also results in increased cellulose production, but only in the presence of wild-type CelR. Based on bacterial two-hybrid assays, CelR forms homodimers but does not interact directly with DivK. In Caulobacter crescentus, DivK is required for polar localization of cell division components; mutants of divK form branched and elongated cells. Similarly, deleting the response regulator in A. tumefaciens results in branched and misshapen cells. The phenotype is fully corrected by complementation with divK, but not with wild-type celR or the constitutively active form of the DGC. These results support our hypothesis that the function of CelR is restricted to stimulating cellulose synthesis, and that the DivK/CelR signaling pathway in Agrobacterium separates production of the polymer from cell cycle checkpoints mediated by DivK.
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Mutating avmA affects UPP production and virulence.

The response regulator DivK impacts cellulose synthesis through CelR.

DivK may connect CelR and AvmA in determining the type of attachment in A. tumefaciens.

Future Directions.

Figures.
Chapter 1

Introduction and literature review

1.1 Taxonomy and physiology of Agrobacterium tumefaciens

Agrobacterium tumefaciens is an alphaproteobacterium within the family Rhizobiaceae (98). This family is organized into three genera—Agrobacterium, in which A. tumefaciens resides, and Rhizobium and Sinorhizobium, containing the nitrogen-fixing bacteria. Originally, these genera were organized based on the pathogenic or mutualistic traits of the bacteria, including tumor formation, hairy root induction and nodulation. However, the taxonomy using this methodology is problematic, as the traits are encoded on plasmids that are transmissible between species (117, 118). Today, there is debate as to whether Rhizobium and Agrobacterium should be combined into one genus (297). This argument is based on 16s RNA similarity, biochemical and phylogenetic analyses and the ability of species from each genus to maintain plasmids from the other (297). However, further examination of these species demonstrates variation between Rhizobium and Agrobacterium, and the names of each genus are accepted in the literature (73). Further, several genomes from representative members in both genera demonstrate that some members of Rhizobium and Agrobacterium are sufficiently different to keep the two groups separate (234), although one group of agrobacteria does display characteristics closer to the Rhizobium (discussed below).

The genus Agrobacterium is best known for the ability of some members to induce cancerous growths on plants. Early classification of species of this genus organized the bacteria based on disease phenotypes (121). Thus, Agrobacterium was
divided into five species: *A. tumefaciens*, which induces crown gall tumors in susceptible plants; *A. rhizogenes*, which causes hairy root disease; *A. rubi*, which induces galls on cane-type plants like raspberries; *A. vitis*, which induces galls on grapevines; and *A. radiobacter*, isolates of which are not pathogenic. With the discovery that these designations were based on traits encoded on mobilizable elements, it became clear that this method of classification did not accurately organize the species. A more traditional taxonomy was developed, based on DNA relatedness and chromosomal-based biochemical properties of each species (98, 114, 122, 285). In this classification, *Agrobacterium* is arranged into three types- biovar I, biovar II and biovar III. Each biotype contains isolates that are pathogenic and non-pathogenic. Recent studies utilizing multilocus sequence analysis confirm that biovars 1 and 3 are distinct from *Rhizobium*, while it was suggested that species in biovar 2 are moved to the *Rhizobium* clade (174). Biovar 3 is significant in that it contains isolates, called *A. vitis*, which induce galls only on grapevines (98, 186). This division into biovars demonstrates the polyphyletic nature of the Rhizobiaceae even within a genus, and supports the separation of the genera *Agrobacterium* and *Rhizobium*.

Members of the genus *Agrobacterium* all are soil-based, rod-shaped, Gram-negative cells measuring around two microns in length (121). These bacteria are motile, driven by one to four peritrichous flagella (121, 241). Members of the genus *Agrobacterium* are aerobic, strictly respiring, chemo-organotrophs, utilizing a wide variety of sugars and plant-based compounds for carbon and energy. These bacteria lack an intact Embden-Meyerhof pathway, and instead catabolize sugars via the Entner-Doudoroff pathway and the tricarboxylic acid cycle (TCA). They also utilize a broad
range of amino and organic acids (85, 292). Like with many alphaproteobacteria, succinate and glutamate are the preferred carbon sources for Agrobacterium, and growth with either compound results in catabolite repression.

The genome of a biovar 1 strain, A. tumefaciens C58, the most studied member of the genus, consists of two chromosomes: a 2.8 Mbp circular chromosome, and a 2.0 Mbp linear chromosome (86). The circular chromosome contains genes and DNA sequences reminiscent of an oriC-based system, similar to the replication system of Escherichia coli (85, 292). Interestingly, the linear chromosome replicates via a plasmid-type repABC system (85, 292), suggesting that chromosome 2 is distantly evolved from larger extrachromosomal elements. All other biovar 1 isolates examined exhibit a similar, if not identical, chromosomal organization. Strain C58 also harbors two large plasmids: a 214 kbp tumor-inducing (Ti) plasmid and a 542 kbp At plasmid (85, 292). Ti plasmids contain genes required for virulence and are essential for the ability of the bacteria to induce crown gall tumors (274, 282). The At family of plasmids encode catabolic pathways for carbon utilization and metabolic flexibility (85, 177).

1.2 Characteristics of crown gall tumors

Agrobacterium tumefaciens was first described as the causal agent of crown galls in 1907 (241). Research since then established that these growths are true tumors. The galls are self-propagating (108), and can be cultured in vitro without added plant hormones (21). Axenically cultured crown gall tissue retains its tumorigenic properties (24, 286), demonstrating that the once tumors are induced, the bacteria are no longer necessary to maintain the plant cells in a transformed state. Further experiments showed
that tumorigenesis involves an inception phase and a development phase, and that the bacteria are not required for the second step (21, 23). These observations suggested that some element, called the “Tumor Inducing Principle” (TIP), was transferred from the bacteria to the plant cell during tumor induction (23, 24). Biochemical studies of the tumors demonstrate that the tissue contains novel carbon compounds called opines, that are not present in normal plant tissues (146, 172). The type of opines present in tumor tissue is specified by the strain of *Agrobacterium* that induced the tumor (18, 193). The opines not only are secreted from the tumors, but they also are translocated throughout the plant (221). The observation that opines serve as specific growth substrates for the inducing bacterial strain (119, 149, 193) led to the “opine concept” (194, 260, 261). This model proposes that the invading bacteria engineer opine synthesis into the host to create a dedicated source of carbon for the bacteria. Furthermore, the tumors effectively increase the number of plant cells that synthesize the opines, which provides a rich source of a nutrient utilized only by the inducing bacteria.

With the relationship between *Agrobacterium* and tumor induction established, researchers sought to identify a substance that induces tumor formation. That the tumor cells continue dividing after removal of the bacteria demonstrates that the inducing factor is systemic and delivered during the initial infection (23, 24). The knowledge that the plant tissue becomes malignant, and the observation that opines are produced by the cells only after infection, suggest that a genetic factor is involved in inducing tumorigenesis (193). Kerr and associates (116, 117) demonstrated that tumorigenicity and the specificity of opines found in the tumors can be transferred from virulent to avirulent agrobacteria. Further, the virulence of C58, and its ability to catabolize opines, could be lost following
growth of the bacteria at elevated temperatures (90), suggesting that a transferrable genetic element is responsible for virulence. Indeed, all virulent strains contain large plasmids, and some of these, called Ti plasmids, confer the ability to induce tumors when transferred to avirulent strains (274, 282, 300). Chilton et al. (42) made the key discovery that a specific region of the Ti plasmid, called the T-region, is found in DNA extracted from crown galls but not normal plant tissues. Subsequently, the segments of DNA, called T-DNA, are integrated into chromosomal DNA in a random manner (42).

The T-DNA encodes the genes for tumorigenesis and the biosynthetic pathway of opines. The oncogenes include ipt, which encodes an enzyme that synthesizes a derivative of the plant hormone cytokinin (2), and iaaH and iaaM, which are involved in the pathway for synthesis of a second hormone, indolacetic acid (228, 263, 264). Expression of these genes from the T-DNA leads to increased levels of the two hormones, resulting in proliferation of plant cells (17). Each T-region also encodes one or more opine biosynthetic pathways, each producing a specific opine (18, 193, 199). There are eight known opine families, generally with one or two families associated with any given Ti plasmid (64, 65). The cohort of opines associated with a Ti plasmid is often used to classify the plasmids into groups. For example, the T-region of pTiC58 contains genes for synthesizing two opine types: nopaline, formed though a 1,4 imine linkage between arginine and α-keto-glutarate, and the agrocinopines A and B, formed by a phosphodiester linkage of either sucrose or glucose to L-arabinose (65, 215). The Ti plasmid also carries the genes that allow strain C58 to utilize these two opine types, and only these two types, as a sole source of carbon.
1.3 Transfer of T-DNA from *Agrobacterium* to plant tissue

The mechanism by which the T-DNA is transferred from *Agrobacterium* to the host involves numerous steps, from assembly of the delivery system, to the transfer and integration of the T-DNA into the plant. Components of this process are encoded by the *vir* regulon, a set of transcriptional units found on Ti plasmids (110, 191). This regulon in pTiC58 consists of seven separate transcribed elements, including the operons *virB*, *virC*, *virD*, *virE*, and *virF*, and the monocistronic genes, *virA* and *virG* (112, 113, 202). In some octopine-type Ti plasmids, the *vir* regulon includes *repABC*, encoding the replication system of the element (43). When the regulon is stimulated, increased transcription of *repABC* results in higher copy number of these plasmids (43). Together, these elements of the regulon encode the regulatory, processing and delivery systems resulting in the transfer to and incorporation of T-DNA into the plant chromosome.

The *vir* regulon is activated by the VirA/VirG/ChvE sensory system. Phenolics, hexose sugars and other environmental cues released from a wounded plant stimulate autophosphorylation of VirA (101). The activated receptor phosphorylates VirG, a transcription factor (192). VirG binds to the *vir* box, a sequence located within the promoters of elements of the *vir* regulon, and activates transcription of the operons (110, 191, 192). In the cases of *virA* and *virG*, upregulation of the promoters results in positive feedback of the system, promoting increased activation of the regulon (248, 290).

Once the *vir* regulon is stimulated, the T-DNA is processed for delivery to the plant. The target region is marked by T-borders, named the left and right border, that are composed of a 14-bp imperfect direct repeat sequence (229, 233, 279, 280). The T-region is processed by two proteins, VirD1 and VirD2, creating the initial T-complex. VirD1
separates the DNA strands for processing (83). VirD2, a tyrosine site-specific recombinase, is a multicomponent protein that nicks one strand of the DNA at the border sequences (247, 250, 276, 295), and forms a covalent bond with the 5´ end of the strand (95, 187, 296). During this processing, the DNA is coated by VirE2, a single-strand DNA-binding protein, protecting the T-complex from degradation (49, 50, 232, 309).

The processed T-complex and ancillary proteins are delivered to the plant cell through a pilus-like type IV secretion system (T4SS). This secretion apparatus is comprised of proteins encoded by the 11-gene \textit{virB} operon (72, 265, 281) and \textit{virD4} (75, 265). The core of the complex is composed of multiple subunits of VirB6, VirB7, VirB8, VirB9 and VirB10, which organize into a pore-like structure through the inner and outer membranes (36, 47, 48). A pilus consisting of VirB2 and VirB5 extends beyond the bacterium from the core complex (134, 224, 299). VirB2 comprises the majority of the pilus, forming a hollow structure (120). VirB5 binds at the end of the T-pilus and along the extended channel, and may be involved in interacting with the plant cell (5, 135, 224, 299). The T4SS is energized by three components, VirB4, VirB11 and VirD4, each containing a Walker A nucleotide-binding domain characteristic of AAA ATPases (8, 59, 76, 251, 275). These proteins provide the energy for transferring the T-complex and protein substrates through the T4SS (8, 218). Translocation of these substrates occurs through ring-like assemblies in the apparatus to the pilus (33, 262).

In addition to translocation of the T-complex into the plant cell, the T4SS delivers several protein substrates involved in nuclear transport and integration of the T-DNA into the plant chromosome. These factors, which include VirE2, VirE3 and VirF, can be
transported separately from the DNA into the host (34). The proteins are thought to translocate through the T4SS, in a manner similar to the T-complex (33).

Once secreted into the plant, the DNA is again coated by VirE2, reforming the T-complex, and the complex is directed towards the nucleus (53, 81, 99, 232, 277, 309). VirD2, VirE2 and VirE3 of the T-complex interact with components of the importin α pathway, including the transport protein VIP1, mediating delivery of the nucleoprotein to the nucleus (51, 133, 270, 301). Both VirD2 and VirE2 are involved in directing transport of the fragment through the nucleopore channel (51, 52, 270). Once the T-complex passes into the nucleus, VirE2 and VIP1 are thought to recognize nucleosomes and bind with the chromatin (132). VirD2 also associates with the TATA binding protein, which may facilitate T-strand/ chromatin binding (13). Once the complex interacts with the nucleosomes, an F-box protein, VirF, targets both VIP1 and VirE2 for degradation, likely to allow recombination between the T-strand and the target (227, 269, 301). Once the transferred DNA is stripped of its accessory proteins, it integrates into the chromosome. Initial reports suggested that the T-DNA was targeted to gene-rich, transcribed, A/T-rich, and/or promoter regions of genes (29, 38, 226). However, these studies based the assay for successful insertions into the plant on expressible and selectable traits, and would not recognize insertions that did not express the selectable phenotype. Later analysis demonstrated that the insertions appear to be random throughout the plant genome (125).

There are currently two models describing the integration of T-DNA (269). In the first model, the homologous recombination system of the plant incorporates the DNA by strand invasion. This would occur by targeting the T-strand via interaction of VirD2 to nicks in the target DNA, then scanning for regions of microhomology between the
bacterial and plant DNA (176, 187). Once associated, the two strands would be incorporated by nicking and rejoining activity of VirD2, followed by synthesis of the complementary strand (266). The second model involves double strand break repair to integrate the T-DNA into the chromosome. Here, the complementary strand of the T-DNA is synthesized to form double stranded DNA, which is integrated into the target at double strand breaks by non-homologous end joining (NHEJ) (144). Genetic analysis in the yeast *Saccharomyces cerevisiae* and the plant *Arabidopsis thaliana* indicate that component proteins of NHEJ are required for integration of the T-DNA (273), and VirE2 inhibits XRCC4, a component of the end joining system, to promote double-stranded breaks (272). To date, there is no consensus as to which of these two systems is used to integrate the T-DNA into the plant chromosome.

1.4 Attachment and colonization of plant tissue by *Agrobacterium*

Cells of *A. tumefaciens* attach to the plant host for several purposes, including association with the nutrient-rich environment of the rhizosphere, delivery of T-DNA for virulence and utilization of opines produced by the crown gall tumors. The bacteria require direct contact with a host to induce tumors (147, 148), and this interaction must persist for at least eight hours in order for tumors to develop (256).

It is generally accepted that *A. tumefaciens* interacts with plant cell surfaces in a two-step process: first, a reversible association between the bacteria and receptors on the surface of the plant, followed by a second interaction involving a tighter binding between the bacteria and the tissue (156, 163). It is likely that the reversible interaction is mediated by some plant factor. Among the possible components are lectins, plant-
associated exopolymers, and receptors on the cell surface (20). Some potential factors were identified by isolating mutants of Arabidopsis thaliana resistant to A. tumefaciens, called rat mutants (304). Among these plant factors are enzymes involved in synthesizing arabinogalactans (80) and a cellulose synthase-like complex (82, 303). However, there is no conclusive evidence that any of these factors are involved in either recognition of plant tissue or binding of the bacteria.

There has been an extensive search for the bacterial factors responsible for the initial attachment of A. tumefaciens to plant cells. However, the complex relationship between these factors, attachment and virulence remains poorly understood. In early studies, lipopolysaccharide (LPS) synthesized by the bacteria was proposed as a potential component. Extracts of the polymer seemingly block the bacteria from attaching to plant tissue, presumably by competitive inhibition (284). In these studies, tumor induction was used as the assay for attachment, and actual binding was not examined. Later, researchers determined that LPS activates host defense systems, thereby inhibiting tumor induction (305).

Smit et al. (238) described a small, Ca²⁺-dependent protein in Rhizobium leguminosarum, called rhicadhesin, that is similar to cadhesins in eukaryotes. This protein, later annotated as RapA1, is important for the colonization of pea shoots by R. leguminosarum (60, 236). RapA1 requires Ca²⁺, likely released from the tissue, to increase attachment of Rhizobium and Bradyrhizobium to plant surfaces (60, 237). In A. tumefaciens, competition assays with purified rhicadhesin from R. leguminosarum resulted in decreased attachment of the bacteria to plant tissue (254). However, there is
no homolog of *rapA1* in *A. tumefaciens*, and the gene is only found in a few species of *Rhizobium* (10, 171).

The synthesis of β-1,2-glucans, exopolysaccharides synthesized by ChvA and ChvB, may contribute to attachment in *A. tumefaciens*. A *chvA* mutant was unable to attach to *Zinnia* leaf tissue (67, 68, 308) and was affected in transfer of T-DNA (255). However, studies of β-1,2-glucans demonstrate that the polysaccharides are key components of the adaptive response to periplasmic osmotic stress (32). Mutants of *chvAB* show pleiotropic effects, including reduced production of flagella, decreased exopolysaccharide synthesis and altered composition of membrane proteins (25). These effects cloud an accurate assessment of the role of *chvAB* in attachment.

A study of transposon mutants of strain C58 revealed a potential genome fragment that, when disrupted, resulted in lower attachment of *Agrobacterium* to the surfaces of cut carrot disks (155). This segment, called the *att* region, contains genes encoding a diverse set of functions, including catabolic pathways, ABC transporters, polysaccharide synthases, peptidases, Mg$^{2+}$ transporters and transcription regulators (164, 165). Deleting one gene in this region, annotated *attR*, resulted in the strongest effect on attachment and virulence (159, 201). However, the *att* region is carried on one of the extrachromosomal plasmids, pAtC58 (85, 292), and curing the plasmid from C58 has little effect on attachment or virulence (177). These observations cast doubt on the role of the *att* region in the attachment process.

Recently, it was reported that *A. tumefaciens* synthesizes a lectin-binding structure, named the unipolar polysaccharide (UPP), that localizes to one pole of the cell (267). The UPP is similar to the holdfast on the tip of the stalk in a related
alphaproteobacterium, *Caulobacter crescentus* (130). The holdfast contains N-acetyl glucosamine (GlcNAc) residues that interact with lectins (168). These polysaccharides are anchored to the holdfast by holdfast attachment (Hfa) proteins localized at the end of the stalk (54, 92, 130, 131). In *A. tumefaciens*, the UPP is likely synthesized by a pathway encoded by *uppABCDEF* (293). Production of the adhesive complex is stimulated by contact of the bacterium with surfaces, suggesting that pressure is a signal for deployment of the structure (143). Interestingly, while the UPP likely contains GlcNAc residues, there are no conserved orthologs of Hfa proteins in *A. tumefaciens* (267). It is possible that other anchoring proteins exist in this structure, although these factors have not been identified. The role of the UPP in attachment and colonization to plant tissue has not been fully determined, although mutants in which the *upp* operon is deleted remain virulent (294). It is important to note, however, that the traditional assays used in these studies involve direct inoculation of suspensions or pastes of bacteria into wound sites on the plant. Under these conditions, the bacteria may initiate tumorigenesis without need for the tight binding associated with the UPP. However, in the real biology of the interaction between *A. tumefaciens* and host plant surfaces, the UPP remains a possible component of initial attachment.

In addition to *A. tumefaciens* and *C. crescentus*, other alphaproteobacteria produce polar binding structures, suggesting that these apparati may be a conserved feature of the family. Isolates of *R. leguminosarum* synthesize a WGA-binding polar polysaccharide, very similar to the UPP, which contributes to polar attachment of the bacteria to pea shoots (139, 140). A majority of sequenced genomes of the Rhizobiaceae contain orthologs of the *upp* genes (NBCI), suggesting that components of the polar
structure are highly conserved within the family. Another alphaproteobacterium, *Asticcacaulis biprosthecum*, produces a holdfast construct similar to that of *C. crescentus* (278). The presence of these adhesion-like attachment structures and their location at the cell pole among a wide range of alphaproteobacteria suggests that polar attachment is a common phenomenon throughout the family. The Rap proteins in *R. leguminosarum*, including rhicadhesin, also are polarly localized (10). Strains of *R. leguminosarum* overproducing RapA1 display increased binding to roots of pea shoots and increased frequency of nodulation (171). However, the *rap* genes are found only in select members of the genus (298), and have not been observed in members of the genus *Agrobacterium* (10).

### 1.5 The role of cellulose synthesis in secondary attachment and biofilm formation

As described above, the interaction of *A. tumefaciens* with plant surfaces occurs in two steps: initial attachment and secondary attachment. The initial attachment phase likely involves unidentified components that associate the bacteria with the target surface. After this initial interaction, the cells form a very tight binding between the surface of the plant cell as well as with other bacteria, resulting in colonization and biofilm formation. This anchoring allows the bacteria to remain within the nutrient-rich space surrounding the plant, and prevents the cells from dispersing into the soil environment. This binding is likely mediated by a number of factors, including several different exopolysaccharides. One of the components in this secondary binding is cellulose, produced by *A. tumefaciens* during formation of microcolonies and biofilms (156, 157, 180).
Cellulose is critical for long-term attachment and biofilm formation. Root colonization by both *Agrobacterium* and *Rhizobium*, but not individual attachment of cells, is dependent upon cellulose synthesis (140, 158, 160). While cellulose synthesis may be critical for colonization and stable binding to the plant, it is not well understood how the fibers affect initial attachment or virulence. Mutants deficient in cellulose production still attach individually to plant tissue (158). Using standard virulence assays, *cel* mutants are fully tumorigenic (156). However, as described above, assays for virulence involve applying the bacteria directly into wound sites on the plant. These methods do not reflect the real-life conditions that cells of the bacterium contend with during contact and interaction with the plant tissue. Indeed, gentle washing of the wound sites inoculated with a *cel* mutant within eight hours post-infection prevents tumorigenesis (256). Thus, the real-life impact of cellulose synthesis on attachment and virulence remains unresolved.

Cellulose is a common component of attachment mechanisms in many proteobacteria, and the components for synthesizing the polymer are highly conserved among these species. Cellulose synthesis in bacteria was first described in a number of alphaproteobacteria, including *Gluconacetobacter* (formerly *Acetobacter*), *Rhizobium* and *Agrobacterium* (27, 180). Later, species of *Salmonella*, *Escherichia*, *Vibrio*, *Pseudomonas* and *Burkholderia* were reported to produce the polymer (16, 244, 307). A system of four genes, called *bcsABCD*, encoding the enzymes for cellulose synthesis was first described in *Gluconacetobacter* (291). The products of these genes include the two subunits of cellulose synthase, BcsA and BcsB; a putative secretion channel through the outer membrane, BcsC; and BcsD, which to date has no known function (223, 288, 291).
Homologs of these genes are present in most, if not all, species known to produce cellulose, with variation in organization and additional components (288).

*Agrobacterium tumefaciens* also contains the genes encoding the pathway for synthesis of cellulose, annotated as the *cel* operons (85, 292). These two operons are organized on the linear chromosome as *celABCG* and *celDE* (Figure 1.1; (85, 162, 292)). Of these genes, *celA*, *celB* and *celC* encode components orthologous to *bcsABC* in other organisms (161, 162, 223, 291). The *celD*, *celE* and *celG* genes are orthologous to other genes in the *cel* operons of other proteobacteria, but have no known function in the pathway (161, 162, 288). This organization of the *cel* operons is conserved in the biovar 1 isolates of *Agrobacterium* (NBCI; (234)), while other members of the family Rhizobiaceae, including the biovar 2 agrobacteria, contain the single *cel(bcs)ABCD* operon, the organization of the genes that is found in the majority of proteobacteria that synthesize cellulose.

*CelA* and *celB* encode the inner membrane-spanning cellulose synthase complex that catalyzes production of the polymer. These components in other systems form a heterodimer, with CelA(BcsA) embedded in the inner membrane, and CelB(BcsB) localized in the periplasmic space (173). The bitopic BcsA contains a glycosyltransferase domain that catalyzes the addition of UDP-glucose to the extending cellulose chain and a domain that recognizes an activating signal (discussed below; (173, 216)). The BcsB subunit assists in translocating the cellulose fibril through the periplasm (173). These complexes often are localized to the poles of rod-shaped cells, including in some species of *Rhizobium* (137, 288).
Less is known concerning the functions of *celC*, *celD*, *celE* and *celG*, the remaining genes in the *cel* operons. CelC is a transmembrane protein that likely localizes to the outer membrane (223). The *celC* gene is required for *in vivo*, but not *in vitro*, synthesis of cellulose, suggesting that the protein acts in exporting the fibril (223). The functions of CelD and CelE remain unknown, although it is hypothesized that the proteins act in synthesizing or recruiting UDP-glucose to the synthase (161, 162). CelG has no known domain structure, but mutating its corresponding gene results in the production of increased levels of cellulose (158), suggesting it has some regulating role in the system.

*Agrobacterium* also synthesizes a second glucose-based polymer, the β-1,3-linked polysaccharide curdlan (178). This polymer is synthesized by CrdS, an enzyme with a catalytic domain structure similar to CelA (249). Curdlan is present in cell free extracts from some species of *Agrobacterium* (249). Recently, curdlan synthesis has been tied to nitrogen starvation, with production of the polymer being increased under conditions of limiting nitrogen (212). Further, curdlan production responds to ppGpp, a small molecule involved in the stringent response associated with starvation and other stress conditions (212). These observations suggest that curdlan synthesis may respond to environmental signals.

There is strong evidence that cellulose synthesis in *A. tumefaciens* is also regulated. Matthysse (158) reported that synthesis of the polymer is induced by the addition of soytone to media, although our laboratory has been unable to replicate this result (Sharik Khan, Personal Communication). Mutational analysis suggests that two genes, *celG* and *celI*, negatively regulate cellulose production; mutating either ORF
results in increased synthesis of the polymer (158). However, attempts to define environmental signals stimulating cellulose synthesis have been unproductive.

While the environmental signal regulating cellulose production in *A. tumefaciens* remains elusive, there is evidence of an intracellular molecule that influences synthesis of the polymer. Benziman (6) reported that in cell-free extracts of the bacteria, cellulose synthesis is stimulated by adding cyclic-di-guanosine monophosphate (c-di-GMP). In *R. leguminosarum*, mutating a gene predicted to synthesize this intracellular signal also affects production of the polymer (11). These observations suggest that c-di-GMP is a factor in regulating cellulose synthesis.

### 1.6 The biology of c-di-GMP regulation

Cyclic-di-GMP is one of the internal signals used by bacteria to regulate intracellular processes (for an overview, see (205)). The signal consists of two molecules of GTP linked together at the 3’ and the 5’ carbons of the deoxyribose sugars (Figure 1.2; (79, 211)). Where determined, cyclic-di-GMP is present at relatively low levels with concentrations around 100-500 nM in *C. crescentus*, although under certain conditions levels of the signal can reach above 1 mM (46). Recent studies of sequenced genomes suggest that up to 85% of gram-negative bacteria use c-di-GMP in some manner (7, 246). Other studies have identified c-di-GMP signaling in *Bacillus subtilis* (39). The field of c-di-GMP signaling has exploded in recent years, with descriptions of numerous processes being regulated by the signal, including virulence, exopolysaccharide production, motility, biofilm production, cell cycle progression and DNA competence (reviewed in (205, 243)).
Interest in c-di-GMP traces directly to studies of the regulation of cellulose biosynthesis in *G. xylinus* (208). In cell-free extracts of this bacterium, adding the signal stimulates production of the polymer (208, 211). Later, Ross et al. (210) demonstrated that c-di-GMP directly stimulates synthesis in purified extracts of the cellulose synthase complex. Further research in *G. xylinus* showed that a class of enzymes called diguanylate cyclases, or DGCs, synthesize c-di-GMP (Figure 1.2; (257)). Diguanylate cyclases are characterized by the presence of a conserved GG(D/E)EF motif at the catalytic site (190, 217, 257). This motif interacts with two molecules of GTP bound to the enzyme and catalyzes the linkage forming c-di-GMP (188, 190). Many DGCs are bitopic, and contain a signal reception domain, suggesting that synthesis of c-di-GMP is itself regulated by sensory pathways (205, 206).

The breakdown of c-di-GMP is catalyzed by two different classes of phosphodiesterases, phosphodiesterase A (PDEA) and HD-GYP (Figure 1.2; (78, 79, 257)). Both enzymes hydrolyze c-di-GMP to pGpG, lowering the intracellular concentration of the signal (79, 225, 231). PDEAs catalyze this reaction through the conserved EAL motif (225, 231), while HD-GYP-type enzymes degrades the molecule using the conserved HDXXXGYP motif (70, 214). These enzymes act in conjunction with DGCs to modulate the intracellular levels of c-di-GMP.

Cyclic-di-GMP is bound by target proteins, with such binding often resulting in regulation of a specific process. The first such protein discovered was PilZ, a small receiver found in species of *Pseudomonas* (7). Proteins containing the PilZ-like domain are present in a large percentage of organisms (7). Since then, a number of c-di-GMP-binding domains have been identified. These additional motifs include modified GGDEF,
EAL or HD-GYP domains, which can bind to c-di-GMP without processing (71, 142) and inhibitory binding sites (I-sites) found in a number of DGCs (35, 181, 188). Of considerable interest, several riboswitches bind c-di-GMP as the folding ligand (141, 253).

As an internal signal, c-di-GMP diffuses easily throughout the cell within milliseconds of production, and could influence other processes beyond the intended target. This issue of spatial organization is controlled in two ways. In the first, receptors of c-di-GMP may show different affinities for the signal and exhibit sensitivity to variations in concentration of the signal. In the second, the molecule may be localized and/or contained within a compartment of the bacterium. In the first model, a given receiver domain has a defined affinity for c-di-GMP, resulting in different phenotypes associated with different concentrations of the signal. Studies of two c-di-GMP-binding proteins in *S. enterica* serovar Typhimurium, YcgR and BcsA, demonstrated that each receptor has a different binding affinity, resulting in either altered motility or cellulose synthesis depending on the *in vivo* concentration of c-di-GMP (197). In the second model, DGCs are localized to specific regions of the cell to prevent the signal from affecting receptors in other locations in the bacterium. The well-studied regulation of cell differentiation and division in *C. crescentus* exemplifies this method, with several DGCs localized to coordinate both processes at one specific pole of the cell (1, 4, 268). These examples suggest that a combination of the two principles - targeting the c-di-GMP synthase and variations in binding affinities of the receptors - are necessary for separating processes regulated by the signal.
In many bacteria, c-di-GMP regulates the transition of the cell from motility to a sessile lifestyle (205). In these organisms, a low concentration of the signal results in production of flagella and a corresponding motile lifestyle (205, 246). As levels of c-di-GMP increase, the bacteria transition to a sessile lifestyle through increased production of exopolysaccharides, upregulation of anchoring systems and transition into biofilms (205).

1.7 Cyclic-di-GMP regulation of cellulose biosynthesis in bacteria

The discovery of c-di-GMP as an intracellular regulator derived from studies of cellulose synthesis. In addition to biochemical studies previously described, purified synthase complexes of *G. xylinus* bound radiolabeled c-di-GMP, suggesting that the enzyme is stimulated by directly interacting with the signal (283). Crystallographic studies later demonstrated that c-di-GMP is bound within the PilZ domain of BcsA in *Salmonella* (74, 173). These results suggest that c-di-GMP activates cellulose synthesis by directly binding with the enzyme.

Genetic studies support the biochemical evidence demonstrating that c-di-GMP regulates cellulose synthesis. Three operons encoding DGCs and PDEAs that affect production of the polymer were identified in *G. xylinus* (257). Each of these two-gene operons, annotated *pde-cdg*, contain a phosphodiesterase (Pde) and a diguanylate cyclase (Dgc) (257), suggesting divergent duplication of an original locus. Later research determined biochemically that the Dgc and Pde proteins possess cyclase and phosphodiesterase activity, respectively (12, 37). These observations suggest that modulation of c-di-GMP levels through these enzymes controls cellulose synthesis in *G. xylinus*.
In many other bacteria, c-di-GMP serves to regulate cellulose synthesis. Genes encoding DGCs that regulate cellulose production have been identified in *Rhizobium* (11), *Salmonella* (307), *Pseudomonas* (57, 245) and *Vibrio* (198). Phylogenetic analysis of these organisms demonstrated that each contains the genes encoding the cellulose synthesis pathway, including BcsA(CelA) (7). In all of these species, this subunit of the synthase contains a conserved PilZ domain (7, 288). The use of c-di-GMP in a diverse group of bacteria suggests a conserved system of regulating production of the polymer in at least the alpha- and gammaproteobacteria.

Regulation of cellulose biosynthesis by a DGC also was described in *R. leguminosarum*, a close relative of *A. tumefaciens*. Using a cellulose-overproducing strain of *R. leguminosarum*, Ausmees *et al.* (11) reported that mutations in a two-gene operon, consisting of a histidine kinase, named *celR1*, and a putative diguanylate cyclase, annotated as *celR2*, negatively affected cellulose production. Complementing the mutations with *celR2* restored cellulose production to levels of the parent, suggesting that the product of the gene stimulated synthesis of the polymer (11). However, while CelR2 contains a GGEEF motif for synthesizing c-di-GMP, at the time the importance of this domain was not known, and therefore the role of c-di-GMP in controlling cellulose production in *R. leguminosarum* was not examined.

### 1.8 Objectives of this research project

Attachment of *A. tumefaciens* to plant cells is required for colonization and proliferation of the bacteria. Additionally, interaction of the bacteria with plants is critical for delivery of T-DNA during induction of crown gall tumors. While the biochemistry of
one component of binding, cellulose, has been studied in some detail in *A. tumefaciens*, less is known about how production of the polymer is regulated. Cyclic-di-GMP stimulates cellulose synthesis in cell-free extracts of *A. tumefaciens* (6), yet the DGC synthesizing the signal has not been identified. Further, despite attempts to determine its function in attachment and virulence, the role of cellulose in these processes remains poorly understood. The effects of other components involved in attachment, including the UPP, also are not yet clear. Characterizing the factors regulating production of components like cellulose and the UPP is necessary to understand the attachment process.

In this study, I propose to determine the role of c-di-GMP in regulating cellulose synthesis and UPP production in *A. tumefaciens*. Biochemical and bioinformatic analyses suggest that production of the polymer is controlled by c-di-GMP (6, 7). In *R. leguminosarum*, overproducing a GGEEF-containing protein, CelR2, results in increased cellulose synthesis (11). Further, deleting specific genes encoding DGCs affects UPP production in *A. tumefaciens* (294). These observations suggest that other GGEEF-containing proteins are involved in regulating cellulose synthesis and UPP formation in *A. tumefaciens*.

In Chapter 2, I examine the effects of two genes encoding DGCs, *atu1060* and *atu1297*, on a number of phenotypes typically affected by c-di-GMP. These two enzymes, similar to their ortholog in *R. leguminosarum*, CelR2, contain two CheY-like receiver domains and a C-terminal GGEEF domain (11). Given the relatedness of Atu1297 and Atu1060 to other DGCs, including CelR2, I hypothesized that one, the other, or both of these two proteins would impact attachment, cellulose synthesis and virulence. Overexpressing either gene results in increased production of the polymer and
increased cell aggregation. A celF mutant, unable to produce cellulose, did not express these phenotypes. Overexpressing atu1297 also impacts cell-cell interactions, attachment to plant tissue, biofilm formation and virulence. Deleting the gene results in a significant decrease in cellulose synthesis, suggesting that atu1297 is necessary to stimulate production of the polymer. However, deleting this gene does not significantly impact other phenotypes affected by overexpressing atu1297, suggesting that other DGCs are involved in regulating these traits. From these results, I concluded that atu1297 directly regulates cellulose synthesis in A. tumefaciens, and named the gene celR.

In Chapter 3, I focus on CelR-mediated regulation of cellulose synthesis. The DGC contains a conserved GGEEF domain and a CheY-like receiver domain, the latter suggesting that the protein is an intermediate component of a larger signal pathway. Site-directed mutagenesis of critical residues in the GGEEF and CheY domains demonstrate that CelR is a functional diguanylate cyclase, and likely requires phosphorylation in the receiver domain for its activation. The signal synthesized by CelR must be received by a target protein, and the cellulose synthase subunit CelA contains a PilZ-type c-di-GMP-binding domain. A site-directed mutation in the PilZ domain of CelA prevents stimulation of cellulose synthesis by CelR, suggesting that CelA requires c-di-GMP for maximum enzymatic activity. Further, a gene encoding a response regulator is located upstream of celR in a two-gene operon. This gene, annotated as divK, is required for full CelR-dependent stimulation of cellulose synthesis, as deleting the response regulator prevents stimulation of production of the polymer by CelR. A constitutively active form of the DGC overproduces cellulose in the divK deletion mutant, suggesting that this response regulator is part of the activation cascade for CelR. The response regulator also
is a component of cell cycle regulation, similar to orthologs of *divK* in other alphaproteobacteria. These observations suggest that DivK contributes to the regulation of progression of the cell cycle and cellulose synthesis through separate mechanisms, while CelR specifically regulates cellulose synthesis in *A. tumefaciens*.

In Chapter 4, I continue examining Atu1060, renamed AvmA, to determine its role in attachment and virulence. Similar to CelR, AvmA contains two CheY-like domains and the GGEEF domain. This observation suggests that some of the phenotypes unaffected by deleting *celR* may be influenced by AvmA. Deleting the gene results in increased UPP production and polar attachment and a decrease in biofilm formation. The mutant phenotypes are complemented by expression of wild-type *avmA*, while overexpressing alleles of the gene altered at either the activation residue or the GGEEF motif failed to replicate the phenotypes displayed when overexpressing the wild-type gene. Interestingly, as compared to the wild-type parent, the *avmA* deletion mutant requires a 10-fold lower dose of bacteria to induce tumors. However, the mutant alleles of *avmA* fully complemented the virulence phenotype of the deletion mutant. These results suggest that, like CelR, AvmA requires activation to synthesize c-di-GMP, and that this signal regulates traits including UPP production and attachment to surfaces. However, the influence of AvmA on virulence is not dependent on the production of c-di-GMP. Combined, these observations suggest that CelR and AvmA regulate the conversion from initial attachment to stable binding in *A. tumefaciens*. 
1.9 Figures

Figure 1.1. The *cel* operons of *A. tumefaciens*. Scale bar represents approximately 1 kb of DNA.
Figure 1.2. A model of cyclic diguanosine monophosphate (c-di-GMP) cycling, with corresponding domains catalyzing the reactions and processes upregulated by the signal.
Chapter 2

CelR, an ortholog of the diguanylate cyclase PleD of Caulobacter, regulates cellulose synthesis in Agrobacterium tumefaciens

2.1 Notes and Acknowledgements

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2.2 Summary

Cellulose fibrils play a role in attachment of Agrobacterium tumefaciens to its plant host. While the genes for cellulose biosynthesis in the bacterium have been identified, little is known concerning the regulation of the process. The signal molecule cyclic diguanosine monophosphate (c-di-GMP) has been linked to the regulation of exopolysaccharide biosynthesis in many bacterial species, including A. tumefaciens. In this study, we identified two putative diguanylate cyclases, celR (atu1297) and atu1060.
that influence production of cellulose in *A. tumefaciens*. Overexpression of either gene resulted in increased cellulose production, while deletion of *celR*, but not *atu1060*, resulted in decreased cellulose biosynthesis. *CelR* overexpression also affected other phenotypes including biofilm formation, formation of a polar adhesion structure, plant surface attachment, and virulence, suggesting that the gene plays a role in regulating these processes. Analysis of *celR* and Δ*cel* mutants allowed differentiation between phenotypes, such as biofilm formation, associated with cellulose production and phenotypes probably resulting from c-di-GMP signaling, which include polar adhesion, attachment to plant tissue and virulence. Phylogenetic comparisons suggest that species containing both *celR* and the cellulose synthase subunit *celA* adapted the CelR protein to regulate cellulose production, while those that lack *celA* use CelR, called PleD, to regulate specific processes associated with polar localization and cell division.

### 2.3 Introduction

The ability to attach to surfaces is critical for the survival and growth of many bacteria in their native environments. Such attachments can provide a protective barrier from harsh environmental conditions and predation, and also are important in establishing a relationship between pathogens and symbionts and their hosts. The interaction of the plant pathogen *Agrobacterium tumefaciens* with its plant host in particular is dependent on such attachment phenomena (147, 148). This bacterium binds to plant cell surfaces and at plant wound sites forming microcolonies and biofilms.

Attachment as biofilms or microcolonies often requires the formation of matrices of complex carbohydrate polymers, with such matrices anchoring the bacteria to each
other as well as to surfaces. One component of the matrix produced by *A. tumefaciens* is cellulose, a β1,4-linked glucose polymer. The cellulose fibrils apparently serve to anchor the bacteria to each other as well as to the plants (157). Mutants deficient in the production of cellulose bind less tightly to plant cell surfaces (156, 160), and do not efficiently establish biofilms (158).

The production of cellulose by *A. tumefaciens* strain C58 is encoded by two closely linked operons, *celABCG* and *celDE*, located on the linear chromosome (162). The two operons encode the cellulose synthase complex, a membrane-bound structure that includes the catalytic complex composed of a CelA/CelB heterodimer. This complex catalyzes the addition of UDP-glucose to the extending cellulose fiber (161, 222, 307). CelC is similar to outer porin-like proteins, and may serve as the complex for secreting the polymer into the extracellular environment (223). The functions of CelD and CelE in the synthesis and secretion of cellulose are unknown (161, 223), while CelG, although of unknown function, apparently contributes to the regulation of cellulose synthesis (158). Based on evidence in other bacteria, the cellulose fibrils are believed to be extruded into the extracellular milieu from the synthase complex imbedded within the membrane of the cells (173, 288). While much is known about the synthesis of the polymer, little is known concerning mechanisms that control cellulose production in *A. tumefaciens*.

In some bacteria, production of cellulose is activated in response to the intracellular signal, cyclic di-guanosine monophosphate (c-di-GMP) (for reviews, see (106, 203, 206, 209)). Synthesis of c-di-GMP is catalyzed by a family of enzymes called diguanylate cyclases (DGC), most of which are characterized by a conserved GG(D/E)EF motif (257). The correct balance of c-di-GMP within the cell is maintained by the
breakdown of the signal molecule mainly by phosphodiesterase A (PDEA), marked by a conserved EAL motif (257) or by the HD-GYP domain (69, 78). The c-di-GMP acts as an allosteric ligand, and is bound by receptor proteins containing one of several identified c-di-GMP binding domains. Such domains include the PilZ domain; modified HD-GYP, EAL and GGDEF motifs; and even riboswitches (7, 35, 181, 204, 216, 253). In recent years, c-di-GMP has been implicated in regulatory systems that control motility, biofilm formation, exopolysaccharide production, and virulence in many bacterial species (for reviews, see (19, 56, 107, 206, 243, 258)).

The production of c-di-GMP regulates at least two attachment processes in the α-proteobacteria. In Caulobacter crescentus, c-di-GMP produced by the diguanylate cyclase PleD regulates the formation and localization of the polarly-localized holdfast stalk, which anchors the cell to surfaces through a terminal adhesive structure (4, 94). Rhizobium leguminosarum bv. Trifoli uses an ortholog of PleD, called CelR2, to regulate production of cellulose (11). There also is evidence that c-di-GMP plays a role in exopolysaccharide production in A. tumefaciens; addition of the signal to cell-free lysates resulted in an increased rate of cellulose synthesis (6). Consistent with this observation, the CelA component of the cellulose synthase of A. tumefaciens contains a PilZ domain (7).

In this study, we identified probable DGCs that influence exopolysaccharide production and examined the effects of such enzymes on cellular processes in A. tumefaciens, including the production of cellulose, the formation of attachment structures and the behavioral consequences of these changes. Our studies indicate that overexpressing two putative DGCs, celR (atu1297) and atu1060, positively affects
cellulose biosynthesis. Deleting celR resulted in a decrease in the production of cellulose, while removal of atu1060 did not affect production of the polymer. Overexpressing celR also influenced other phenotypes, including biofilm formation, formation of a polar attachment structure, and virulence, suggesting that the protein or the c-di-GMP signal plays a role in regulating these processes as well.

2.4 Materials and Methods

2.4.1 Strains, cultures and growth conditions. The strains used in this study are listed in Table 2.1. Strains of Escherichia coli were grown on Luria- Bertani (LB, Invitrogen) agar plates with appropriate antibiotics at 37°C. Strains of A. tumefaciens were maintained on nutrient agar (NA, Fisher) or AB minimal medium agar (41) supplemented with 0.2% mannitol (ABM) with appropriate antibiotics at 28°C. Cultures of E. coli were grown in LB broth with the corresponding antibiotics at 37°C with shaking. Cultures of A. tumefaciens were grown in MG/L (100) complex medium with appropriate antibiotics at 28°C with shaking. For some experiments, cultures of A. tumefaciens were grown in AB minimal medium- based vir induction medium (100) supplemented with 0.2% mannitol and 200 µg/ml acetosyringone (ABIM). Antibiotics used include ampicillin (100µg/ml for E. coli), carbenicillin (50 µg/ml for A. tumefaciens), kanamycin (50 µg/ml for E. coli and A. tumefaciens), gentamicin (50 µg/ml for E. coli, 25 µg/ml for A. tumefaciens), and tetracycline (10 µg/ml for both E. coli and A. tumefaciens). When necessary, Congo red (50 µg/ml) or aniline blue (50 µg/ml) was added to ABM plates for assessing production of exopolysaccharides.
2.4.2 Strain construction. Production of overexpression strains: Genomic DNA was prepared from an overnight culture of *A. tumefaciens* NTL7 as described previously (84). Genes to be cloned were amplified by PCR using *Pfu* polymerase (Stratagene) and the following primer sets: *atu1297*-f (5′- CGGGATCCCATATGACGGCGAGAGTTCT-3′) and *atu1297*-r (5′- CGGATCCTCAGGCGGCGGCGCCACGACGCG-3′), *atu1060*-f (5′- CGGATCCCATATGCAGGATAAAATCCTTCTG-3′) and *atu1060*-r (5′- CGGATCCCTCAGGCCGTTCAAGCCGAT-3′), *atu0826*-f (5′- CGGGATCCCATATGCAGGATAAAATCCTTCTG-3′) and *atu0826*-r (5′- CGGATCCCTCAGGCCGTTCAAGCCGAT-3′), *atu2228*-f (5′- CGGGATCCCATATGACGGCGAGAGTTCT-3′) and *atu2228*-r (5′- CGGATCCTCAGGCCGTTCAAGCCGAT-3′), and *atu4490*-f (5′- CGGGATCCCATATGACGGCGAGAGTTCT-3′) and *atu4490*-r (5′- CGGATCCCTCAGGCCGTTCAAGCCGAT-3′). The PCR products were digested with *BamHI* and ligated into *BamHI*- digested pUC18. The resulting ligation products were introduced into DH5α by CaCl₂ transformation, with selection on LB plates containing ampicillin. Resistant colonies were selected, the plasmids purified and digested with *NdeI* and *BamHI*. The resulting fragments were ligated into the expression vector pZLQ (Table 2.1), placing the gene under the transcriptional control of the *lac* promoter, and transformed into DH5α. After selecting for kanamycin resistance, the plasmids were isolated, analyzed, and the correct constructs were electroporated into the appropriate strains of *A. tumefaciens*.

Deletion of the *A. tumefaciens* chromosomal *cel* locus: Removal of the *cel* locus was performed by Dr. Shengchang Su. An 800 bp *BamHI*-HindIII fragment of *celD*
(atu3302) and a 1 kb HindIII-SpeI fragment containing sequences of celC (atu3307), celG (atu8186) and celB (atu3308) were amplified by PCR from NTL4 genomic DNA using Pfu DNA polymerase and two pairs of primers: celD/Bm (5′-CGGGATCCATGCGCATCGATATC-3′ and celD/Hind 5′-CCCAAGCTTTCGCCGAACCACAGC-3′); celC/Hind (5′-CCCAAGCTTACGGATTGACCACCG-3′ and celB/Sp 5′-GCTCTAGAACTAGTTGGATGAAGCGGAAT-3′), respectively. The above two PCR products were treated with the appropriate restriction endonucleases, mixed with a 1.6 kb HindIII fragment carrying the tetA gene from pBBR1MCS-3, and inserted between the BamHI and SpeI sites of pSR47s (Table 2.1). The resulting ligation products were transformed into S17-1 λ pir. A ligation product, pSRΔcel, in which the tetA gene was flanked on one side by the first 500 bp of celC and the last 800 bp of celD on the other side (Figure 2.1), was identified and mated into NTL7 as previously described (55). NTL7 carrying the chromosomal disruption in the cel gene cluster was selected by plating on medium containing the appropriate antibiotics and 5% sucrose. Allelic exchange of the altered cel region was verified by PCR using additional primers located further upstream and downstream from the original fragments.

**Mutation of celR by allelic exchange:** Two flanking regions of celR were amplified from genomic DNA of strain NTL7 using pfu DNA polymerase and two pairs of primers: celRdel1-f (5′-GCTCTAGAGGGCCCACGTAGCCAACCATACTCCG-3′) and celRdel1-r (5′-CGCGGCATGCCTTTACGAGGCGAAACATGC-3′); celRdel2-f (5′-CGCGGCATGCCTTTACGAGGCGAAACATGC-3′) and celRdel2-r (5′-CGGGATCCACTAGTCGTTGAAATAAAGGCAGAGC-3′), respectively. The
fragments were digested using XbaI and SmaI for fragment celRdel1 and SphI and BamHI for fragment celRdel2, and the fragments were then inserted separately into pUC18. The resulting ligation products were transformed into DH5α. The plasmids were identified for the correct insertions and digested again with XbaI and SmaI for fragment celRdel1 and SphI and BamHI for fragment celRdel2. A gentamicin resistance cassette from pMGm (Table 2.1) was digested using SmaI and SphI, and the fragment was ligated between the two flanking regions of celR, forming pUCcelRdel (Figure 2.2). The new construct was digested with XbaI and BamHI and ligated into pWM91 (Table 2.1), a suicide vector containing sacB, and transformed into S17-1 λpir by electroporation. Successful constructs were selected for by resistance to ampicillin and gentamicin, and the plasmids were isolated, analyzed and electroporated into A. tumefaciens. Initial transformants were selected for resistance to gentamicin and 5% sucrose, followed by screens for sensitivity to carbenicillin. Potential marker-exchange mutants were confirmed using PCR and Southern analysis.

Indel mutation of atu1060: The atu1060 gene was replaced with a kanamycin resistance cassette using a protocol modified from that of Datsenko and Wanner (61). Briefly, a set of primers, atu1060frt-f (5′-GCGTTTTTGTGCCTAGAGACTAGAGCTGAGCGTTGCCGCGGCCTGTGTAGGCAGGAGCTGCCCTTC-3′) and atu1060frt-r (5′-GAGGAAAGACTGGGGAGACGGGCCAGGGGGCTTGGGACGGCCCATATGAACTGCTCTTA-3′), were used to amplify the kanamycin cassette from pKD4 (Table 2.1) by PCR, and the product was treated with DpnI to blunt the ends. Additionally, a 3.6 kb fragment containing atu1060 was amplified from NTL7 genomic DNA using the
primers atu1060region-f (5′-GGGGTACCGCGATTGTGCATGCTAAAGA-3′) and atu1060region-r (5′-GGGGTACCGCGCCCTCATCTATGTCATT-3′). The fragment was digested with KpnI and cloned into pRK415, creating the construct pRKatu1060region. The construct was introduced into DH5α by CaCl2 transformation, and the plasmid was purified and analyzed by restriction digest and sequencing. The kanamycin fragment was then electroporated into an E. coli strain harboring both the red recombinase plasmid pKD46 (Table 2.1) and pRKatu1060region. The transformants were selected for resistance to kanamycin, and plasmids were purified and examined for replacement of atu1060 by restriction digest and PCR. The correct plasmids containing the replaced gene were digested with KpnI, the modified atu1060 gene was cloned into pWM91 (Table 2.1), producing the construct pWMatu1060kan, and this plasmid was transformed into S17-1 λpir. Successful constructs were selected for resistance to ampicillin and kanamycin, and a verified plasmid was electroporated into A. tumefaciens. Initial transformants were selected for resistance to kanamycin and 5% sucrose, followed by screening for sensitivity to carbenicillin. Potential mutants were confirmed using PCR and Southern analysis.

Complementation of NTL7ΔcelR::Gm: Wild-type celR is the second gene in an operon with atu1296, an ortholog of divK in Caulobacter crescentus ((93); Figure 2.2). To delete atu1296 and keep celR under regulation of its native promoter, a 400 bp region containing the promoter of the divK/celR operon was amplified using Pfu DNA polymerase and the primers prcelR-f (5′-GCGGATCCTGGCCGGCATTGCTTTTGGT-3′) and prcelR-r (5′-GCGGATCCCATATGGTGCCGAGTCCCCGTTC-3′), digested with BamHI, and cloned into pUC18. The promoter fragment and the cloned celR gene
were digested with \textit{NdeI} and \textit{BamHI}, and ligated together to form pUCprcelR (Figure 2.2). The clone was purified and confirmed by sequence analysis. In this construct, \textit{divK} is deleted and \textit{celR} is driven directly by the \textit{divK- celR} promoter. The correct clone was digested with \textit{BamHI} and the \textit{prcelR} fragment was inserted into pUC18-miniTn7T-Km (44) to form pUCTn7T-prcelR. The new construct, as well as the transposase plasmid pTNS2 (Table 2.1; (44)), were electroporated into NTL7Δ\textit{celR::Gm}, with selection for resistance to kanamycin. Potential mini-Tn7 integrants were confirmed by PCR analysis.

\textbf{2.4.3 Cellulose extraction assays.} Cellulose was quantified following extraction using a modified Updegraff protocol (271). Briefly, cells were grown in 12 ml of MG/L with appropriate antibiotics overnight at 28°C with shaking. From this culture, a 10 ml volume was centrifuged at 3000 \(x\) \(g\) at 4°C for 10 minutes, while the remaining 2 ml were reserved for protein concentration determinations. The cell pellets were resuspended in 3 ml of 85% acetic/ 5% nitric acid, and the suspension was boiled for 30 minutes. The resulting suspension was centrifuged for 30 minutes, the pellet was washed with 5 ml of double distilled water (ddH2O), and collected by centrifugation for 30 minutes. The resulting pellet, which represents the remaining acid-stable carbohydrate polymers, was resuspended in 5 ml 67% H\textsubscript{2}SO\textsubscript{4} and incubated for 1 hour at room temperature. The acid-digested samples were diluted 1:5 into ddH\textsubscript{2}O, mixed with 3 volumes of anthrone reagent [50 mg of anthrone (Sigma-Aldrich) per 1 ml H\textsubscript{2}SO\textsubscript{4}], boiled for 15 minutes, and chilled to room temperature. Absorbance of the solution at 620 nm was determined using a BioRad SmartSpec\textsuperscript{TM} Plus spectrophotometer. Absorbance values were compared to a standard curve created from a stock solution of pure cellulose (Sigma-Aldrich) dissolved in 67% H\textsubscript{2}SO\textsubscript{4}. 

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The amount of anthrone-reactive material was standardized based on the soluble protein concentration of the sample. Cells in the remaining 2 ml of sample were collected by centrifugation, resuspended in 100 µl of 0.9% NaCl solution and disrupted by sonication. The insoluble components were removed by centrifugation, the remaining soluble protein was assayed using Coomassie Plus™ Assay Reagent (Thermo Scientific), and absorbance of Coomassie-bound protein was measured at 595 nm. Statistical analysis was performed using the Student t-test using a one-sided distribution model.

2.4.4 Cellulase treatment. Cells from cultures grown as described above were collected by centrifugation at room temperature for 10 minutes at 3000 x g, and resuspended in 3 ml of LTE buffer (10 mM Tris-Base, 1.2 mM EDTA, pH 8.0). Purified cellulase (Sigma-Aldrich) was added to the cell suspension to a final concentration of 20 µg/ ml, and the suspension was incubated for 1 hour at 37°C with shaking. The cells were recovered by centrifugation, and the amount of glucose-containing polymer remaining associated with the cells was quantified as described above.

2.4.5 Microscopy and lectin-binding assays. Cells, grown in liquid culture for two days at 28°C, were collected by centrifugation for 5 minutes before resuspending in 0.9% NaCl to a final OD$_{600}$ of 0.4. For lectin staining, the resuspended cells were incubated with 100 µg per ml of Alexafluor633-WGA (Thermo Scientific) for 15 minutes, and washed three times with 0.9% NaCl by centrifugation. Cells were visualized by differential interference contrast (DIC) microscopy at the Institute for Genomic Biology-Microscopy and Imaging Facility (University of Illinois) using a Zeiss Axiovert™ 200M microscope equipped with an Apotome Structured Illumination Optical Sectioning System set at 63x/1.40 objective magnification, and images were captured
using a Zeiss MRc 5 camera. Where cells were treated with lectin, samples were excited at 633nm and observed for fluorescence at 647 nm. Images were compiled and analyzed using Zeiss Axiovision™ software. For statistical analysis, four randomly-chosen images containing cells were compiled, and the number of cells in each image and their arrangement/lectin labeling were counted. The data was analyzed for statistical significance using the Chi-squared test.

2.4.6 Microscopic analysis of bacterial attachment to Arabidopsis. SEM analysis of bacterial attachment was performed in the laboratory of Dr. Lois Banta, Williams College, MA. Seeds of Arabidopsis thaliana ecotype Columbia (Col-0) were surface-sterilized with a solution of 50% bleach/0.1% SDS, and sown onto solid Gamborg’s B5 media containing 100 mg/l tricarcillin (Research Products International). Seeds were incubated for 2 days at 4°C, and then germinated and grown at room temperature for 16 days. Bacterial strains were grown in MG/L medium at 28°C overnight, with addition of antibiotics if required. Strains of A. tumefaciens were subcultured into ABIM containing either 100 or 200 µM acetosyringone and grown on a rotary shaker at 22°C to mid-exponential phase (OD₆₀₀ ≈ 0.5). Sterile forceps were used to wound leaves excised from seedlings before co-cultivating with bacterial cells for 2 days at 21°C. Co-cultivated leaf pieces were rinsed three times in ABIM with gentle vortexing (20 sec/wash) to remove any unattached bacteria. Samples were fixed in 3% glutaraldehyde (in 0.1 M HEPES; pH 7.1) for 3 days and rinsed three times in 0.1M HEPES (pH 7.1) before postfixing in 1% OsO₄ for 1-2 hours. Samples were subsequently rinsed with distilled H₂O, sequentially dehydrated in 70, 80, 90 and 100% ethanol, and immediately dried in a Ladd critical-point drying apparatus under CO₂. Samples were
loaded on aluminum specimen holders, sputter-coated with gold-palladium using a Polaron SEM autocoating unit, and viewed on a FEI Quanta 400 Series scanning electron microscope. Three to five leaves were examined for each bacterial strain per assay, and several representative images per leaf were captured for analysis. Analysis of the SEM samples was performed “blind” (i.e. without knowing the identity of the sample) to ensure a lack of observer bias. For statistical analysis, the number of cells in each image and the number of polarly-bound cells were counted. The data was analyzed for statistical significance using the Student t-test.

2.4.7 Biofilm assays. Cells were grown overnight with shaking in MG/L at 28°C with appropriate antibiotics, and diluted 1/1000 into 2 ml of MG/L with antibiotics. The diluted samples were incubated for 5 days at room temperature without shaking in 13 X 100 mm sterile borosilicate tubes. After incubation, 1 ml of 0.1% crystal violet was added to each sample and the cultures were incubated at room temperature for 15 minutes. Supernatants were carefully decanted, and the inside walls of the stained tubes were gently washed three times with 2 ml of ddH2O. The remaining adherent crystal violet stain was solubilized using 1 ml of ice-cold 70% ethanol. The absorbance of the ethanolic samples was measured at 540 nm using a BioRad SmartSpec™ Plus spectrophotometer.

2.4.8 Virulence assays. Two different assays were utilized on different host plants. For Kalanchöe daigremontiana, bacteria were grown for 2 days at 28°C, collected by centrifugation, and resuspended in 1 ml of 0.9% NaCl. The population sizes of the resuspended cells were standardized to an OD$_{600}$ of 1.0, and the suspensions were then diluted 1:10 and 1:100 in 1 ml of 0.9% NaCl. Kalanchöe leaves were wounded using a thin syringe needle, and 2 µl volumes of cell suspensions from each dilution were
inoculated into the wound sites. At least six leaves on three different plants were wounded and inoculated in this manner. Tumors were visualized and photographed 3 to 5 weeks after inoculation, depending on day length and plant growth rates.

For virulence assays on Solanum lycopersicum (tomato), bacterial cultures were grown and standardized as described above. The suspensions were diluted in ten-fold increments from $10^{-1}$ to $10^{-5}$. Twenty mm-long wounds between the primary leaves and the first set of secondary leaves were produced using a razor blade. As above, 2 µl volumes of the bacterial suspensions were inoculated into the wound sites, and the plants were incubated in the greenhouse for 3 to 5 weeks, depending on day length and plant growth rates. At least four different plants per condition (sample strain and dilution amount) were wounded and inoculated in this manner. Total tumor mass was determined by excising the segment of stem, cutting just above and below the wound site. The stem pieces were weighed individually, and the tumor mass was removed by cutting with a cork borer and weighed. The tumor mass was averaged between the four plants. The experiments were repeated at least three times, and the total average of the samples, as well as standard error, was calculated from these experiments. Statistical analysis was performed using the Student t-test with a one-sided distribution model.

2.5 Results

2.5.1 Overexpressing different putative diguanylate cyclases in Agrobacterium tumefaciens has varying effects on exopolysaccharide production. In A. tumefaciens, the stimulation of cellulose production in cell extracts by the addition of exogenous c-di-GMP (6) suggests that a diguanylate cyclase (DGC) is involved in
regulating production of this polymer. Annotation indicates that the genome of *A. tumefaciens* strain C58 may encode as many as 32 proteins with DGC activity (85, 292). Of these candidates, five genes—*atu0826, atu1060, atu1297, atu2228* and *atu4490*, were chosen for testing based on the association of the GGDEF motif with a signaling domain (Figure 2.3). Of particular interest was *atu1297*, annotated as *pleD*, which, in *C. crescentus*, is known to synthesize c-di-GMP (188, 294). We tested these genes to determine if any, when overexpressed, resulted in changes in the production of cellulose.

For the initial examination, the five GGDEF-containing ORFs were cloned into the overexpression vector pZLQ (Table 2.1) and introduced into strain NTL7. Overexpression of *atu4490* had no effect on colony morphology on solid medium or growth in liquid media (Figure 2.4A, B). Strains overexpressing either *atu0826* or *atu2228* formed smaller colonies on solid medium (Figure 2.4A), although the strains were unaffected in growth in liquid culture (Figure 2.4B). However, overexpression of *atu1060* and *atu1297* resulted in the formation of small, hard colonies on agar surfaces (Figure 2.4A). When tested on solid medium containing Congo red, colonies of these strains, but not those expressing the other three genes, incorporated more dye than did the strain lacking an overexpression construct (Figure 2.4A). Unlike the parent, when grown in liquid media, cells of both NTL7(pZLQatu1297) and NTL7(pZLQatu1060) formed large aggregates (Figure 2.4B) and these aggregates were difficult to disrupt by physical means. These results suggested that *atu1060* and *atu1297* play a role in exopolysaccharide production and in cell-cell interactions.

**2.5.2 Increasing the expression of *atu1060* and *atu1297* affects the production of cellulose.** Congo red binding is indicative of exopolysaccharides and some amyloid
proteins (259, 302), and suggests that *atu1060* and *atu1297* influence the production of such products. To test if the exopolysaccharide induced by overexpression of *atu1297* or *atu1060* is cellulose, the effects of pZLQ*atu1297* and pZLQ*atu1060* on NTL7 were examined by genetic manipulation and by quantification of total anthrone-positive material. First, the constructs were introduced into NTL7Δ*cel* (Table 2.1), a mutant prepared by Dr. Shengchang Su in which components of the *cel* locus have been deleted. Overexpressing either of the two genes in NTL7Δ*cel* did not result in any of the phenotypes displayed during overexpression in the wild-type parent, including hard colony formation, Congo Red binding and aggregation in liquid media (Figures 2.5A and B). These results suggest that at least some of the phenotypes associated with overexpression of *atu1060* and *atu1297* involve production of cellulose.

Strain C58, the parent of NTL4 and NTL7, produces at least two polyglucose-type exopolysaccharides: β1,4 linked cellulose and β1,3 linked curdlan (249). To determine if curdlan biosynthesis is affected by the overexpression of these genes, the strains were grown on solid ABM medium containing aniline blue, a dye that binds to the β1,3 polymer but not to cellulose (178). Colonies overexpressing either of the two genes were no more intensely blue than those of the wild-type NTL7, while a curdlan-overproducing strain, *Agrobacterium sp*. ATCC31749 (91; Table 2.1), grew as dark blue colonies (Figure 2.6). Moreover, NTL7Δ*cel* yielded colonies that bound amounts of aniline blue similar to NTL7 (Figure 2.6). Notably, colonies of ATCC31749 yielded darker red colonies on Congo red plates as compared to NTL7 (Figure 2.6), suggesting that Congo red binding is indicative of both cellulose and curdlan production. These results suggest that overexpression of *atu1060* or *atu1297* does not affect curdlan biosynthesis.
We next quantified the amount of anthrone-positive exopolysaccharide material produced by our strains using the Updegraff protocol (271; see Materials and Methods). Wild-type NTL7 produced, on average, 998 µg, while the Δcel mutant produced 545 µg of anthrone-positive material per milligram of soluble protein (Figure 2.7). Strains overexpressing either atu1060 or atu1297 produced two to three times as much anthrone-positive material, respectively, as compared to NTL7 (Figure 2.7 and Table 2.2). NTL7Δcel overexpressing either atu1060 or atu1297 produced amounts of anthrone-positive material comparable to the levels produced by the parent cel- mutant (Figure 2.7 and Table 2.2).

To confirm that the anthrone-reacting material recovered by the Updegraff protocol is cellulose, the cultures were pretreated with purified cellulase prior to analysis, as described in Materials and Methods. In cells of NTL7 pretreated with the enzyme, the amount of anthrone-positive material recovered dropped to levels comparable to those seen in NTL7Δcel (Table 2.2). The decrease in the amount of material collected from wild-type cells suggests that the difference between NTL7 and NTL7Δcel cultures represents the amount of cellulose produced by the wild-type bacteria (Table 2.2). The strain overexpressing atu1297 also showed lower amounts of anthrone-reacting material after cellulase treatment (Table 2.2). The results taken as a whole suggest that overexpression of atu1297 and atu1060 causes increased production of cellulose by NTL7, and that this increase in cellulose production requires genes of the cellulose synthesis locus. Based on these results, we suspect that the anthrone-positive material produced by NTL7Δcel is curdlan and perhaps other yet to be identified glucose-containing exopolysaccharides. In subsequent experiments we express levels of anthrone-
positive material in relative units normalized against the amounts detected from NTL7Δcel set at a value of zero.

2.5.3 _atu1297_, but not _atu1060_, is a positive regulator of cellulose production.

Overexpressing DGCs in bacteria often results in pleiotropic phenotypes (4, 152, 179, 216). This effect suggests that not only are multiple regulatory systems dependent on c-di-GMP, but that the signal produced by overexpression of any active DGC can crosstalk with other c-di-GMP responding systems. To determine if Atu1060 and Atu1297 both are directly involved in regulating cellulose production, the genes were deleted by allelic replacement with either a kanamycin- or a gentamicin- resistance cassette. The resulting mutants NTL7Δatu1297::Gm and NTL7Δatu1060::Km were tested for changes in Congo red binding and levels of cellulose production. On medium containing Congo red, neither mutant showed a visible difference in dye binding compared to wild-type NTL7 (Figure 2.8A). When assessed quantitatively, the _atu1297_ mutant produced significantly less cellulose as compared to NTL7, its wild-type parent (Figure 2.8B). The _atu1060_ mutant, however, showed no significant difference in the amounts of cellulose produced as compared to NTL7 (Figure 2.8B), suggesting that _atu1297_, but not _atu1060_, has a direct regulatory effect on production of the polymer. Based on these results, we focused our studies on _atu1297_.

To confirm that the deletion of _atu1297_ is responsible for the decrease in cellulose production, NTL7Δatu1297::Gm was complemented by mini-Tn7-mediated insertion of the wild-type gene expressed at unit copy number from its native promoter into a single site downstream of the _glmS_ gene on chromosome 1 (44, 45). The complemented _atu1297_ mutant produced levels of cellulose comparable to that of wild-type NTL7.
(Figure 2.8B). These results confirm that *atu1297* is required for production of wild-type levels of cellulose in *A. tumefaciens*. Based on this evidence, we renamed the *atu1297* gene *celR* (cellulose regulator).

### 2.5.4 Overexpression of *celR* affects the aggregation phenotype of individual cells

Cellulose produced by *A. tumefaciens* is involved in stabilizing colonization of plant surfaces (156-158, 160). In addition, production of cellulose may impact interactions of the cells with one another. To determine if *celR* is responsible for the aggregation phenotype seen in liquid medium, the overexpression and mutant strains were visualized using DIC microscopy. Cells of NTL7 overexpressing *celR* formed dense masses at a much higher frequency as compared to wild-type NTL7 (compare Figures 2.9A and B). Interestingly, cells of NTL7(pZLQ*celR*) that were separated from these large masses often were arranged in rosettes, with three to five cells connected to each other at one pole (Figure 2.9B, Table 2.3). NTL7Δ*cel*(pZLQ*celR*) produced fewer and smaller aggregates (Figure 2.9C). However, a number of cells were still associated with rosettes (Figure 2.9D, Table 2.3). We conclude from these results that the aggregation phenotype, but not rosette formation, is due to overproduction of cellulose resulting from overexpression of *celR*.

### 2.5.5 Overexpression of *celR* affects polar lectin binding

In *Caulobacter crescentus* the diguanylate cyclase PleD, an ortholog of CelR, regulates production and localization of the stalk with its holdfast structure (4, 94, 190). A similar, but stalk-less lectin-binding holdfast structure was recently described in *A. tumefaciens*, with the structure forming at one pole of the cell (143, 267, 294). This unipolar polysaccharide (UPP) structure also may play a role in initial attachment of the bacteria to plant cells.
To explore the role, if any, of CelR in the formation of the UPP, strains altered in expression of celR were examined for the polar adhesive.

The UPP in A. tumefaciens can be visualized using fluorescently labeled lectin conjugates, which bind the glucomannan fibers that constitute the adhesive. Similar to previous studies (267), when strain NTL7 was incubated with WGA-Alexafluor-633, a small subset of the cells showed polar binding of the lectin label (Figure 2.10A, Table 2.3). In contrast, cells of NTL7 overexpressing celR were labeled over the entirety of the aggregates (Figure 2.10B), making it difficult to identify the location of the lectin on individual cells. When cells of NTL7(pZLQcelR) were separated from the aggregate, about three times as many exhibited polar lectin binding (Table 2.3), suggesting that overexpression of celR results in increased formation of the UPP. Problems arising from the aggregation phenotype associated with overexpressing celR in a cel+ strain were resolved by overexpressing the gene in the Δcel background. Such cells continued to show an increase in polar lectin binding (Figure 2.10C, Table 2.3). Additionally, the rosettes produced by the Δcel strain overexpressing celR showed polar labeling at the center of the clustered cells. NTL7ΔcelR::Gm, on the other hand, did not display any alteration in lectin binding or in cellular aggregation as compared to wild-type cells (Figure 2.10D, Table 2.3). Consistent with the report by Xu et al. (294), these observations suggest that a DGC may play a role in UPP formation in A. tumefaciens, although it is likely that CelR is not directly involved in regulating this phenotype.

2.5.6 Modification of celR expression affects the attachment of cells to plant tissue. While cellulose helps to stabilize the attachment of the bacteria to plants, its role in attachment per se is not entirely understood. To assess the effects of altering celR
expression on primary attachment to plant tissue, the laboratory of Dr. Lois Banta incubated wild-type and mutant strains with *Arabidopsis* leaves, and binding was visualized using SEM. In comparison to NTL7, the cells of the *celR* mutant exhibited a statistically significant increase in attachment to plant tissue (Figures 2.11A and B; Table 2.4). In addition, compared to the wild-type parent, a significantly greater number of Δ*celR* cells attached to the surface in a polar orientation (Figure 2.11B; Table 2.4). These results suggest that deleting *celR* and its resultant negative effect on cellulose production impacts the initial attachment of the bacteria to plant cell surfaces.

To determine if CelR affected initial cellulose-independent attachment, the *celR* gene was overexpressed in the Δ*cel* background. NTL7Δ*cel* bound to the plant tissue at numbers comparable to those of NTL7 (Figures 2.11A and C). On the other hand, in comparison to the *cel* parent, NTL7Δ*cel* overexpressing *celR* was more sparsely bound to the plant tissue (Figures 2.11C and D). Interestingly, when viewing the cultured material by light microscopy before fixation for SEM, NTL7Δ*cel*(pZLQ*celR*) formed large aggregation patches on the surfaces of the plant tissue (data not shown). These patches are fragile and were disrupted by gentle washing before the fixation process. When attached to the plant cells, the overexpressing strain also displayed altered cell morphologies, forming branched structures and elongated cells (Figures 2.11E and F). These effects on cell morphology suggest that increasing expression of *celR* can alter cell division programming in the cells.

2.5.7 Overexpression of *celR* affects production of biofilms. Both the production of the UPP and cellulose influence attachment by *A. tumefaciens* to surfaces (143, 158, 267). To test the influence of *celR* on the ability of the bacteria to form
biofilms, a crystal violet staining protocol was used as a metric to quantify the number of cells bound to borosilicate glass. Strain NTL7(pZLQcelR) exhibited a significant decrease in crystal violet staining as compared to its parent (Figure 2.12), indicating that overexpressing celR negatively affects biofilm formation. The amount of bound crystal violet was increased in the Δcel strain, regardless of whether or not celR was overexpressed (Figure 2.12). This increase in biofilm production by the Δcel mutant suggests not only that *A. tumefaciens* does not require cellulose for attachment to glass surfaces, but also that the production of this polymer inhibits the process. This phenomenon has been noted in other studies, where strains of *A. tumefaciens* that overproduced cellulose appeared to form elevated biofilms on tomato roots, but the aggregates were easily dislodged and therefore represented unanchored masses of cells (158). Deleting celR resulted in increased crystal violet staining as compared to the wild-type parent (Figure 2.12), with the ΔcelR mutant binding to glass at the same level as the Δcel mutant of NTL7 (Figure 2.12).

### 2.5.8 celR overexpression severely attenuates virulence in *Agrobacterium tumefaciens*

*tumefaciens*. Pathogenic isolates of *A. tumefaciens* induce tumors on wounded plants, with tumor induction requiring attachment of the bacteria to the plant cells (147, 148). To examine the effects of altering celR expression or other putative DGCs on tumorigenesis, cultures of strains to be tested were inoculated onto wounded leaves of *Kalanchöe daigremontiana* and onto wounded tomato stems, and virulence was quantified as described in Materials and Methods. Overexpressing *atu0826, atu2228* or *atu4490* had no detectible effect on virulence on *Kalanchöe* leaves (Figure 2.13A). However, NTL7 overexpressing either celR or *atu1060* was strongly attenuated on both host plants.
(Figures 2.13A and B). Overexpressing celR or atu1060 in the cellulose-deficient background led to the same attenuated phenotype (Figure 2.13B), while the parent Δcel strain remained fully virulent (Figure 2.13B). These results suggest that cellulose production is not a contributing factor to the loss of tumorigenicity in the overexpressing strains. Interestingly, NTL7ΔcelR::Gm produced slightly larger tumors on tomato stems as compared to NTL7, although this difference was not statistically significant (Figure 2.13B). These results suggest that putative DGCs may play some role in tumor induction. However, this effect on virulence is not mediated through cellulose biosynthesis, and is not dependent on celR.

2.6 Discussion

2.6.1 CelR controls cellulose synthesis in A. tumefaciens. Our results clearly show that the putative diguanylate cyclase CelR regulates cellulose production in A. tumefaciens. Overexpressing the gene resulted in increased production of the exopolysaccharide, while deleting celR led to a substantial decrease in cellulose production (Figure 2.8). Complementation of the null mutant with a single copy of the gene expressed from its native promoter restored cellulose production to wild-type levels, further supporting the requirement of celR for inducing synthesis of the polymer (Figure 2.8).

Several lines of evidence support our hypothesis that CelR is an active c-di-GMP synthase. First, c-di-GMP stimulates synthesis of cellulose in cell extracts of A. tumefaciens (6). Coupled with the observation that CelA, the catalytic subunit of the
cellulose synthase, contains a PilZ domain, this result supports the notion that the nucleotide signal controls activity of the enzyme. This conclusion is, in turn, consistent with the role of c-di-GMP in regulating the activity of the cellulose synthase purified from *Gluconacetobacter xylinus* (74, 166). Second, Xu *et al.* (294) report that extracts of *E. coli* overexpressing CelR, which they called PleD, from *A. tumefaciens* contained a 46-fold greater amount of c-di-GMP as compared to extracts from cells in which the gene was not overexpressed. Third, we show that overexpressing CelR alters several phenotypes, most of which are not directly affected by the protein when expressed at normal levels. This observation is consistent with overproduction of a soluble and promiscuous intracellular signal molecule. However, definitive proof of its activity awaits further analysis of CelR.

*Agrobacterium tumefaciens* also synthesizes curdlan, another poly-glucose polymer (178, 249), and it is conceivable that production of this polysaccharide is impacted by c-di-GMP. Curdlan binds both aniline blue and Congo red (178), an observation that suggests that increased staining of colonies by Congo red is indicative of higher levels of glucose-based polymers in general, and is not specific to cellulose. Indeed, colonies of the curdlan-overproducing strain ATCC31749 bound both aniline blue and Congo red (Figure 2.6). However, overexpressing either celR or atu1060 in strain NTL7 only resulted in increased binding of Congo red, and not aniline blue (Figure 2.6), suggesting that neither protein stimulates curdlan production. Alignments of CelA, the cellulose synthase of *A. tumefaciens*, and the curdlan synthase, CrdS (Atu3056), show that while both share similar catalytic sites for polymer elongation, only CelA has the conserved PilZ c-di-GMP binding domain (data not shown; (7)). Moreover, CrdS does
not contain any other known c-di-GMP binding domains. Given that curdlan is a glucose-based polymer, we consider it likely that production of this polysaccharide accounts for at least some of the residual anthrone-positive material produced by the Δcel mutant (Figure 2.7). Additionally, these results demonstrate that of the two reagents, Congo red binding is the better in situ assay for cellulose production.

2.6.2 CelR, but not Atu1060, directly controls production of cellulose.

Overexpression of a second gene, \textit{atu1060}, also resulted in increased cellulose production. Only overexpression of \textit{atu1060} and \textit{celR}, and not the other three putative DGCs studied, affected these phenotypes. Atu1060 has a domain structure similar to that of CelR (Figure 2.3), which when combined with the similar phenotypes observed when either gene is overexpressed, suggests that these two potential synthases can crosstalk to their respective target pathways. However, while deleting \textit{celR} resulted in decreased levels of cellulose, deleting \textit{atu1060} had no such effect (Figure 2.8), suggesting that at native levels of expression \textit{celR}, but not \textit{atu1060}, is a regulator in the pathway. Consistent with this interpretation, \textit{celR} is conserved in all members of the Rhizobiaceae that produce cellulose, while \textit{atu1060} is found only in the genomes of biovar 1 agrobacteria.

Assuming that CelR is an active DGC, and that overexpressing \textit{celR}, but not three of the other potential DGCs studied, affects cellulose production suggests these enzymes or their signal product can be compartmentalized. Overexpression of \textit{atu0826} and \textit{atu2228}, while having no effect on Congo red binding, did result in greatly reduced colony sizes (Figure 2.4A). That overexpressing other putative DGCs affects different
phenotypes suggests that the role of a particular DGC may not impact processes outside of that specific system. Of the genes examined, only *atu1060* appears to crosstalk with *celR*. These observations suggest that signal compartmentalization, as well as some level of specificity, are held in common by the two proteins.

### 2.6.3 The impact of *celR* overexpression on other phenotypes suggests multiple processes are regulated by c-di-GMP in *A. tumefaciens*. Overexpressing *celR* affected phenotypes in addition to cellulose synthesis, including colony size, cell morphology, polar UPP production, rosette formation and virulence. The overexpression of either *celR* or *atu1060* in NTL7 resulted in greatly reduced colony size, similar to the effect of overexpressing *atu0826* and *atu2228* (Figure 2.4A). However, overexpression of *celR* or *atu1060* in NTL7Δcel resulted in colonies of a size comparable to those of either wild-type NTL7 or NTL7Δcel (Figure 2.5A). The Δcel mutant overexpressing *atu0826* or *atu2228* continued to grow as small colonies (data not shown). These observations suggest that the effects of *celR* and *atu1060* on the size of colonies is a result of cellulose production, and that *celR* and *atu1060* do not crosstalk to the cellular processes affected by *atu0826* and *atu2228*.

Overexpressing *celR* affected the morphology of wild-type NTL7, with cells forming branches and elongated rods. This effect was observed in the cellulose-deficient background, indicating that the effects on cell morphology are not due to alterations in production of cellulose. If CelR is an active DGC, it is likely that the signal produced by overexpressing the enzyme influences systems involved in controlling cell division. This conclusion is supported by the absence of such morphological alterations in the *celR*
indel mutant, and supports the hypothesis that in A. tumefaciens c-di-GMP is a critical intracellular signaling component in cell cycle regulation (124). However, as with several other phenotypes, the signal produced by overexpressing celR may be cross talking to some non-cognate system.

The influence of celR on rosetting, a unipolar attachment phenomenon first described by Braun and Elrod (22), is interesting. Overexpressing the gene in both the wild-type and the Δcel mutant resulted in increased frequency of rosettes, suggesting that celR is associated with this patterning phenomenon. However, the celR mutant demonstrated wild-type levels of rosetting, ruling out a role for this protein in the process. The overexpression of celR also increased UPP production in both backgrounds, a phenotype seen in other studies (124, 294). However, as with rosetting, deleting celR did not negatively impact the number of cells expressing UPP as compared to the wild-type (Figure 2.10), suggesting that celR is not the regulating protein.

Attenuation of virulence associated with overexpressing celR or atu1060 also is not due to overproduction of cellulose. Overexpressing either celR or atu1060 in NTL7Δcel resulted in the same attenuated phenotype as observed in wild-type NTL7 (Figure 2.13B). Furthermore, overexpression of the two putative DGCs that affected colony size, atu0826 and atu2228, had no impact on virulence (Figure 2.13A). Deleting celR did not influence virulence (Figure 2.13B), suggesting that this protein does not contribute to regulating this process. This result is consistent with the observation that celT mutants of A. tumefaciens are fully virulent (158), but these results do suggest that an
unknown DGC controls some process important for tumorigenesis. The relevant protein and its target remain to be identified.

**2.6.4 Altering the expression of celR affects biofilm formation and attachment to plants, exclusive of cellulose production.** Overexpressing celR in wild-type NTL7 dramatically decreased biofilm formation (Figure 2.12) and attachment to leaf surfaces (Figure 2.11). This result is in contrast to the results reported by Xu et al. (294), and may be due to differences in the methodology of the experiments. NTL7Δcel overexpressing celR yielded wild-type levels of crystal violet staining (Figure 2.12), suggesting that the failure to form biofilms on glass is due to overproduction of cellulose in the wild-type strain. This inhibition of biofilm formation mirrors the effects reported with two other cellulose-overproducing mutants (158). The overproduction of cellulose may affect biofilm formation by increasing cell-cell aggregation, which could inhibit strong interactions between the cells and other surfaces. A similar effect likely occurs during the attachment of cells of NTL7Δcel overexpressing celR to plant tissue, with the cells aggregating through some other means rather than through cellulose production.

Deleting celR resulted in increased polar attachment of individual bacteria to plant tissue (Figure 2.11), an unexpected result given the loss of cellulose production and lack of effect on the UPP. This effect on attachment is similar to the increase in single cell binding to root hairs reported for the celR2 mutant of R. leguminosarum (11). Further, strains of Rhizobium produce aggregate caps at root hair tips, with the formation of these caps dependent on cellulose (239). Based on this evidence, the alteration in cell attachment exhibited by the celR indel mutant of A. tumefaciens most probably is due to
an inability to aggregate as efficiently as wild-type cells, resulting in increased single cell attachment to the plant tissue.

2.6.5 CelR orthologs within the alphaproteobacteria have diverged to regulate separate processes. Interestingly, in those bacteria where c-di-GMP contributes to regulating cellulose biosynthesis, the cellulose synthase complex contains a subunit with the PilZ domain (7, 216). In both *Gluconacetobacter xylinus* and *Salmonella enterica* serovar Typhimurium, BcsA, the PilZ-containing ortholog of CelA from *A. tumefaciens*, directly binds c-di-GMP, and this binding activates the enzyme (74, 197, 216). Additionally, the genomes of a number of other bacterial species encode a putative cellulose synthase with a PilZ domain orthologous to CelA (7). Conservation of this domain suggests that regulation of cellulose synthesis by c-di-GMP is a common if not universal phenomenon in the Proteobacteriaceae.

The DGC responsible for regulating cellulose production has been identified in a number of species. In *Salmonella* and *Escherichia* species, two orthologous genes, *adrA* and *yaiC*, control cellulose biosynthesis, resulting in the rdar morphotype (207, 307). In *Gluconacetobacter xylinus*, a member of the Acetobacteraceae of the alphaproteobacteria, three DGCs, annotated as Dgc, are involved in controlling cellulose production (257). Moreover, other families of the alphaproteobacteria, including the Rhodobacteraceae, encode a *celA* gene, and contain the *dgc* operons (Table 2.5). These three enzymes, which are related to each other, are not orthologous to AdrA/YaiC. In both *A. tumefaciens* and *R. leguminosarum*, the probable DGCs CelR and its ortholog CelR2 regulate cellulose production (11). However, apart from shared GGDEF domains,
CelR and its orthologs are structurally distinct from the Dgc proteins of G. xylinus, and AdrA/YaiC in the Enterobacteraceae. These observations suggest that distinct c-di-GMP-dependent signaling pathways utilizing different DGCs have evolved within the Proteobacteriaceae as a whole, and even among the alphaproteobacteria.

Putative DGCs with a domain structure essentially identical to CelR are found throughout many families of the alphaproteobacteria (NCBI), and have been identified in at least two members of the gammaproteobacteria, *Pseudomonas aeruginosa* (57, 97) and *Pseudomonas fluorescens* (152, 245). Within the alphaproteobacteria, these CelR orthologs are usually organized as the second gene of a two-gene operon with the first encoding a small CheY-like receiver protein, generally annotated as *divK* (Table 2.5, Figure 2.14). Studies of *divK* in alphaproteobacteria, including *Caulobacter*, *Brucella* and *Agrobacterium*, link this gene to the polar localization of cell division proteins (4, 89, 103, 124). These observations suggest that *divK* is a conserved component of cell cycle regulation among a number of the alphaproteobacteria.

While the organization of *divK* and *celR* as an operon is conserved in many families of the alphaproteobacteria, the GGDEF-containing protein does not always regulate cellulose synthesis. In the Caulobacteraceae, for example, the *celR* ortholog, named *pleD*, plays a role in the production and polar localization of the holdfast stalk during differentiation (4, 94). Interestingly, this group of bacteria apparently does not produce cellulose; genome analyses indicate that the Caulobacteraceae, as well as several other families of the alphaproteobacteria that contain the *divK/celR(pleD)* gene set do not encode a *celA* gene or any other genes associated with cellulose biosynthesis (Table 2.5; 56)
(153)). However, the genomes of other taxa within the alphaproteobacteria, including families as diverse as the Rhizobiaceae, Bradyrhizobiaceae, Pelagibacteriaceae and Phyllobacteriaceae, contain both the divK/celR operon and a cel system that includes a PilZ-containing CelA subunit (Table 2.5). Furthermore, celR is not a component of cell cycle regulation in A. tumefaciens (124). Our results support the notion that, at least among the Rhizobiaceae, the celR/celR2 gene product is dedicated to controlling polymer production and does not participate directly in regulating cell cycle events or polar localization. Taken together, these observations suggest that the divK/celR(pleD) regulatory system has evolved along at least two independent tracts; controlling polar localization and adhesion, as in the case of Caulobacteraceae, and regulating cellulose production, as seen in the Rhizobiaceae. It is possible that this separation occurred with the acquisition of cellulose biosynthesis among the Rhizobiaceae.

2.6.6 Cellulose biosynthesis in A. tumefaciens is regulated at several levels.

Our work as well as previous studies (158) suggest that in A. tumefaciens, cellulose production is regulated at least two levels. CelR contains a pair of CheY-like domains (Figure 2.3), suggesting that the activity of this protein is controlled by some unknown upstream signal. In addition, mutations in two other genes in A. tumefaciens, celG and celI, result in overproduction of the polymer (158). While CelG has no predictable structure, CelI is a putative member of the MarR/ArsR family of transcriptional regulators, suggesting that production of cellulose is also regulated at the level of transcription. These two genes, with celG located in the cel gene cluster and celI located elsewhere on the chromosome, are conserved within other members of the Rhizobiaceae, including R. leguminosarum. Based on this evidence, cellulose production in the
Rhizobiaceae may be regulated by transcriptional control of the cel cluster, possibly through celI, and by modulating the rate of cellulose synthesis in the cell through allosteric regulation of the synthase.

The impact of celR on cellulose production in A. tumefaciens suggests that there is a signaling cascade involved in regulating synthesis of the polymer. One of the CheY-like domains in CelR contains a conserved aspartate residue, which in PleD of C. crescentus is a target for phosphorylation (190). This observation suggests that CelR can be activated by phosphorylation by some unidentified kinase. The identity of this kinase and how its activity is regulated remains to be determined. Continuing to examine the components of the pathway regulating the function of CelR in cellulose biosynthesis may help us to better understand the interaction of A. tumefaciens and the host plant.
### 2.7 Tables and Figures

**Table 2.1. Strains and plasmids used in this studya.**

<table>
<thead>
<tr>
<th>Strain or Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
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<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44ΔlacU169(ϕ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>(219)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S17-1 λpir</td>
<td>Pro⁺ Res⁺ Mod⁺ recA; integrated RP4· Tc⁺::Mu-Kan::Tn7, Mob⁺;Smr λ::pir</td>
<td>(62)</td>
</tr>
<tr>
<td><strong>A. tumefaciens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTL4</td>
<td>A derivative of C58, ΔtetRS, lacks pTiC58</td>
<td>(150)</td>
</tr>
<tr>
<td>NTL7</td>
<td>A derivative NTL4 with pTiC58 reintroduced</td>
<td>(150)</td>
</tr>
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<td>NTL7 with celC and celDE deleted, Tc⁺</td>
<td>Shengchang Su, This Study</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>ATCC31749</td>
<td>Curdlan-overproducing strain</td>
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<td>NTL7 celR::Gm⁺</td>
<td>This Study</td>
</tr>
<tr>
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<td>This Study</td>
</tr>
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<td>Cloning vector, Ap⁺</td>
<td>Invitrogen</td>
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<td>celR gene cloned into pUC18</td>
<td>This Study</td>
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<td>atu1060 gene cloned into pUC18</td>
<td>This Study</td>
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<td>atu0826 gene cloned into pUC18</td>
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<tr>
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Table 2.1. (cont.)

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<td>pUCcelRdel</td>
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<td></td>
</tr>
<tr>
<td>pUCatu1060region</td>
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<td></td>
</tr>
<tr>
<td>pUCpr</td>
</tr>
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<td>pUCprcelR</td>
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<td>pMGm</td>
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<tr>
<td>pZLQ</td>
</tr>
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<td>pZLQcelR</td>
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<td>pZLQatu0826</td>
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<tr>
<td>pZLQatu2228</td>
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<td>pZLQatu4490</td>
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Table 2.1. (cont.)

<table>
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<tr>
<th>Plasmid</th>
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<tr>
<td>pUC18mini-Tn7T-Km</td>
<td>Tn7 carrier vector containing Km cassette, Ap&lt;sup&gt;f&lt;/sup&gt;, Km&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>pTNS2</td>
<td>Tn7 helper plasmid encoding the TnsABC+D specific transposition, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pUCTn7-Km-prcelR</td>
<td>prcelR fragment cloned into BamHI site of pUC18mini-Tn7T-Km</td>
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<tr>
<td>pRK415</td>
<td>InP1α broad host range cloning vector, Tc&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td>pRKatu1060region</td>
<td>The atu1060 region inserted into pRK415</td>
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<tr>
<td>pRKatu1060kan</td>
<td>Allelic replacement of atu1060 with a kanamycin cassette on pRKatu1060region</td>
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<td>pWM91</td>
<td>λpir-dependent cloning vector, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>celRdel fragment cloned into BamHI site of pWM91</td>
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<tr>
<td>pWMatu1060kan</td>
<td>atu1060kan fragment cloned into BamHI site of pWM91</td>
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<tr>
<td>pKD46</td>
<td>Lambda Red recombinase helper plasmid, Ap&lt;sup&gt;f&lt;/sup&gt;</td>
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<td>pKD4</td>
<td>Template plasmid of kanamycin cassette for chromosomal exchange, Ap&lt;sup&gt;f&lt;/sup&gt;, Km&lt;sup&gt;f&lt;/sup&gt;</td>
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This Study
<table>
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<tr>
<th>Vector</th>
<th>Description</th>
<th>Resistance(s)</th>
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<td>pBBR1MCS-3</td>
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<td>pSR47s</td>
<td>Suicide vector, $\text{Ap}^r$</td>
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<td>(169)</td>
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</table>

*aAbbreviations: $\text{Ap}^r$, resistance to ampicillin; $\text{Cm}^r$, resistance to chloramphenicol; $\text{Gm}$, resistance to gentamicin; $\text{Km}^r$, resistance to kanamycin; $\text{Sm}^r$, resistance to streptomycin; $\text{Tc}^r$, resistance to tetracycline.*
Table 2.2. The increase in anthrone-reacting material from \textit{atu1297} overexpression is due to cellulose.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Extractable Anthrone-Positive Material$^a$</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-Cellulase</td>
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<tr>
<td>NTL7</td>
<td>-</td>
<td>782±76$^b$</td>
</tr>
<tr>
<td>NTL7</td>
<td>pZLQatu1297</td>
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<tr>
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<tr>
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<td>pZLQatu1297</td>
<td>620±68</td>
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</table>

$^a$ Expressed as µg/mg of protein.

$^b$ Average values of four experiments, with ± number indicating standard error range.
Table 2.3. Overexpressing CelR affects lectin binding and rosetting.

<table>
<thead>
<tr>
<th></th>
<th>Total Cells</th>
<th>% Labeled&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Rosette&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>1</td>
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<sup>a</sup> Percentage of cells examined that displayed polar lectin binding.

<sup>b</sup> Percentage of cells examined that were observed in rosettes.

<sup>c</sup> p ≤ 0.005 compared to NTL7 using Chi-squared analysis.
Table 2.4. Deleting *celR* affects attachment of the bacteria to *Arabidopsis* leaf surfaces\(^a\).

<table>
<thead>
<tr>
<th>No. of cells attached(^b)</th>
<th>No. of polar-attached cells(^c)</th>
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<tbody>
<tr>
<td><strong>NTL7</strong></td>
<td>80±19</td>
</tr>
<tr>
<td><strong>NTL7ΔcelR::Gm</strong></td>
<td>120±26*</td>
</tr>
</tbody>
</table>

\(^a\)Samples were prepared and examined by SEM by Dr. Lois Banta, Williams College, MA. Attached cells were enumerated from the micrographs by D. M. Barnhart.

\(^b\)Average number of cells per field from 10 (NTL7) or 6 (NTL7ΔcelR::Gm) randomly-chosen fields from 4 leaves inoculated with either strain. Second value represents standard error.

\(^c\)Average number of cells from one randomly-chosen field from each of 3 independent leaves incubated with either strain. Standard error is represented with the second value.

*Samples statistically significant (p < 0.05) from the parent strain using Student t-test with one-sided distribution.
Table 2.5. Genes present in members of the alphaproteobacteria.

<table>
<thead>
<tr>
<th>Family</th>
<th>Contains the divK/celR operon</th>
<th>Contains CelA subunit with a PilZ domain</th>
<th>Uses CelR to regulate</th>
<th>Cell differentiation</th>
<th>Cellulose synthesis</th>
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<td>Y</td>
<td>U(^d)</td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Bradyrhizobiaceae</td>
<td>Y(^b)</td>
<td>Y(^c)</td>
<td>U</td>
<td>U</td>
<td>U</td>
</tr>
<tr>
<td>Brucellaceae</td>
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<td>U</td>
<td></td>
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<td>Rhizobiaceae</td>
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<td>Rhodobacteriaceae</td>
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\(^a\) No members of this family contain these genes or enzymes.

\(^b\) Several members in this family contain these genes or enzymes.

\(^c\) Only *Bradyrhizobium* species contain celA; all other members of the Bradyrhizobiaceae lack the gene.

\(^d\) U: No available literature.
Figure 2.1. Organization of the cel locus of *A. tumefaciens* strain C58 and construction of NTL7Δcel. The figure represents the gene organization of a 10.5 kb segment of chromosome 2 of *A. tumefaciens* strain C58. Arrows indicate each gene and its orientation. The region within the dotted lines represents the section of the cel locus that was replaced with tetA.
Figure 2.2. Organization of the \textit{divK-celR} locus of \textit{A. tumefaciens} strain C58 and construction of the NTL7\textDelta celR::Gm mutant. The gene organization of a 3.6 kb segment of chromosome 1 of \textit{A. tumefaciens} strain C58 that encodes the \textit{divK-celR} operon. Arrows indicate each gene and its orientation. The fragments flanking the gentamicin cassette (Gm\textsuperscript{r}) represent the organization of the insert in pUCcelRdel used for recombination into the chromosome of strain NTL7. The 300 bp fragment containing the promoter region of \textit{divK-celR} is fused directly to the \textit{celR} gene in pUCprcelR.
Figure 2.3. Domain structure of GGDEF-containing proteins examined in this study.

The domains of the five studied proteins as derived by analysis of amino acid sequences in the NCBI databases. CheY: CheY-like receiver domain, GGDEF: diguanylate cyclase domain, EAL: phosphodiesterase A domain, CBS: cystathionine beta synthase domain, HAMP: linker domain of receptor histidine kinase and methyl-accepting proteins, PAS: oxygen-sensing heme-bound domain, TAT: TAT secretion system signal, Chase4: low molecular weight ligand-binding domain. The number of amino acids in each protein is shown at the end of each protein structure.
Figure 2.4. Overexpressing GGDEF domain proteins results in varied phenotypes on solid and liquid media. Strain NTL7 with constructs expressing genes coding for GGDEF domain proteins were grown for two days at 28°C A) on ABM plates containing Congo red, and B) in MG/L, with shaking. Both media contained the appropriate antibiotics.
Figure 2.5. Overexpressing atu1297 and atu1060 does not affect exopolysaccharide production in NTL7Δcel. Strains NTL7 and NTL7Δcel, with or without constructs overexpressing either atu1297 or atu1060, were grown for two days at 28°C A) on ABM plates containing Congo red, and B) in MG/L, with shaking. Both media contained the appropriate antibiotics.
Figure 2.6. Overexpressing *atu1060* and *atu1297* does not affect curdlan production.

Strains of *A. tumefaciens* were grown for two days at 28°C on ABM plates containing (A and B) aniline blue or (C) Congo red.
Figure 2.7. Overexpressing *atu1297* and *atu1060* results in increased production of anthrone-reacting material. Strains NTL7 and NTL7Δ*cel* with constructs overexpressing either *atu1297* or *atu1060* were grown in MG/L with the appropriate antibiotics for two days at 28°C with shaking. The cells were harvested and assessed for production of anthrone-reacting material as described in the Materials and Methods. Each experiment was repeated four times. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 2.8. The *atu1297* gene, but not the *atu1060* gene, positively regulates cellulose production. Derivatives of NTL7 with mutations in *atu1297* or *atu1060* were grown A) on ABM plates containing Congo red for two days at 28°C, and B) in MG/L, harvested and assayed for extractable cellulose as described in the Materials and Methods. The total amount of anthrone-reactive material from each strain was normalized by comparison to the amount of material extracted from NTL7Δcel, which was set to zero. Each strain was tested four times and the data was averaged, with the error bars representing the standard error of the experiments.
Figure 2.9. Overexpression of *celR* affects aggregation and rosetting. Cultures of NTL7 or NTL7Δ*cel* and their derivatives were grown in MG/L and samples were viewed by DIC microscopy as described in Materials and Methods. 

A) NTL7. B) NTL7(pZLQ*celR*). 

C) NTL7Δ*cel*(pZLQ*celR*). D) NTL7Δ*cel*(pZLQ*celR*) in rosettes. Scale bars represent 5 µm.
Figure 2.10. Cells overexpressing celR display increased polar binding of lectins.

Cells grown in MG/L with the appropriate antibiotics were collected, incubated with WGA-Alexafluor 633 and observed by fluorescence microscopy as described in Materials and Methods.  A) NTL7.  B) NTL7(pZLQcelR).  C) NTL7Δcel(pZLQcelR).  D) NTL7ΔcelR::Gm. Circled cells represent examples of polar-bound lectin.
Figure 2.11. Cells altered in the expression of \textit{celR} are affected in plant attachment.

Strains were cultured and inoculated onto \textit{Arabidopsis thaliana} leaves, and the interactions were visualized by SEM as described in Materials and Methods by Dr. Lois Banta. \textbf{A)} NTL7. \textbf{B)} NTL7\textDelta celR::Gm. \textbf{C)} NTL7\textDelta cel. \textbf{D)} NTL7\textDelta cel(pZLQcelR). \textbf{E} and \textbf{F)} Higher magnification images of NTL7\textDelta cel(pZLQcelR).
Figure 2.12. Overexpressing celR decreases biofilm formation on glass surfaces.

Cultures were grown in MG/L with the appropriate antibiotics in borosilicate tubes and assayed for adherence to the glass surface by crystal violet staining as described in Materials and Methods. Each strain was grown in triplicate, and each experiment was repeated three times. The values represent the average of the nine total samples, with error bars representing the standard error of the experiments.
Figure 2.13. Overexpression of celR and atu1060 severely attenuates virulence. A.

Derivatives of NTL7 containing constructs overexpressing one of the GGDEF-containing proteins were inoculated onto leaves of Kalanchoë daigremontiana as described in Materials and Methods. B. NTL7 altered in expression of either celR or atu1060 were inoculated onto tomato stems and assessed for tumor induction as described in Materials and Methods. Each experiment was repeated three times, with five samples per experiment. The data indicate the averages of samples for each strain tested, with error bars representing standard error.
Figure 2.14. Comparison of the divK/celR(pleD) region in the genomes of three members of the α-proteobacteria. The figure represents the gene organization of the divK-celR operons in A. tumefaciens C58 (At) and R. leguminosarum bv. viciae 3841 (Rl), and the divK-pleD operon in C. crescentus NA1000 (Cc), as well as the surrounding genes and their annotations. Identical shading indicates orthologous genes. The scale bar indicates 1 kb.
Chapter 3

A signaling pathway involving the diguanylate cyclase CelR and the response regulator DivK controls cellulose synthesis in Agrobacterium tumefaciens

3.1 Notes and Acknowledgements

This chapter is adapted from the Journal of Bacteriology article, entitled “A signaling pathway involving the diguanylate cyclase CelR and the response regulator DivK controls cellulose synthesis in Agrobacterium tumefaciens”, March 2014, Volume 196, Pages 1257-1274, with authors D. M. Barnhart, S. Su, and S. K. Farrand. Additionally, I would like to thank Dr. Peter Orlean and Dr. Lois Banta for their guidance in this chapter. Of note, development of the strain NTL7ΔcelA::lacZ was done by Dr. Shengchang Su, University of Illinois.

3.2 Summary

The production of cellulose fibrils is involved in the attachment of Agrobacterium tumefaciens to its plant host. Consistent with previous studies, we reported recently that a putative diguanylate cyclase, celR, is required for synthesis of this polymer in A. tumefaciens. In this study, the effects of celR and other components of the regulatory pathway of cellulose production were explored. Mutational analysis of celR demonstrated that the cyclase requires the catalytic GGEFF motif, as well as the conserved aspartate residue of a CheY-like receiver domain, for stimulating cellulose production. Moreover, a site-directed mutation within the PilZ domain of CelA, the catalytic subunit of the cellulose synthase complex, greatly reduced cellulose production. In addition, deletion of
divK, the first gene of the divK-celR operon, also reduced cellulose production. This requirement for divK was alleviated by expression of a constitutively active form of CelR, suggesting that DivK acts upstream of CelR activation. Based on bacterial two-hybrid assays, CelR homodimerizes but does not interact with DivK. The mutation in divK additionally affected cell morphology, and this effect was complementable by a wild-type copy of the gene, but not by the constitutively active allele of celR. These results support the hypothesis that CelR is a bona-fide c-di-GMP synthase and that the nucleotide signal produced by this enzyme activates CelA via the PilZ domain. Our studies also suggest that the DivK/CelR signaling pathway in Agrobacterium regulates cellulose production independent of cell cycle checkpoint systems that are controlled by divK.

3.3 Introduction

The interaction of the plant pathogen Agrobacterium tumefaciens with its host is dependent on attachment (147, 148), which requires the production of anchoring factors. One component of the attachment matrix produced by the bacterium is cellulose, a \( \beta1,4 \)-linked glucose polymer. The cellulose fibrils serve to anchor the bacteria to each other as well as to stabilize the interaction of the bacteria to the plant cells (157). Mutants deficient in the production of cellulose bind less tightly to plant cell surfaces (156, 160), and, in addition, do not efficiently establish biofilms (158).

The production of cellulose by A. tumefaciens strain C58 is encoded by two closely linked operons, celABC and celDE, located on the linear chromosome (85, 162, 292). Two of the genes, celA and celB, encode the heterodimeric cellulose synthase
complex (161). The CelD and CelE proteins likely are responsible for the synthesis of UDP-glucose, a process that involves a lipid-glucose intermediate (161). The CelA-CelB complex then catalyzes the addition of the activated glucose to the extending cellulose fiber (223, 307). Based on evidence in other bacteria, the cellulose fibrils are extruded into the extracellular milieu from the synthase complex, perhaps by an exporter encoded by CelC, imbedded within the membrane of the cells (173, 287).

Cellulose synthesis in *A. tumefaciens* resembles production of this polymer in the model bacterium *Gluconoacetobacter xylinus* (291). In this system, the synthase complex, coded for by *bcsA* and *bcsB*, responds to the secondary signal molecule cyclic-di-guanosine-monophosphate (c-di-GMP) (208, 211), with the BcsA subunit recognizing the nucleotide via a PilZ domain, one of several known c-di-GMP-binding domains (7, 216, 283). The sequences of CelA and BcsA are strongly conserved, including the C-terminal PilZ domain. Consistent with this conserved signal binding domain, Amikam and Benziman (6) reported that in cell-free extracts of *A. tumefaciens*, addition of c-di-GMP substantially increased the rate of cellulose synthesis. These observations strongly suggest that cellulose synthesis in *A. tumefaciens* is regulated in part by c-di-GMP.

In previous studies, we reported that production of cellulose in *A. tumefaciens* is stimulated by a putative diguanylate cyclase (DGC) we named CelR (14). Overexpression of *celR* resulted in increased production of the polymer, while deleting the gene greatly reduced the amount of cellulose extractable from the cells (14). These results suggest that *celR* is involved in activating synthesis of cellulose. The *celR* gene is part of the *divK-celR* operon, which is strongly conserved in many alphaproteobacteria (Figure S1, (14)). Interestingly, in *Caulobacter crescentus*, this operon, annotated as *divK*
and pleD, controls events at the poles of the cell. PleD, the ortholog of CelR, is involved in regulating the formation of the holdfast stalk, while DivK localizes cell cycle regulators, including factors for activating PleD, to specific poles of the cell during division (4, 93, 94, 136, 268). The divK gene also controls polar localization during the cell cycle of Brucella abortus (89), and in Agrobacterium, divK functions in a phosphorelay cascade that regulates cell division (124). These observations suggest that the divK-celR(pleD) operon and its component genes may play different roles among the diverse species of the alphaproteobacteria.

While we have demonstrated that celR influences cellulose production in A. tumefaciens, the mechanism by which it does so is still unknown. Based on biochemical and mutational analysis, PleD of C. crescentus has diguanylate cyclase activity (4, 188, 190). However there is limited evidence that CelR, while containing the conserved c-di-GMP synthesis motif, is a functional DGC (14, 124). There are several examples of proteins with GGDEF domains that do not exhibit c-di-GMP synthesis activity (reviewed in (205, 243)). It is conceivable, then, that celR stimulates cellulose synthesis by some alternate mechanism.

In this study, we examined the effects of targeted mutations in both divK and celR on cellulose synthesis in A. tumefaciens. Our results indicate that CelR activity requires an intact GGEEF motif, as well as a conserved aspartate residue in the first of two CheY domains of the protein. In turn, a substitution mutation in a residue associated with c-di-GMP binding in the PilZ domain of CelA greatly reduces the amount of cellulose synthesized by the mutant. We also show that stimulation of cellulose biosynthesis by CelR is dependent on DivK. However, divK also influences cell division in A.
tumefaciens, and this influence is independent of celR. Our results are consistent with the notion that divK continues to regulate cell division in A. tumefaciens, as in other alphaproteobacteria, while the divK-celR operon has diverged to also regulate cellulose production among the Rhizobiaceae.

3.4 Materials and Methods

3.4.1 Strains, culture and growth conditions. The bacteria used in this study are listed in Table 3.1. Strains of Escherichia coli were grown on Luria-Bertani (LB, Invitrogen) agar plates with appropriate antibiotics at 37°C. Strains of A. tumefaciens were grown on nutrient agar (NA, Fisher) or on plates of AB minimal medium (41) supplemented with 0.2% mannitol (ABM) with appropriate antibiotics at 28°C. Cultures of E. coli were grown in LB broth with the required antibiotics at 37°C with shaking. Cultures of A. tumefaciens were grown in MG/L medium (31) with appropriate antibiotics at 28°C with shaking. Antibiotics used include ampicillin (100 µg/ml for E. coli), carbenicillin (50 µg/ml for A. tumefaciens), kanamycin (50 µg/ml for E. coli and A. tumefaciens), gentamicin (50 µg/ml for E. coli, 25 µg/ml for A. tumefaciens), and tetracycline (10 µg/ml for both E. coli and A. tumefaciens). When necessary, Congo red (50 µg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG; 400 mM) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 80 µg/ml) was added to plates for phenotype assessment.

3.4.2 Strain construction. Primers used for PCR amplification are listed on Table 3.2.
Construction of overexpression strains: Genomic DNA was prepared from an overnight culture of *A. tumefaciens* NTL7 as described previously (84). To create the overexpression plasmid pKK38celR, the *celR* gene was amplified by PCR using *Pfu* polymerase (Stratagene) and the primers *celR*-fnco and *celR*-r. The amplicon was digested with *BamHI* and ligated into *BamHI*-digested pUC18. The resulting ligation products were introduced into DH5α by CaCl₂ transformation with selection on LB plates containing ampicillin. The plasmids were purified, and the resulting fragment was ligated into *NcoI-* and *BamHI*-digested pKK38ASH (Table 3.1) and transformed into DH5α. After selecting for tetracycline resistance, the plasmid was isolated, analyzed, and the correct construct was electroporated into the appropriate strains of *A. tumefaciens*. To express *celA* from a controlled promoter, the gene was amplified by PCR using *Pfu* polymerase and the primers *celA*-f and *celA*-r. The PCR product was digested with *BamHI*, ligated into *BamHI*-digested pUC18, and the resulting ligation products were transformed into DH5α. Colonies resistant to ampicillin were selected, the plasmids purified and digested with *Ndel* and *BamHI*. The resulting fragment of *celA* was ligated into the expression vector pSRK-Gm (Table 3.1) and transformed into DH5α. After selecting for gentamicin resistance, the plasmid was isolated, analyzed, and the correct construct was electroporated into the appropriate strains of *A. tumefaciens*.

Production of Tn7 insertion vectors. A 400 bp segment containing the promoter region of the *divK-celR* operon along with additional DNA encoding either *divK* or *divK-celR* was amplified using *Pfu* polymerase and the primers *prdivKcelR*-f and *divK*-r, or *prdivKcelR*-f and *celR*-r. The amplicons were digested and cloned into *BamHI*-digested pUC18. The resulting ligation products were transformed into DH5α, transformants were
isolated on LB plates containing ampicillin, and the recombinant plasmids were purified and confirmed by sequence analysis. The correct clones were digested with BamHI, and the fragments were ligated into pUC18-miniTn7t-Gm to form pUCTn7-prdivK and pUCTn7-prdivKcelR. The resulting plasmids were transformed into DH5α, selected for resistance to ampicillin and gentamicin, purified and tested for the appropriate insertion by restriction digest analysis.

Site-directed mutagenesis: The target fragments cloned into either pUC18, pUC-miniTn7t-Gm/Km, or pSRK-Gm were amplified by PCR using *Pfu* polymerase with primers containing point mutations at the target residue (See Table 3.2). The resulting amplified plasmids were digested with *DpnI* to remove the original template DNA, and the remaining plasmids were transformed into DH5α. After selecting for resistance to the appropriate antibiotic, the plasmids were isolated and the mutations were confirmed by sequence analysis. The altered plasmids were introduced into the appropriate strains by electroporation or by Tn7 insertion, as described below. Fragments containing the correct mutation on pUC18 were recovered by digesting with the appropriate restriction enzymes, and then ligating into either pZLQ (Table 3.1) or pKK38ASH.

Disruption of the chromosomal celA gene. A nonpolar deletion of *celA* (*atu3309*) was constructed as follows by Dr. Shengchang Su. A 500 bp internal fragment of *celA* was amplified by PCR from genomic DNA of strain NTL7 using *Pfu* DNA polymerase and primers *celA*/Eco and *celA*/Xb. The resulting amplicon was digested with *Eco*RI and *Xba*I and cloned between the corresponding sites on pVIK112 (111). The resulting ligation products were transformed into S17-1λ*pir* with selection for resistance to kanamycin. The resulting plasmid, pVIKcelA, was identified and transformed into NTL7
by electroporation. NTL7 carrying the disruption of celA resulting from a single
crossover event was selected by plating on media containing the appropriate antibiotics.
Insertional disruption of celA was verified by PCR using additional primers located
further upstream and downstream from the original fragments. The resulting mutant
creates a celA::lacZ transcriptional fusion.

Deleting celR by allelic exchange in the celA mutant. The celR gene was removed
from NTL7ΔcelA::lacZ using the procedure described in Barnhart et al. (14).

Indel mutation of divK: The divK gene was replaced with a kanamycin resistance
cassette using a protocol modified from that of Datsenko and Wanner (61). Briefly, a set
of primers, divKfRT-f and divKfRT-r was used to amplify the kanamycin cassette from
pKD4 (Table 3.1) by PCR, and the product was treated with DpnI to blunt the ends.
Additionally, a 3.9 kb fragment containing divK was amplified from NTL7 genomic
DNA using the primers divKregion-f and divKregion-r. The fragment was digested with
KpnI and cloned into pRK415 (Table 3.1), creating the construct pRKdivKregion. The
construct was introduced into DH5α by CaCl₂ transformation, and the plasmid was
purified and confirmed by restriction analysis and sequencing. The PCR-generated
kanamycin fragment was electroporated into an E. coli strain harboring both the red
recombinase plasmid pKD46 (Table 3.1) and pRKdivKregion, transformants were
selected for resistance to kanamycin, and plasmids were purified and examined for
replacement of divK on pRK415 by restriction analysis and PCR. The correct plasmids
containing the replaced gene were digested with KpnI, the modified divK gene was
cloned into the pir-dependent vector pWM91 (Table 3.1), producing the construct
pWMdivKkan, and this plasmid was transformed into S17-1/λpir. Successful constructs
were selected for resistance to ampicillin and kanamycin, and a verified plasmid was electroporated into *A. tumefaciens*. Initial transformants were selected for resistance to kanamycin and sucrose, followed by screening for sensitivity to carbenicillin. Potential mutants were confirmed using PCR and Southern analysis.

Allelic exchange of *divK* and *celR*: The *divK* and *celR* operon was deleted using a different modification to the protocol from Datsenko and Wanner (61). A set of primers, *divK* and *celR*, were used to amplify the kanamycin cassette from pKD4 by PCR, and the product was treated with *DpnI* to blunt the ends. Additionally, a 4.2 kb fragment containing *divK* and *celR* was amplified from NTL7 genomic DNA using the primers *divK* and *celR*. The fragment was digested with *KpnI* and cloned into pRK415, creating the construct pRK*divK* and *celR*. The construct was introduced into DH5α by CaCl2 transformation, and the plasmid was purified and verified by restriction digestion and sequencing. The fragment containing the kanamycin cassette was electroporated into an *E. coli* strain harboring both the *red* recombinase plasmid pKD46 and pRK*divK* and *celR*, transformants were selected for resistance to kanamycin, and plasmids were purified and examined for replacement of the operon with the kanamycin cassette by restriction digest and PCR. The correct plasmid containing the replaced operon, renamed pRK*divK* and *celR* kan, was electroporated into an *E. coli* strain containing the plasmid pCP20, which expresses the *flp* recombinase gene. A construct in which the kanamycin cassette was deleted was identified by screening for resistance to tetracycline but sensitivity to kanamycin, and the plasmids were purified and examined for loss of the kanamycin cassette by restriction digest. The correct plasmids were digested with *KpnI*, the modified region was cloned into pWM91, producing the construct pWM*divK* and *celR* del,
and this plasmid was transformed into S17-1/λpir. Successful constructs were selected for resistance to ampicillin, and a verified plasmid was electroporated into A. tumefaciens. Initial transformants were selected for resistance to ampicillin for single recombination events, followed by growth in MG/L and screening for resistance to sucrose and sensitivity to carbenicillin. Potential marker-exchange mutants were confirmed using PCR and Southern analysis.

_Complementation of NTL7ΔcelR::Gm, NTL7ΔdivK::Km and NTL7ΔdivKcelR._ To complement disruptions of _divK_ and _celR_, appropriate Tn7 constructs, as well as the transposase plasmid pTNS2 (Table 3.1), were electroporated into the target mutant at a 1:2 ratio of Tn7 vector to pTNS2. The transformed cells were selected for resistance to gentamicin, and mini-Tn7 integrants were confirmed by PCR analysis using the primers Tn7insert-L and either pTn7-L, _divK-r_ or _celR-r_.

_Two-hybrid constructs:_ Both _divK_ and _celR_ genes were amplified from genomic DNA of strain NTL7 using Pfu polymerase and primers specific for insertion into two-hybrid expression vectors. The appropriate alleles of _divK_ and _celR_ were amplified from their recombinant plasmids using Pfu polymerase and the above primers. Fragments of _divK_ were digested with _XhoI_ and _KpnI_, while fragments of _celR_ were digested with _BamHI_ and _KpnI_. The fragment encoding _divK_ was ligated into pSR658, transformed into DH5α, and selected for on medium containing tetracycline. The fragment encoding _celR_ was ligated into pSR659, transformed into DH5α, and selected for on medium containing ampicillin. The plasmids were recovered, purified and examined for the correct insertion by restriction digest and sequencing. Plasmids containing the correct insert were
transformed into either *E. coli* strains SU101 or SU202, and were selected for resistance to kanamycin and either tetracycline or ampicillin.

### 3.4.3 *E. coli* two-hybrid assays.

Bacterial two-hybrid assays were performed as described (58). Strains SU101 containing plasmids with alleles of *celR-lexA* or SU202 containing plasmids with alleles of *divK* and *celR* fused to the DNA-binding fragment of *lexA* were plated on LB medium containing the appropriate antibiotics, IPTG and X-gal. Plates were incubated at 28°C for three days to determine if the *lacZ* gene of the test strain was repressed. Interactions between fusion proteins yield light blue colonies due to repression of *lacZ* driven by a LexA-regulated promoter (58). All experiments were repeated at least three times, with negative controls of the parent strains harboring the empty vectors pSR658 or pSR659, and the parent strains harboring the positive control plasmids pMS604 or pDP804 (66), each containing full-length *lexA* for dimerization.

### 3.4.4 Cellulose assays.

Cellulose was quantified following extraction using a modified Updegraff protocol (271) as previously described (14). The amount of anthrone-reactive material extractable from a sample was standardized to the number of cells as determined by OD₆₀₀ measurement. *Cel* mutants of strain NTL7 still produce anthrone-positive material, probably curdlan (14). Because of this, the average amount of cellulose extractable per 10⁹ cells was then normalized by subtracting out the amount of anthrone-positive material extracted from either NTL7Δ*cel* or NTL7Δ*celA::lacZ*, depending on the experiment, all as we described previously (14). Statistical analysis was performed using the Student t-test using a one-sided distribution model.

### 3.4.5 β-galactosidase assays.

β-galactosidase activity was quantified using a microtiter assay as described by Slauch and Silhavy (235). Cells grown overnight at 28°C
in MG/L with appropriate antibiotics were subcultured into 2 ml of ABM medium, grown for 24 hours to mid-exponential phase, collected by centrifugation, and resuspended in 1.5 ml of Z-buffer, pH 7.1 (235). Volumes of 250 µl of each suspension were removed for turbidity measurements at 600 nm. Fifteen µl of 1% SDS and 30 µl of chloroform was added to the remaining volumes, the samples were vortexed for 10 seconds, incubated for 15 minutes at room temperature and 60 µl volumes of the lysates were distributed to polystyrene microtiter wells (Corning). The final volume in each well was adjusted to 200 µl with Z-buffer. The reaction was initiated by adding 50 µl of o-nitrophenyl-3-D-galactopyranoside (ONPG; 10 mg/ml) in Z-buffer without added 3-mercaptoethanol and the absorbance at 420 nm was monitored in a Cambridge Biotechnology Model 700 microplate reader. Readings were taken at 3-minute intervals for 24 minutes. β-galactosidase activity was calculated as described (235). Each strain was assayed nine times, and the reaction rates were averaged and statistically analyzed using the Student t-test with a one-sided distribution model.

3.4.6 Microscopy. Cells were grown in MG/L with appropriate antibiotics overnight at 28°C with shaking. Cells from each culture grown to the same OD600 were examined by phase contrast microscopy using an Olympus BH-2 Research Microscope (Olympus) with an Olympus Camedia Digital Camera (Olympus). Two image-fields were recorded for each sample, and the total number of cells and the number of malformed cells were counted in each field. The percentage of malformed cells was calculated from two repetitions (four fields total), with statistical analysis performed using the Student t-test with a one-sided distribution model.
3.5 Results

3.5.1 Alteration of key residues affects the influence of CelR on cellulose production. Previously, we showed that celR is required to stimulate cellulose biosynthesis (14). Bioinformatic analysis of this protein identified two N-terminal CheY-like receiver domains, the first containing a conserved aspartate residue, as well as a C-terminal catalytic GGEEF motif associated with synthesis of c-di-GMP (Figure 3.1). Proteins with CheY domains often transition between activated and inactive forms based on the phosphorylation state of a conserved aspartate residue (4, 35, 152). Given its domain structure, these observations suggest that CelR is a c-di-GMP synthase, and that its activity is modulated by modification of the Asp residue in the CheY domain.

In a set of preliminary experiments, we overexpressed celR mutated at Asp53 in the CheY domain (celRD53A) or at a key residue of the enzymatic GGEEF motif (celRE373A) in wild-type strain NTL7. We assessed the effect of these mutations on cellulose production by colony color on Congo red plates (302) and by growth properties in liquid media. As we described previously (14), when compared to the parent, overexpressing wild-type celR resulted in increased Congo red binding (Figure 3.2A) and also pronounced aggregation in liquid media (Figure 3.2B). NTL7 overexpressing either celRD53A or celRE373A exhibited little change in Congo red binding (Figure 3.2A), and showed virtually no observable aggregation during growth in liquid media (Figure 3.2B). In quantitative assays, NTL7 overexpressing celR produced a significantly higher level of extractable cellulose as compared to the parent strain (Figure 3.3A). In contrast, overexpressing either celRD53A or celRE373A had no significant effect on the amount of the polymer produced (Figure 3.3A). These results suggest that, to stimulate cellulose
synthesis, CelR requires an intact GGEEF motif and the conserved aspartate residue in the CheY domain.

3.5.2 CelR regulates cellulose biosynthesis through its activation and synthesis of c-di-GMP. While overexpressing the D53A and E373A mutants of CelR has no effect on phenotypes associated with cellulose synthesis, these results do not establish the role of these residues in stimulating production of the polymer. We therefore tested celRD53A and celRE373A for their ability to complement the indel mutant of celR, NTL7ΔcelR::Gm. As previously described (14), the ΔcelR mutant does not produce cellulose as compared to wild-type NTL7 (Figure 3.3B). Expressing wild-type celR from its native promoter in the mutant restored cellulose production to wild-type levels (Figure 3.3B). Complementing the indel mutant with either celRD53A or celRE373A failed to restore cellulose synthesis as compared to the uncomplemented mutant (Figure 3.3B). Taken together, the data indicate that CelR requires both the conserved aspartate in the CheY domain and the conserved glutamate in the GGEEF motif for stimulating cellulose production.

3.5.3 An Asp53 → Glu mutation in the first CheY domain of CelR results in increased cellulose production. Analysis of the D53A mutant of CelR indicates that this amino acid is critical for the proper function of the protein. In proteins containing similar CheY-like receiver domains, the aspartate residue can be phosphorylated (220), and converting this residue to a glutamate often results in a constitutively-active form of the protein (190). We examined the role of Asp53 in CelR by mutating this residue to glutamate and assessing the effect on cellulose synthesis. Overexpressing celRD53E in NTL7 resulted in increased cellulose production, even as compared to a strain
overexpressing wild-type celR (Figure 3.4). Complementing the ΔcelR mutant with celRD53E inserted at unit copy number and expressed from its native promoter also resulted in increased levels of cellulose synthesis as compared to the mutant complemented with wild-type celR (Figure 3.4). These observations suggest that substituting glutamate for aspartate at position 53 results in a more active form of celR. Taken together, our results are consistent with the notion that CelR is a bona fide c-di-GMP synthase, and that it is part of a signaling pathway that involves phosphorylation at Asp53 in the CheY domain.

3.5.4 An intact PilZ domain of CelA is required for cellulose biosynthesis.

The presence of a PilZ domain on CelA suggests that full activity of the cellulose synthase depends upon binding c-di-GMP. Altering a conserved serine in the PilZ domain of other proteins can result in a significant decrease in the binding affinity of the nucleotide signal (127, 167). Based on these observations, we tested if an intact PilZ domain of CelA is necessary by comparing levels of cellulose produced by a celA mutant complemented with either the wild-type gene or celAS594A, an allele with a mutation in the conserved serine (Figure 3.1, (162)). As expected, the celA::lacZ mutant did not produce detectable levels of cellulose (Figure 3.5A). Complementing the indel mutant with wild-type celA expressed from pSRK-Gm restored cellulose production to wild-type levels (Figure 3.5A), confirming that the celA mutation is not deleteriously polar on celB and celC. However, expressing the celAS594A allele failed to restore cellulose production to levels observed in NTL7ΔcelA::lacZ complemented with the wild-type gene (Figure 3.5A). Further, while overexpressing wild-type celA in NTL7 led to high levels of cellulose production, overexpressing celAS594A in this strain led to
considerably lower levels of the polymer (Figure 3.5A). This result suggests that celAS594A exerts a modest dominant negative effect on the wild-type gene. Moreover, these results support a requirement for the PilZ domain of CelA.

In carefully studied systems, the mutation of the serine residue in the PilZ domain lowers, but does not eliminate, the binding affinity of c-di-GMP (167). It is conceivable, then, that the effect of the mutant PilZ domain on cellulose synthesis can be compensated for by overexpressing celR. We tested this possibility by overexpressing celR in NTL7ΔcelA::lacZ complemented with either wild-type celA or the celAS594A allele. Overexpressing celR in the celA mutant complemented with wild-type celA resulted in increased levels of cellulose as compared to the parent strain (Figure 3.5B). Further, overexpressing celR increased cellulose production in the celA mutant complemented with celAS594A, although not to the levels of the mutant complemented with wild-type celA (Figure 3.5B). The results suggest that increasing the concentration of c-di-GMP can partially compensate for the mutation in the PilZ domain of CelA.

3.5.5 Altering celR expression does not affect transcription of celA. While our results suggest that the c-di-GMP signal produced by CelR stimulates cellulose synthesis via the PilZ domain of CelA, it is conceivable that CelR or its nucleotide signal product induces transcription of the celA gene. To determine if expression of celA is affected by CelR, we utilized the lacZ transcriptional fusion in NTL7ΔcelA::lacZ. As shown in Figure 3.5C, the reporter mutant expresses celA at a modest level. Overexpressing celR in the celA mutant resulted in a two-fold decrease in β-galactosidase activity compared to the parent strain (Figure 3.5C), while deleting celR in the reporter mutant resulted in slightly lower levels of β-galactosidase activity as compared to the parent strain (Figure
3.5C). These results suggest that celR, expressed at its normal levels, has little or no direct effect on the transcription of celA.

### 3.5.6 The response regulator DivK affects production of cellulose through CelR

CelR. In *A. tumefaciens*, celR is the distal gene in a two-gene operon that begins with *atu1296*, a gene generally annotated as divK (Figure 3.1). The product of the well-studied founder ortholog, divK in *Caulobacter crescentus*, is required for localizing factors involved in cell division as well as activation of PleD, the ortholog of CelR (183, 189, 268). Given the operonic structure of divK-celR and the function of divK in other alphaproteobacteria, these observations suggest the possibility that divK plays a role in stimulating cellulose synthesis.

We first assessed the role of divK in regulating cellulose production by deleting the gene, creating NTL7ΔdivK::Km. The mutant accumulated significantly lower amounts of the polymer as compared to the wild-type, and matched the amount of cellulose produced by the ΔcelR mutant (Figure 3.6A). This effect on cellulose production most likely is due to the polar nature of the ΔdivK indel mutation on celR. When introduced into this mutant at single copy, celR under the control of its native promoter increased cellulose synthesis appreciably, although not to wild-type levels (Figure 3.6A). Interestingly, this strain produced levels of cellulose equivalent to the amounts produced by NTL7ΔcelR::Gm complemented with the D53A allele of celR (Figure 3.6A), suggesting that DivK plays a role in the pathway for activation of CelR. Complementing NTL7ΔdivK::Km with a chromosomally-inserted construct containing both divK and celR (Tn7-prdivKcelR) restored cellulose production to wild-type levels (Figure 3.6A). This result suggests that it is the absence of celR in the divK-celR deletion...
mutant that is responsible for most, but not all, of this decrease in levels of cellulose observed in NTL7ΔdivK::Km.

The influence of DivK suggests that this CheY-like protein, a putative response regulator (85, 292), is required for CelR-mediated stimulation of cellulose synthesis. If this is correct, the full effect of celR overexpression should require an expressed copy of divK. To test this hypothesis, pKK38celR was introduced into NTL7ΔdivK::Km complemented with either Tn7-prcelR or Tn7-prdivKcelR. Overexpressing celR did lead to a modest increase in cellulose production in NTL7ΔdivK::Km or in NTL7ΔdivK::Km/Tn7-prcelR (Figure 3.6B). That increased celR expression failed to fully compensate for the loss of divK further demonstrates the importance of the CheY homolog in the signaling pathway. When celR was overexpressed in the divK deletion mutant complemented with Tn7-prdivKcelR, levels of cellulose accumulation were significantly higher, exceeding even the levels observed in wild-type NTL7 overexpressing celR (Figure 3.6B).

These results support the involvement of DivK in the signaling pathway that activates CelR. To test this requirement for DivK, we determined if the constitutively-active form of celR stimulates cellulose production in the divK mutant. As described earlier, compared to wild-type celR, overexpression of celRD53E in NTL7 resulted in a significant increase in cellulose production (Figure 3.4A). While introducing wild-type celR into NTL7ΔdivK::Km did not restore cellulose production to wild-type levels (Figure 3.6C), introducing the constitutively-active form of the DGC yielded significantly higher levels of cellulose in the divK mutant (Figure 3.6C). We conclude from these
results that the constitutively-active form of CelR can compensate for the loss of \textit{divK}, and from this, that DivK is involved in the pathway for activation of CelR.

3.5.7 The aspartate residues in the CheY domains of both DivK and CelR are important for cellulose production. The effects of altering the conserved aspartate residues of \textit{divK} and \textit{celR} suggest that the two residues are critical for stimulating cellulose synthesis. To examine the roles of these two residues, the \textit{divKcelR} operon was deleted, and the resulting strain, NTL7\textit{Δ}divK\textit{celR}, was complemented with wild-type and mutant alleles of \textit{divK} and \textit{celR} using the mini-Tn7 chromosomal insertion system.

Deleting \textit{divK} and \textit{celR} resulted in a 5-fold decrease in cellulose production as compared to the wild-type parent (Figure 3.7A). Complementing the mutant with Tn7-\textit{prdivKcelR}, in which both genes are wild-type, restored cellulose production to wild-type levels, while complementation with either \textit{divK} or \textit{celR} alone did not fully restore synthesis of the polymer (Figure 3.7A). These results are consistent with our complementation analysis of the \textit{divK} mutant with either gene (Figure 3.6). Complementing the \textit{Δ}divK\textit{celR} mutation with \textit{divKD53A} or \textit{divKD53E} resulted in modest increases in levels of cellulose production (Figure 3.7B), suggesting that the presence of \textit{divK} has a minor effect on polymer production. Introducing \textit{celRD53A} into the \textit{Δ}divK\textit{celR} mutant also failed to fully restore cellulose synthesis (Figure 3.7B). On the other hand, complementing the mutation with \textit{celRD53E} restored cellulose production to greater than wild-type levels (Figure 3.7B). That constitutively-active CelR can compensate for the loss of the putative response regulator, while wild-type CelR does not suggests that DivK is necessary for CelR to fully stimulate cellulose production.
To more fully evaluate the role of the aspartate residues of both celR and divK, the divKcelR operon, with either divK or celR mutated at D53, was reintroduced into the divK-celR double mutant at unit copy number using the Tn7 system. NTL7ΔdivKcelR complemented with wild-type divK and celRD53A failed to increase cellulose production as compared to the parent mutant (Figure 3.8A). However, complementation with wild-type divK and celRD53E resulted in a 2-fold increase in cellulose synthesis as compared to levels of the polymer produced by wild-type NTL7 (Figure 3.8A). Complementing the double mutant with wild-type celR and divKD53A restored cellulose production to wild-type levels (Figure 3.8A), while complementation with wild-type celR and divKD53E resulted in cellulose levels 2-fold greater than those observed in NTL7 (Figure 3.8A). These data indicate that the D53E alleles of both celR and divK stimulate cellulose production.

The effect of mutating aspartate to glutamate on both DivK and CelR suggests that altering the residue in both proteins also would increase cellulose production. To test this hypothesis, the divKcelR deletion mutant was complemented with a mini-Tn7 insertion carrying both genes mutated at Asp53. Insertions with either divKD53A/celRD53A or divKD53E/celRD53A did not result in significantly higher levels of cellulose as compared to the parent mutant (Figure 3.8B), suggesting that divK alone does not stimulate cellulose synthesis. When divKD53A/celRD53E or divKD53E/celRD53E were inserted into the double mutant, the complemented strains produced amounts of cellulose comparable to or somewhat greater than amounts produced by wild-type NTL7 (Figure 3.8B). These results suggest that constitutively-
active CelR stimulates cellulose production, regardless of the allelic nature of Asp53 in divK.

3.5.8 Deleting divK affects cell morphology. In C. crescentus, DivK acts as a critical checkpoint regulator for cell cycle progression (1, 189). DivK also is involved in regulating cell division in A. tumefaciens; deleting the gene results in morphologically abnormal cells (124). We assessed the effect of deleting both divK and celR on cell morphology by phase-contrast microscopy. As compared to the parent, the ΔdivKcelR mutant displayed a greater number of cells with morphological defects, including branched and elongated forms (compare Figures 3.9A and B; Table 3.3). Complementing the mutation with Tn7-prdivKcelR restored the frequency of abnormal cell morphologies to that of the wild-type (compare Figures 3.9A and 8C; Table 3.3). As expected from the report by Kim et al. (124), complementing the double mutant with divK alone lowered the frequency of the cell morphology defects to wild-type levels (Figure 3.9F; Table 3.3). Further, the constitutively-active form of divK complemented the defects in NTL7ΔdivKcelR in the presence or absence of celR (Figures 3.9G and H, Table 3.3). However, complementing the ΔdivKcelR mutant with only prcelR or prcelRD53E did not reduce the number of cells exhibiting morphological defects, (Figure 3.9D and E; Table 3.3), suggesting that celR has no effect on processes that control morphology. These results support the involvement of divK in cell division of A. tumefaciens, but suggest that celR does not play a role in cell cycle regulation.

3.5.9 CelR forms homodimers but does not interact with DivK. That DivK contributes to the regulation of cellulose production through CelR suggests that the CheY-like protein is a component of the pathway for activation of the DGC. Since DivK
functions by binding with its targets in other species of alphaproteobacteria (189), it is possible that the protein interacts directly with CelR. We tested this hypothesis using a bacterial two-hybrid assay as described in Materials and Methods. In this assay, interaction between two proteins leads to repression of lacZ, resulting in light blue colonies when grown on medium containing X-gal. The two-hybrid strain expressing celR from pSR659 formed light blue colonies in the presence of X-gal, suggesting that the DGC interacts with itself to generate an active hybrid LexA repressor (Figure 3.10A). Mutating Asp53 to Ala or Glu had no effect on this reaction (Figure 3.10A), suggesting that although CelR homodimerizes, this interaction is not dependent on signal-induced modifications at Asp53. Strains expressing wild-type CelR and DivK on separate plasmids hydrolyzed X-gal (Figure 3.10B), indicative of a failure of the two proteins to interact. Mutating Asp53 to alanine or glutamate on both DivK and CelR did not result in a positive reaction for protein interaction (Figures 3.10B and C).

3.6 Discussion

3.6.1 CelR regulates cellulose synthesis through production of c-di-GMP.

Previously, we reported that the putative diguanylate cyclase CelR is required for stimulating cellulose production in A. tumefaciens (14). Given that CelR contains both a CheY-like response receiver domain and a catalytic GGEEF domain (Figure 3.1), we hypothesized that CelR is a part of a signaling system that controls cellulose synthesis. Recent biochemical studies showed that CelR likely produces c-di-GMP (294), but did not link this signal to regulating cellulose synthesis. Our results reported here demonstrate that CelR requires the catalytic GGEEF motif to stimulate production of the
polymer; a mutant modified in this motif failed to complement strains in which celR had been deleted (Figure 3.4). This observation, coupled with results from Xu et al. (294), supports our hypothesis that CelR is a bona fide cyclase, and that the cyclic dinucleotide signal produced by this enzyme influences cellulose synthesis.

3.6.2 CelR requires the conserved aspartate residue in the CheY domain to stimulate cellulose production. Conservation of an aspartate in the CheY domain of CelR and its orthologs in other species (Figure 3.11) suggests that the residue is important for activation of the protein, likely as a target for phosphorylation (220). Our current results support this hypothesis; mutating aspartate 53 to an alanine abolished CelR-dependent stimulation of cellulose production either when overexpressed or when the mutant allele was used to complement a celR deletion mutant (Figure 3.4). A similar mutation in the conserved aspartate in the CheY domain of PleD of C. crescentus prevented cells from building the polarly-located holdfast stalk (4, 94, 188).

In the case of PleD of C. crescentus, mutating the conserved aspartate in the CheY domain to glutamate creates a constitutively-active form of the protein; expressing the mutant allele results in increased formation of holdfast structures and deformed cells (4). In other proteins with CheY-related receiver domains, the conversion of the aspartate residue to glutamate often but not always mimics the activated state (126, 138). These observations suggest that a similar mutation on celR will result in a constitutively-active form of the protein. Indeed, either overexpressing celRD53E or complementing the ΔcelR mutant with the mutant allele resulted in increased amounts of cellulose synthesis as compared to the deletion mutant expressing wild-type celR (Figure 3.4). Taken together,
our analysis of the two Asp53 mutants of CelR suggests that for its activity, the protein requires activation, likely by phosphorylation of the conserved aspartate residue.

That CelR requires activation, likely by a kinase, suggests that cellulose synthesis is controlled by some as yet unidentified environmental cue that results in delivery of c-di-GMP from the DGC to a target further along the pathway. To date, our attempts to identify the environmental signal that stimulates cellulose production have not been successful (data not shown). Further work will be needed to identify both the upstream signal and the kinase that activates CelR.

3.6.3 The PilZ domain of CelA is necessary for cellulose synthesis. Given that CelR is a bona-fide c-di-GMP synthase, there must be a downstream signal receptor that is involved in cellulose synthesis. In many organisms, the catalytic subunit of the cellulose synthase, including CelA of *A. tumefaciens*, contains a c-di-GMP-binding PilZ domain (7). Certain residues in PilZ-containing proteins, including a serine within a DxsxxxG motif, are required for binding c-di-GMP (196, 230). CelA in *A. tumefaciens* contains this motif, and mutating Ser594 to alanine prevented full complementation of a celA deletion mutant (Figure 3.5), suggesting that the serine residue within this motif is required for cellulose synthesis. In *in vitro* studies of both PlzD of *Vibrio cholerae* and Alg44 of *P. aeruginosa* (167, 196), mutating this serine lowers the binding affinity for c-di-GMP. One might expect that increasing the levels of the signal could compensate for the lower affinity of the altered PilZ domain in CelA. Indeed, overexpressing celR somewhat stimulated cellulose synthesis in the celA deletion mutant complemented with celAS594A (Figure 3.5), further supporting the role of c-di-GMP in regulating production of the polymer.
We considered the possibility that CelR, in some manner, regulates transcription of the cel regulon. Based on our assays of a celA::lacZ fusion, expression of the synthase from its native promoter is relatively low (Figure 3.5). Mutating or overexpressing celR did not significantly affect the level of celA expression, suggesting that the cyclase does not affect transcription of the celABC operon. This result further supports our hypothesis that CelR stimulates cellulose production through activation of CelA via c-di-GMP.

Taken together, our observations are consistent with a pathway in which CelR produces c-di-GMP, and that this signal stimulates cellulose synthesis by activating CelA. Biochemical analysis of BcsA in S. typhimurium, a homolog to CelA, demonstrated that c-di-GMP binding activates cellulose synthesis, and that mutating critical binding residues in the PilZ domain abolished signal binding and enzymatic activity (184, 197). According to the current model, signal binding at the PilZ domain of BcsA allosterically controls cellulose production by triggering conformational changes in the complex that result in increased enzymatic activity (184, 197). Moreover, because CelR requires activation to synthesize c-di-GMP, the combined results suggest that any stimulation of polymer production by CelA requires an upstream signaling cascade.

3.6.4 DivK participates in regulating cellulose synthesis through CelR. The operonal organization of celR with divK suggests that both genes are involved in regulating cellulose synthesis. Previous studies demonstrated that DivK of C. crescentus is a CheY-type response regulator, and is phosphorylated by the hybrid kinase DivJ (136, 189). Our studies of DivK in A. tumefaciens support a role for this putative response regulator in stimulating production of the polymer through CelR. In a divK deletion mutant, wild-type CelR failed to fully stimulate cellulose production (Figure 3.6).
However, a constitutively-active form of the c-di-GMP synthase compensated for the loss of the response regulator; expressing celRD53E restored polymer synthesis to the divK mutant. These results suggest that DivK is important for the Asp53-dependent activation of CelR and subsequent stimulation of cellulose synthesis.

That DivK is composed of a single CheY-like domain (Figure 3.1) with its conserved aspartate residue suggests that the protein is activated in a manner similar to CelR. Indeed, conversion of Asp53 in DivK to alanine prevented full stimulation of cellulose synthesis (Figure 3.7). On the other hand, complementing the ΔdivK mutant with the divKD53E allele resulted in higher levels of cellulose as compared to the deletion mutant complemented with wild-type divK (Figures 3.6 and 3.8). Consistent with this interpretation, the levels of cellulose produced by the divKD53E mutant were comparable to the levels observed from strains expressing celRD53E (Figure 3.8B). These results support the hypothesis that DivK, like many other members of the CheY family, can be activated, probably by phosphorylation at Asp53, and that this activation is part of a signaling pathway that results in activating CelR.

In *C. crescentus*, phosphorylated DivK interacts with its target, the master response regulator CtrA, resulting in localization of the CtrA-DivK complex to the target pole of the cell (104, 105, 189). Based on our two-hybrid analyses, DivK does not directly interact with CelR (Figure 3.9), although our two-hybrid analysis indicates that CelR likely homodimerizes. This latter result is consistent with our observation that the celRD53A mutation exerts dominant negativity (Figure 3.3), as well as with crystallographic and biochemical studies of PleD, the CelR ortholog in *C. crescentus* (35,
Interestingly, the DGC activity of PleD requires dimerization (188). Clearly, DivK must interact with some upstream kinase, and in its phosphorylated form must interact with some downstream target that affects the phosphorylation state of CelR. While we have no evidence that DivK directly interacts with CelR, the response regulator may localize other components of the signal pathway. Further studies of these interactions will be necessary to determine the mechanism by which DivK participates in the regulation of cellulose synthesis.

3.6.5 DivK but not CelR functions in pathways for both cellulose synthesis and cell division. In addition to regulating cellulose synthesis, a recent study by Kim et al. (124) clearly showed that DivK functions in regulating processes important for cell division in *A. tumefaciens*. Our results support this hypothesis; deleting *divK* resulted in cell division defects, illustrated by elongated and branched cells (Figure 3.9). In several alphaproteobacteria, including *A. tumefaciens* and the related *Sinorhizobium meliloti*, *divK* and its orthologs are linked to both polar localization of cell division proteins (4, 89, 103, 124, 195), and initiation of S-phase through localization and degradation of CtrA (1). Coupled with our observations that DivK is required for activating CelR, it is clear that the CheY homolog of *A. tumefaciens* retains its role in regulating cell division while also modulating cellulose synthesis.

Such a role for DivK in other alphaproteobacteria is not without precedent. In *C. crescentus*, activated DivK stimulates construction of the stalk and holdfast assembly, as well as initiating cell cycle progression. The response regulator apparently participates in these two pathways by stimulating the PleC and DivJ hybrid kinases associated with the
two systems (1, 189, 268). The activated forms of PleC and DivJ either phosphorylate or dephosphorylate cell division proteins as well as PleD, which signals the construction of the stalk (268). Given the relatedness of DivK in *A. tumefaciens* to its orthologs (Figure 3.12), the effects of mutating *divK* on cell division in a number of bacteria (4, 89, 103, 124, 195), and the presence of homologs of *divK* in a large number of the alphaproteobacteria (26), it is likely that the protein plays a critical role in regulating cell division in all of the alphaproteobacteria that carry the gene. Further, in *C. crescentus*, DivK interfaces stalk production to cell division by activating PleD through stimulation of the activating kinase, DivJ (189, 268). In this manner, activated DivK not only drives the cell cycle through S-phase, but also initiates stalk construction concurrently with cell cycle progression, through a separate signal transduction pathway.

Our results demonstrate that DivK maintains a dual function in *A. tumefaciens*, but unlike *C. crescentus*, it regulates these pathways independently. CelR mutants show no morphological or developmental defects ((14); Figure 3.9), suggesting that the DGC, although requiring DivK for activity, does not influence cell division. Furthermore, expressing *divKD53E* stimulated cellulose synthesis but did not affect cell morphology. That the regulatory effects of DivK on production of the polymer are independent from the controls on cell division suggest that DivK regulates both processes, but does not necessarily link the two pathways.

While the role of DivK in cell cycle regulation is conserved among many alphaproteobacteria, its adaption to a secondary regulatory function seems dependent upon the corresponding DGC in the operon. The observation that CelR has adapted to
stimulate cellulose synthesis in *A. tumefaciens* and *R. leguminosarum* (11), rather than regulating polar adhesion (11, 14), suggests that the role of the DGC is based on the biological system needed by the bacteria. In this manner, the *divK-celR(pleD)* operon functions as a system to regulate species-specific processes through established signal transduction pathways. The dual roles of DivK likely allow some species of alphaproteobacteria to connect cell division to specific needs, such as attachment or biofilm formation. DivK may also respond to different signal inputs, resulting in activation of each pathway based on these cues.

Given the number of processes regulated by c-di-GMP in *A. tumefaciens* (14, 294), production and distribution of the signal must be tightly controlled to prevent crosstalk with other systems. The requirement for c-di-GMP binding by CelA resulted in adapting the transduction pathway containing CelR for regulating production of cellulose. However, some but not all DGCs can influence cellulose synthesis when overexpressed (14). Therefore, the interaction of c-di-GMP and CelA must be spatially and temporally controlled, either through direct interaction of the DGC with the synthase, or by localization of CelR near the cellulose synthase complex. Further analysis of CelR localization will help determine how the c-di-GMP signal is targeted to CelA.
### 3.7 Tables and Figures

#### Table 3.1. Strains and Plasmids used in this study

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<td>NTL7ΔcelR::Gm with Tn&lt;sup&gt;7&lt;/sup&gt;-Km-prcelRD53A inserted at the glmS site</td>
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<tr>
<td><strong>NTL7ΔcelR::Gm/Tn&lt;sup&gt;7&lt;/sup&gt;-prcelRD53E</strong></td>
<td>NTL7ΔcelR::Gm with Tn&lt;sup&gt;7&lt;/sup&gt;-Km-prcelRD53E inserted at the glmS site</td>
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<td><strong>NTL7ΔdivK::Km</strong></td>
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<tr>
<td><strong>NTL7ΔdivK::Km/Tn&lt;sup&gt;7&lt;/sup&gt;-prdivK</strong></td>
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<td><strong>NTL7ΔdivKcelR</strong></td>
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<tr>
<td>Strain Description</td>
<td>Description</td>
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<tr>
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<td><strong>Table 3.1. (cont.)</strong></td>
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<tr>
<td></td>
<td><em>glmS</em> site</td>
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**Plasmid**

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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>pUC18</td>
<td>Cloning vector, Ap&lt;sup&gt;i&lt;/sup&gt;</td>
<td>Invitrogen</td>
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<tr>
<td>pUCcelR</td>
<td><em>celR</em> gene cloned into pUC18</td>
<td>(14)</td>
</tr>
<tr>
<td>pUCcelRE373A</td>
<td>pUCcelR altered at E373 to an alanine</td>
<td>This Study</td>
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<tr>
<td>pUCcelRD53A</td>
<td>pUCcelR altered at D53 to an alanine</td>
<td>This Study</td>
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<tr>
<td>pUCcelRD53E</td>
<td>pUCcelR altered at D53 to a glutamate</td>
<td>This Study</td>
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<tr>
<td>pUCcelA</td>
<td><em>celA</em> gene cloned into pUC18</td>
<td>This Study</td>
</tr>
<tr>
<td>pUCcelAS594A</td>
<td>pUCcelA altered at S594 to an alanine</td>
<td>This Study</td>
</tr>
<tr>
<td>pUCprdivKcelR</td>
<td>pUC18 containing the <em>divKcelR</em> operon</td>
<td>This Study</td>
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<tr>
<td></td>
<td>and 400 bp of the promoter region</td>
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<td>pUC18 containing <em>celR</em> and 400 bp of the</td>
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<td><strong>pUCprcelRD53A</strong></td>
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<td>This Study</td>
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<tr>
<td><strong>pUCprcelRD53E</strong></td>
<td>pUCprcelR altered at D53 to a glutamate</td>
<td>This Study</td>
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<tr>
<td><strong>pUCprdivK</strong></td>
<td>pUC18 containing divK and 400 bp of the promoter region</td>
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<td><strong>pZLQ</strong></td>
<td>pBBR1MCS-2 based expression vector, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(151)</td>
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<td><strong>pZLQcelR</strong></td>
<td>celR gene from pUCcelR cloned into NdeI/BamHI sites of pZLQ</td>
<td>(14)</td>
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<tr>
<td><strong>pZLQcelRE373A</strong></td>
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<td>This Study</td>
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<tr>
<td><strong>pZLQcelRD53A</strong></td>
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<tr>
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<tr>
<td><strong>pUC18mini-Tn7t-Km</strong></td>
<td>Tn7 carrier vector containing Km cassette, Ap&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(44)</td>
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<tr>
<td><strong>pUC18mini-Tn7t-Gm</strong></td>
<td>Tn7 carrier vector containing Gm cassette, Ap&lt;sup&gt;r&lt;/sup&gt;, Gm&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(44)</td>
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<td><strong>pTNS2</strong></td>
<td>Tn7 helper plasmid encoding the TnsABC+D specific transposition pathway, Ap&lt;sup&gt;r&lt;/sup&gt;</td>
<td>(44)</td>
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<tr>
<td>Description</td>
<td>Detailed Description</td>
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<tr>
<td>pUCTn7-Km-prcelR</td>
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<tr>
<td>pUCTn7-Gm-prcelRD53E</td>
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<tr>
<td>pUCTn7-prdivKcelR</td>
<td>prdivKcelR fragment cloned into BamHI site of pUC18mini-Tn7-Gm</td>
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<tr>
<td>pUCTn7-prdivKD53AcelR</td>
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<tr>
<td>pUCTn7-prdivKD53EcelR</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of divK to a glutamate</td>
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<td>pUCTn7-prdivKcelRD53A</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of celR to an alanine</td>
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<td>Expression</td>
<td>Description</td>
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<tr>
<td>pUCTn7-prdivKcelRD53E</td>
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<td>This Study</td>
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<td>pUCTn7-prdivKcelRD53A</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of divK and celR to an alanine</td>
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<tr>
<td>pUCTn7-prdivKcelRD53E</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of divK to an alanine and at D53 of celR to a glutamate</td>
<td>This Study</td>
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<tr>
<td>pUCTn7-prdivKcelRD53E</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of divK to a glutamate and at D53 of celR to an alanine</td>
<td>This Study</td>
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<tr>
<td>pUCTn7-prdivKcelRD53E</td>
<td>prdivKcelR fragment in pUC18mini-Tn7t-Gm, altered at D53 of divK and celR to a glutamate</td>
<td>This Study</td>
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<tr>
<td>pKK38ASH</td>
<td>Broad-host-range IncP cloning vector, tac promoter, Tc^r</td>
<td>(145)</td>
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<tr>
<td>pKK38celR</td>
<td>celR gene cloned into pKK38ASH at BamHI site</td>
<td>This Study</td>
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<tr>
<td>pKK38celRD53E</td>
<td>celRD53E allele cloned into pKK38ASH at BamHI site</td>
<td>This Study</td>
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<tr>
<td>pRK415</td>
<td>IncP1a broad host range cloning vector, Tc^r</td>
<td>(115)</td>
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### Table 3.1. (cont.)

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<tr>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;Kregion</td>
<td>The &lt;i&gt;divK&lt;/i&gt; gene and flanking DNA inserted into pRK415</td>
<td>This Study</td>
</tr>
<tr>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;region</td>
<td>The &lt;i&gt;divK&lt;sub&gt;celR&lt;/sub&gt;&lt;/i&gt; operon and flanking DNA inserted into pRK415</td>
<td>This Study</td>
</tr>
<tr>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;Kan</td>
<td>Allellic replacement of &lt;i&gt;divK&lt;/i&gt; with a kanamycin cassette on pRK&lt;sup&gt;divK&lt;/sup&gt;Kregion</td>
<td>This Study</td>
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<tr>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;Kan</td>
<td>Allellic replacement of &lt;i&gt;divK&lt;sub&gt;celR&lt;/sub&gt;&lt;/i&gt; with a kanamycin cassette on pRK&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;region</td>
<td>This Study</td>
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<tr>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;del</td>
<td>pRK&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;Kan with kanamycin cassette removed by &lt;i&gt;flp&lt;/i&gt; recombinase</td>
<td>This Study</td>
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<tr>
<td>pWM91</td>
<td>λpir-dependent cloning vector, Ap&lt;sup&gt;r&lt;/sup&gt;Suc&lt;sup&gt;s&lt;/sup&gt; (170)</td>
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<td>pWM&lt;sup&gt;divK&lt;/sup&gt;Kan</td>
<td>&lt;i&gt;divK&lt;/i&gt;Kan fragment cloned into BamHI site of pWM91</td>
<td>This Study</td>
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<tr>
<td>pWM&lt;sup&gt;divK&lt;/sup&gt;K&lt;sub&gt;celR&lt;/sub&gt;del</td>
<td>&lt;i&gt;divK&lt;sub&gt;celR&lt;/sub&gt;&lt;/i&gt;del fragment cloned into BamHI site of pWM91</td>
<td>This Study</td>
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<td>pWM&lt;sup&gt;celR&lt;/sup&gt;del</td>
<td>&lt;i&gt;celR&lt;/i&gt;del fragment cloned into BamHI site of pWM91 (14)</td>
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<td>pSRK-Gm</td>
<td>Broad host range expression vector, &lt;i&gt;ara&lt;/i&gt; promoter, Gmr&lt;sup&gt;f&lt;/sup&gt; (123)</td>
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<td>pSRK&lt;i&gt;celA&lt;/i&gt;</td>
<td>&lt;i&gt;celA&lt;/i&gt; inserted at the NdeI/BamHI sites of pSRK-Gm</td>
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### Table 3.1. (cont.)

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<tr>
<td>pSRK&lt;sub&gt;celAS594A&lt;/sub&gt;</td>
<td>S594A allele of &lt;i&gt;celA&lt;/i&gt; cloned into pSRK-Gm at the NdeI/BamHI sites</td>
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<tr>
<td>pKD46</td>
<td>Lambda Red recombinase helper plasmid, Ap&lt;sup&gt;r&lt;/sup&gt; (61)</td>
</tr>
<tr>
<td>pKD4</td>
<td>Template plasmid of kanamycin cassette for chromosomal exchange, Ap&lt;sup&gt;r&lt;/sup&gt;, Km&lt;sup&gt;r&lt;/sup&gt; (61)</td>
</tr>
<tr>
<td>pCP20</td>
<td>Temperature-sensitive replicon containing the FLP recombinase, Ap&lt;sup&gt;r&lt;/sup&gt;, Cm&lt;sup&gt;r&lt;/sup&gt; (40)</td>
</tr>
<tr>
<td>pSR658</td>
<td>Expression plasmid for LexA dimerization system, Tc&lt;sup&gt;r&lt;/sup&gt; (58)</td>
</tr>
<tr>
<td>pSR659</td>
<td>Expression plasmid for LexA dimerization system, Ap&lt;sup&gt;r&lt;/sup&gt; (58)</td>
</tr>
<tr>
<td>pMS604</td>
<td>Expression plasmid carrying a LexA-WT-Fos fusion positive control for dimerization, Tc&lt;sup&gt;r&lt;/sup&gt; (66)</td>
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<tr>
<td>pDP804</td>
<td>Expression plasmid carrying a LexA-Jun fusion positive control for dimerization, Ap&lt;sup&gt;r&lt;/sup&gt; (66)</td>
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<td>pSR659&lt;sub&gt;celR&lt;/sub&gt;</td>
<td>pSR659 with &lt;i&gt;celR&lt;/i&gt; cloned at the BamHI and KpnI sites</td>
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<td>pSR659&lt;sub&gt;celRD53A&lt;/sub&gt;</td>
<td>pSR659 with &lt;i&gt;celRD53A&lt;/i&gt; cloned at the BamHI and KpnI sites</td>
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This Study
Table 3.1. (cont.)

<table>
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<td>pSR659&lt;sub&gt;celRD53E&lt;/sub&gt;</td>
<td>pSR659 with &lt;i&gt;celRD53E&lt;/i&gt; cloned at the &lt;i&gt;BamHI&lt;/i&gt; and &lt;i&gt;KpnI&lt;/i&gt; sites</td>
<td>This Study</td>
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<tr>
<td>pSR658&lt;sub&gt;divK&lt;/sub&gt;</td>
<td>pSR658 with &lt;i&gt;divK&lt;/i&gt; cloned at the &lt;i&gt;XhoI&lt;/i&gt; and &lt;i&gt;KpnI&lt;/i&gt; sites</td>
<td>This Study</td>
</tr>
<tr>
<td>pSR658&lt;sub&gt;divKD53A&lt;/sub&gt;</td>
<td>pSR658 with &lt;i&gt;divKD53A&lt;/i&gt; cloned at the &lt;i&gt;XhoI&lt;/i&gt; and &lt;i&gt;KpnI&lt;/i&gt; sites</td>
<td>This Study</td>
</tr>
<tr>
<td>pSR658&lt;sub&gt;divKD53E&lt;/sub&gt;</td>
<td>pSR658 with &lt;i&gt;divKD53E&lt;/i&gt; cloned at the &lt;i&gt;XhoI&lt;/i&gt; and &lt;i&gt;KpnI&lt;/i&gt; sites</td>
<td>This Study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Abbreviations: Ap<sup>f</sup>, resistance to ampicillin; Cm<sup>f</sup>, resistance to chloramphenicol; Gm, resistance to gentamicin; Km<sup>f</sup>, resistance to kanamycin; Sm<sup>f</sup>, resistance to streptomycin; Suc<sup>a</sup>, sensitivity to sucrose; Tc<sup>f</sup>, resistance to tetracycline.
Table 3.2. PCR primers used.

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<td>\textit{celR-r}</td>
<td>\texttt{CGGATCCTCAGGCCGCAGCGCCACAGCG}</td>
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<tr>
<td>\textit{celA-f}</td>
<td>\texttt{GCGGATCCCATATGAACAAGGCCATCACAGTC}</td>
</tr>
<tr>
<td>\textit{celA-r}</td>
<td>\texttt{GCGGATCCTCAGGCCGCAGCGCCACAGCG}</td>
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<td>\textit{divKregion-f}</td>
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<td>\textit{divKregion-r}</td>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<tr>
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</tr>
<tr>
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Table 3.2. (cont.)

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<th>Sequence</th>
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<tr>
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<td>celR_E373A-r</td>
<td></td>
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<tr>
<td>celR_D53E-r</td>
<td></td>
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</tr>
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<td></td>
<td>CATCCTCATGGAAATCCAGCTGCC</td>
</tr>
<tr>
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<td></td>
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</tr>
<tr>
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Table 3.3. The loss of *divK* results in increased frequency of branched and misshapen cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Complementing Tn7 Insertion</th>
<th>Total Cell Count</th>
<th>Total Misshapen Cells</th>
<th>Percent Misshapen</th>
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</thead>
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<tr>
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<td>None</td>
<td>962</td>
<td>5</td>
<td>0.52%</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td>None</td>
<td>736</td>
<td>29</td>
<td>3.91%*</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prdivKcelR</em></td>
<td>1057</td>
<td>9</td>
<td>0.87%</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prcelR</em></td>
<td>631</td>
<td>17</td>
<td>2.63%*</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prcelRD53E</em></td>
<td>799</td>
<td>21</td>
<td>2.62%*</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prdivK</em></td>
<td>938</td>
<td>7</td>
<td>0.74%</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prdivKD53E</em></td>
<td>789</td>
<td>6</td>
<td>0.78%</td>
</tr>
<tr>
<td>NTL7Δ<em>divKcelR</em></td>
<td><em>prdivKD53EcelR</em></td>
<td>764</td>
<td>5</td>
<td>0.69%</td>
</tr>
</tbody>
</table>

*Denotes significant difference from NTL7, p < 0.05.
Figure 3.1. The *divK* and *celR* genes are organized in an operon on the circular chromosome. Organization of the *divKcelR* operon of *A. tumefaciens* C58. The protein products of *divK* and *celR* both contain conserved aspartate residues at position 53 within a CheY-like receiver domain. CelR contains the GGEEF motif at residues 369-374. CelA contains the conserved serine at 594 in the PilZ domain.
Figure 3.2. Overexpressing mutant alleles of *celR* does not induce Congo red binding and aggregation on solid and liquid media. Strain NTL7 and its constructs expressing genes coding for altered alleles of *celR* were grown for two days at 28°C A) on ABM plates containing Congo red, and B) in MG/L, with shaking. Both media contained the appropriate antibiotics.
Figure 3.3. Mutant alleles of *celR* do not stimulate production of cellulose. Strains NTL7, NTL7Δ*cel* and NTL7Δ*celR*:Gm and their constructs A) overexpressing alleles of *celR* or B) expressing alleles introduced by Tn7 insertion were grown in MG/L with the appropriate antibiotics for two days at 28°C with shaking. The cells were harvested and assessed for production of anthrone-reacting material as described in Materials and Methods. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 3.4. Mutating Asp53 of celR to glutamate increases production of cellulose.

Strains NTL7, NTL7Δcel or NTL7ΔcelR::Gm either A) overexpressing celRD53E or B) expressing celRD53E from Tn7 insertions were grown in MG/L with the appropriate antibiotics for two days at 28°C with shaking. The cells were harvested and assessed for production of cellulose as described in Materials and Methods. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 3.5.
Figure 3.5. (cont.)

Figure 3.5. The cellulose synthase subunit CelA requires an intact PilZ domain to fully promote cellulose production. A) Alleles of celA expressed on pSRK-Gm were introduced into strains NTL7 and NTL7ΔcelA::lacZ and the constructs were examined for cellulose production as described in Materials and Methods. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments. B) The wild-type celR gene was overexpressed in either NTL7 or NTL7ΔcelA::lacZ containing alleles of celA, and assessed for production of cellulose as described in Materials and Methods. The values represent the average of four experiments with the error bars representing the standard error of the experiments. C) Strains of NTL7 and NTL7ΔcelA::lacZ were grown in MG/L containing the appropriate antibiotics overnight. The cells were harvested and examined for β-galactosidase activity all as described in Materials and Methods. Each experiment was repeated nine times, and error bars represent the standard error of the experiments.
Figure 3.6.
Figure 3.6. (cont.)

**Figure 3.6. DivK is required for full stimulation of cellulose production by CelR.**

A) Strains with mutations in *divK* and *celR* were examined for cellulose production as described in the Materials and Methods. Each experiment was repeated four times. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments. 

B) The *celR* gene was overexpressed in NTL7, NTL7ΔcelA::lacZ or NTL7ΔdivK::Km and the constructs were assessed for production of cellulose as described in Materials and Methods. Each experiment was repeated four times. The values represent the average of the four samples of each strain, and the error bars represent the standard error of the experiments.

C) Either *celR* or *celRD53E* was introduced into NTL7 and NTL7ΔdivK::Km by Tn7 insertion, and the constructs were examined for cellulose production as described in Materials and Methods. The experiments were repeated four times. The values represent the average of four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 3.7.

(A) 

(B)
Figure 3.7. (cont.)

Figure 3.7. Stimulation of cellulose synthesis requires both celR and divK. Strain NTL7ΔdivKcelR was complemented by Tn7 insertion with A) wild-type divK, celR or divKcelR, and B) alleles of divK or celR altered at Asp53. Strains were assessed for production of cellulose as described in Materials and Methods. The values represent the average of four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 3.8.

A

Genotype

Inserted Genes

NTL7  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR


μg cellulose / 10^6 cells

B

Genotype

Inserted Genes

NTL7  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR  ΔdivKcellR


μg cellulose / 10^6 cells
Figure 3.8. An aspartate at position 53 in both DivK and CelR is necessary for stimulating cellulose production. Strain NTL7ΔdivKcelR was complemented by Tn7 insertion with prdivKcelR containing A) an altered form of divK or celR at Asp53 to either alanine or glutamate, or B) with both divK and celR mutated at Asp53 to either alanine or glutamate. The cells were grown, harvested and assessed for production of cellulose as described in Materials and Methods. The values represent the average of four samples of each strain, and the error bars represent the standard error of the experiments.
Figure 3.9.
Figure 3.9. Deleting \textit{divK} affects cell morphology. Cultures of NTL7 and its derivatives were grown in MG/L, and samples were viewed by phase-contrast microscopy as described in Materials and Methods. \textbf{A)} NTL7. \textbf{B)} NTL7\textit{ΔdivKcelR}. \textbf{C)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{prdivKcelR}. \textbf{D)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{pcelR}. \textbf{E)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{prcelRD53E}. \textbf{F)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{prdivK}. \textbf{G)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{prdivKD53E}. \textbf{H)} NTL7\textit{ΔdivKcelR}/Tn7-\textit{prdivKD53EcelR}. Scale bars represent 10 µm. Arrows mark branched or elongated cells.
Figure 3.10. CelR forms homodimers, but does not interact with DivK. A) Reporter strain SU101 with positive (pDP804) and negative controls (pSR659) and with two-hybrid expression vectors containing alleles of celR. B) Reporter strain SU202 containing positive (pDP804/pMS604) and negative controls (pSR658/pSR659) and two-hybrid expression vectors with wild-type divK and altered alleles of celR, or celR and altered alleles of divK. C) Reporter strain SU202 containing positive (pDP804/pMS604) and negative controls (pSR658/pSR659) and two-hybrid expression vectors with altered alleles of divK and celR. All strains were grown on LB agar plates containing the appropriate antibiotics, IPTG and X-gal overnight at 28°C.
Figure 3.11. Amino acid sequence alignment of CelR of *A. tumefaciens*, CelR2 of *R. leguminosarum* and PleD of *C. crescentus*. The catalytic GGEEF motif is boxed, and the conserved aspartate residue and the altered glutamate in the GGEEF motif are marked with arrows. Identical residues between the three genes are purple, conserved residues between two genes are blue, similar residues are in red, and non-conserved residues are in white. The Textbox graphic was produced from the ClustalW multiple sequence alignment routine of SDSC Biology Workbench (SDSC).
Figure 3.12. Amino acid sequence alignment of DivK of *A. tumefaciens*, CelR1 of *R. leguminosarum* and DivK of *C. crescentus*. The conserved aspartate residue is marked with an arrow. Identical residues between the three genes are purple, conserved residues between two genes are blue, similar residues are in red, and non-conserved residues are in white. The Textbox graphic was produced from the ClustalW multiple sequence alignment routine of SDSC Biology Workbench (SDSC).
Chapter 4

AvmA, a homolog of the cyclic-di-GMP synthase CelR, modulates unipolar polysaccharide production and virulence in Agrobacterium tumefaciens through different mechanisms

4.1 Notes and Acknowledgements


Additionally, I would like to thank Dr. Lois Banta for contributing to the SEM and qPCR data provided in this chapter, as well as to Dr. Xavier Nesme for his analysis of biovar 1 genomovars for this project.

4.2 Summary

Agrobacterium tumefaciens must attach to plant tissue to establish microcolonies and biofilms, as well as to initiate tumorigenesis. This process is likely mediated by several mechanisms, including the formation of a unipolar polysaccharide, or UPP. Recent studies suggest that the intracellular signal c-di-GMP regulates production of this polar structure. Here, we provide evidence that one diguanylate cyclase, AvmA, not only is an active enzyme that synthesizes c-di-GMP, but is directly involved in regulating production of the UPP. Mutants of avmA display increased UPP synthesis and polar attachment to surfaces, suggesting that the product of this gene negatively influences
production of the structure. Deleting the gene also results in reduced biofilm formation, while increasing individual cell attachment. These observations suggest that the DGC influences establishment of microcolonies after the bacteria associate with a surface. While overexpressing \textit{avmA} strongly attenuated virulence, deleting the gene significantly lowered the number of bacteria necessary to initiate crown gall tumors. Interestingly, this effect on tumorigenesis did not require the GGEEF and CheY motifs, suggesting that although AvmA appears to negatively modulate tumorigenesis, the enzyme does not affect virulence through synthesis of c-di-GMP.

4.3 Introduction

Attachment to surfaces is critical for the survival and growth of many pathogenic bacteria. \textit{Agrobacterium tumefaciens}, the causative agent of crown gall disease in plants, must attach in order to initiate infection (147, 148), a process that involves transfer of DNA and proteins from the bacteria directly to the plant host (47, 77). Interaction with the plant is likely through a two-step process, beginning with a reversible interaction between the bacteria and the surface, followed by a tighter, irreversible binding (156, 163).

Recently, an attachment structure named the unipolar polysaccharide (UPP) was identified as a potential component for anchoring cells of \textit{A. tumefaciens} to plant tissue (267). This glucomannan-based adhesin is typically located at one pole of the cell (267), that associates with the attached surface (143). The UPP is similar to attachment structures seen in other alphaproteobacteria, including the holdfast adhesive located at the end of the stalk of \textit{Caulobacter crescentus} (168), and a polar adhesion structure found in
*Rhizobium leguminosarum* (10, 139). The presence of these polar anchoring structures in a number of alphaproteobacteria suggests a common theme of polar attachment within this family.

Attachment to surfaces by bacteria is often tightly controlled to allow ordered transitions between motile and sessile forms of the cell, and studies of the UPP in *A. tumefaciens* suggest that production of this structure is also regulated. Formation of UPP apparently is triggered by physical contact of the cell with a surface, thereby anchoring the cell in a polar orientation (143). In addition, synthesis of the UPP responds to levels of the small intracellular signal molecule cyclic di-guanosine monophosphate (c-di-GMP) (14, 294). This molecule is synthesized by a class of enzymes, called diguanylate cyclases (DGC), identified by a conserved GG(D/E)EF motif within the catalytic domain (106, 206). Cyclic-di-GMP is utilized by bacteria for regulating a number of cellular processes, one being the conversion from motile to sessile cells (106, 205, 206). In *A. tumefaciens*, Xu et al. (294) identified three DGCs, encoded by *atu1257*, *atu1691*, and *atu2179*, that affect UPP formation and subsequent attachment.

In this study, we identify another DGC encoded by *atu1060* that influences UPP production, biofilm formation, attachment to plant surfaces and virulence of *A. tumefaciens*. Based on the effects of this gene, we renamed the ORF as attachment and virulence modulator A, or *AvmA*. Deleting *avmA* increases lectin binding and surface attachment, suggesting that the DGC negatively regulates UPP formation and attachment. Furthermore, deleting *avmA* lowers the effective number of cells necessary to induce tumors, suggesting that this DGC is involved in regulating some event involved in virulence. The phenotypes associated with deleting *avmA* on attachment, UPP production
virulence suggest that this protein may interface initial attachment of the bacteria to plant tissue with later events specific to virulence.

4.4 Materials and Methods

4.4.1 Strains, cultures and growth conditions. The strains used in this study are listed in Table 4.1. Strains of *Escherichia coli* were grown on LB agar plates with appropriate antibiotics at 37°C. Strains of *A. tumefaciens* were maintained on NA or ABM (41) plates with appropriate antibiotics at 28°C. Cultures of *E. coli* were grown in LB broth with the corresponding antibiotics at 37°C with shaking. Cultures of *A. tumefaciens* were grown in MG/L (31) minimal medium with appropriate antibiotics at 28°C with shaking. For some experiments, cultures of *A. tumefaciens* were grown in AB minimal medium-based *vir* induction medium (ABVIM) (31) supplemented with 0.2% glucose and 200 µg/ml acetosyringone. Antibiotics used include ampicillin (100 µg/ml for *E. coli*), carbenicillin (50 µg/ml for *A. tumefaciens*), kanamycin (50 µg/ml for *E. coli* and *A. tumefaciens*), gentamicin (50 µg/ml for *E. coli*, 25 µg/ml for *A. tumefaciens*), and tetracycline (10 µg/ml for *E. coli* and *A. tumefaciens*). When necessary, Congo red (50 µg/ml) was added to ABM plates for assessing production of exopolysaccharides (302).

4.4.2 Strain construction. The deletion mutant NTL7ΔavmA::Km was constructed as described (14).

Complementation of NTL7ΔavmA::Km: A 2.2 kb fragment containing both the promoter and open reading frame (*atu1060*) of *avmA* was amplified using Pfu DNA polymerase and the primers pravmA-f (5’-GCGGTACCCAAAACCGCCTGAAACATT-3’) and avmA-r (5’-
CGGATCCTCAGCCGTTCAGCCCGAT-3\'). The PCR product was digested with
BamHI and KpnI, and cloned into pUC18. After introduction into DH5α, potential
constructs were purified and confirmed by restriction analysis. The correct clone was
digested with BamHI and KpnI, and the fragment was inserted into pUC18-miniTn7t-Gm
to form pUCTn7t-pravmA. The new construct, as well as the transposase plasmid pTNS2,
were co-electroporated into NTL7ΔavmA::Km (Table 4.1), with selection for resistance
to kanamycin and gentamicin. Potential mini-Tn7 integrants were confirmed by PCR
analysis.

Site-directed mutagenesis of avmA. Single nucleotide substitutions in avmA were
introduced by a PCR-based site-directed mutagenesis protocol (15) using pUCavmA and
pUCTn7-Gm-pravmA as the target plasmids. To alter the aspartate residue at position 59
to an alanine, the adenosine at position 158 was changed to a cytosine using the primers
avmA_D59A-f (5'-TCAACCTGCCCGCTGCGCCCAAGGG-3') and avmA_D59A-r (5'-
CCCTTGGGCGCAGCGGGCAGGTTGA-3'). The second glutamate residue in the
GGEEF motif, located at position 338 of the amino acid sequence, was also converted to
an alanine by exchanging the adenosine located at position 1118 to a cytosine using the
primers avmA_E338A-f (5'-TCGGCGGTGAGGCATTTGCCGTGTT-3') and avmA_E338A-r (5'-
AACACGGCAAATGCCTCACCGCCGA-3'). The resulting
amplified plasmids were digested with DpnI and transformed into DH5α (219). After
selecting for resistance to the appropriate antibiotic, the plasmids were isolated and the
mutations were confirmed by sequence analysis. The altered alleles were recloned into
pZLQ and were introduced into the appropriate strains by electroporation. Alleles
constructed in the mini-Tn7 vector were introduced into the bacterial chromosome by transposition (15).

4.4.3 Microscopy and lectin-binding assays. Cells, grown in liquid culture for two days at 28°C, were collected by centrifugation at 4000x g for five minutes before resuspending in 0.9% NaCl to a final OD$_{600}$ of 0.4. For lectin staining, the resuspended cells were incubated with 100 µg per ml of Alexafluor633-WGA (Thermo Scientific) for 15 minutes, and washed three times with 0.9% NaCl by centrifugation. Cells were visualized by differential interference contrast (DIC) microscopy at the Microscopy and Imaging Facility, Institute for Genomic Biology (University of Illinois) using a Zeiss Axiovert™ 200M microscope equipped with an Apotome Structured Illumination Optical Sectioning System set at 63x/1.40 objective magnification, and images were captured using a Zeiss MRc 5 camera. Where cells were treated with lectin, samples were excited at 633 nm and observed for fluorescence at 647 nm. Images were compiled and analyzed using Zeiss Axiovision™ software. For statistical analysis, four images containing cells were compiled, and the number of bacteria in each image, and their arrangement and lectin labeling were counted, with statistical analysis performed using the Chi-squared test.

4.4.4 Microscopic analysis of bacterial attachment to Arabidopsis surfaces. Scanning Electron Microscopy (SEM), performed in the laboratory of Dr. Lois Banta (Williams College, MA), was used to assess attachment of the bacteria to plant tissue. Seeds of Arabidopsis thaliana ecotype Columbia (Col-0) were surface-sterilized with a solution of 50% bleach/0.1% SDS, and sown onto solid Gamborg’s B5 medium containing 100 µg/ml tricarcillin (Research Products International). Seeds were incubated
for two days at 4°C, and then germinated and grown at room temperature for sixteen days. Strains were grown in MG/L medium with appropriate antibiotics at 28°C overnight. The cells were subcultured into ABVIM containing either 100 or 200 μM acetylsyringone and grown on a rotary shaker at 22°C to mid-exponential phase (OD600 ≈ 0.5). Sterile forceps were used to wound leaves excised from seedlings before co-cultivating with bacterial cells for two days at 21°C. Co-cultivated leaf pieces were rinsed three times in ABVIM with gentle vortexing to remove any unattached bacteria. Samples were fixed in 3% glutaraldehyde (in 0.1 M HEPES; pH 7.1) for three days, then rinsed three times in 0.1 M HEPES (pH 7.1) before postfixing in 1% OsO4 for one to two hours. Samples were subsequently rinsed with distilled H2O, sequentially dehydrated in 70, 80, 90 and 100% ethanol, and immediately dried in a Ladd critical-point drying apparatus under CO2. Samples were loaded on aluminum specimen holders, sputter-coated with gold-palladium using a Polaron SEM autocoating unit, and viewed on a FEI Quanta 400 Series scanning electron microscope. Three to five leaves were examined for each bacterial strain per assay, and several representative images per leaf were captured for analysis. Analysis of the SEM samples was performed “blind” (i.e. without knowing the identity of the sample) to ensure a lack of observer bias. For statistical analysis, the number of bacterial cells in each image and the number that were attached in a polar orientation were counted. The data were analyzed for statistical significance using the Student t-test.

4.4.5 Biofilm assays. Cells were grown overnight at 28°C with appropriate antibiotics in borosilicate glass tubes under shaking conditions, then diluted 1/1000 into 2 ml of MG/L with antibiotics. The diluted samples were incubated in borosilicate tubes for
five days at room temperature without shaking. After incubation, 1 ml of 0.1% crystal violet was added to each sample and the tubes were allowed to sit at room temperature for fifteen minutes. The liquid phase of the culture was carefully decanted, and the stained tubes were gently washed three times with 2 ml ddH₂O. The remaining crystal violet stain was solubilized using 1 ml of ice-cold ethanol, and the absorbance of the ethanolic samples was measured at 540 nm using a BioRad SmartSpec™ Plus spectrophotometer. Values were compared to the average absorbance of crystal violet solutions prepared from similarly grown cultures of wild-type strain NTL7 included as a positive control.

4.4.6 Virulence assays. Bacteria were grown for two days at 28°C, collected by centrifugation, and resuspended in 1 ml of 0.9% NaCl. The population sizes of the resuspended cells were standardized to an OD₆₀₀ of 1.0, and the suspensions were diluted to ratios of 1:10, 1:100, 1:1000, 1:10,000 and 1:100,000. Twenty mm-long wounds were produced between the primary leaves and the first set of secondary leaves on stems of Solanum lycopersicum (tomato). Two-µl volumes of cell suspensions at each dilution were inoculated into the wound sites, and the plants were incubated in the greenhouse for three to five weeks, depending on day length and plant growth rates. Each dilution of each strain was tested on four plants. Total tumor mass was determined by excising a segment of the stem, cutting above and below the wound site. The stem pieces were weighed individually, and the tumor mass was removed by cutting with a cork borer and weighed. The tumor mass was averaged between the four plants. The experiments were repeated at least three times, and the total average of the samples, as well as standard
error, were calculated from these experiments. Statistical analysis was performed using the Student t-test using a one-sided distribution model.

4.4.7 Expression analysis of virB4 by qPCR. Expression of the virB operon was assessed by quantitative PCR in the laboratory of Dr. Lois Banta as follows. Bacterial cultures were grown in ABVIM for from six to 18 hours at 28°C with shaking. Total RNA was isolated (Qiagen), reverse transcribed into cDNA, and amplified by qPCR using primers specific for virB4 or the A. tumefaciens housekeeping gene, purA, as a control. The fold change in virB4 expression was determined by comparing the difference in cycle threshold (Ct) values of the gene to values obtained for purA. Each experiment was performed in triplicate, each experiment was repeated four times, and the average of the experiments with standard error was calculated.

4.5 Results

4.5.1 AvmA affects aggregation and interaction of A. tumefaciens with surfaces. Previously, we reported that overexpressing celR results in increased cellulose synthesis and lectin binding, while decreasing biofilm formation and virulence (14). Further analysis demonstrated that CelIR is a positive regulator of cellulose production, but this DGC does not regulate lectin binding or virulence (14, 15). Overexpressing a related DGC encoded by atu1060, which we rename avmA, also resulted in increased cellulose synthesis, but does not directly regulate production of the polymer (14). These observations prompted a more thorough analysis of the role of avmA in phenomenon that involve attachment.
Our previous results indicate that when overproduced, AvmA can crosstalk with CelR to stimulate cellulose synthesis (14), suggesting that when overexpressed both DGCs can influence the same systems. Since deleting celR does not affect polar attachment to surfaces or lectin binding, AvmA instead may directly influence UPP production and adhesion to surfaces. To assess the effects of overexpressing and deleting avmA on individual bacteria, we examined cells of NTL7 altered in expression of the gene using DIC microscopy. Wild-type NTL7 grew in liquid as dispersed individual bacteria (Figure 4.1A). When wet mounts were prepared for microscopy, some cells interact with the slide, binding in both polar and lateral orientations (Figure 4.1A). Overexpressing avmA resulted in dense masses of cells unassociated with the glass surface, as compared to the wild-type NTL7 (Figures 4.1A and B). The clumping decreased when avmA was overexpressed in NTL7Δcel (Figures 4.1C and D), demonstrating that increased synthesis of cellulose is responsible for the massive aggregation. Deleting avmA did not affect cellulose-based aggregation as compared to wild-type NTL7 (Figures 4.1A and F). Interestingly, as compared to its parent, a significantly greater number of cells of the avmA mutant were attached to the glass microscopic slide in a polar orientation (Figure 4.1F; Table 4.2). This increase in polar attachment exhibited by the mutant suggests that avmA is affecting how the bacteria interact with surfaces.

Among cells not associated with masses, both NTL7 and NTL7Δcel overexpressing avmA formed rosettes, with three to five cells interacting with each other at the poles of the bacteria (Figures 4.1D and E; Table 4.2). Neither wild-type NTL7 nor the cel+ mutant formed detectable numbers of rosettes (Table 4.2), indicating that
overexpressing avmA stimulates formation of these arrangements, regardless of cellulose synthesis. The avmA deletion mutant displayed a slight but statistically insignificant change in the frequency of rosetting as compared to the wild-type parent (Table 4.2). These observations suggest that overexpressing avmA increases rosetting by the bacteria.

CelR, a close homolog to AvmA, requires the conserved GGEEF motif and the conserved aspartate in the CheY-like receiver domain to regulate cellulose synthesis (15). To determine if AvmA is a bona fide c-di-GMP synthase, we utilized cellulose-based aggregation as a measurement of activity of mutants of the DGC altered at the aspartate residue (avmAD59A) or at a key residue of the GGEEF motif (avmAE338A). In cultures grown in MG/L, overexpressing avmAE338A in NTL7 did not increase aggregation to levels comparable to overexpressing wild-type avmA in NTL7 (Figures 4.1B and G). There was some aggregation when avmAD59A was overexpressed in NTL7, as compared to the parent (Figures 4.1A and H), but much less than we observed for NTL7 overexpressing the wild-type gene (Figures 4.1B and H). These observations suggest that like CelR, AvmA synthesizes c-di-GMP, and requires the conserved aspartate residue in the CheY domain for maximum activity.

4.5.2 Modification of avmA expression results in increased polar lectin binding. Previously, we considered the possibility that CelR regulates other mechanisms of attachment beyond cellulose synthesis. However, deleting cellR had no effect on lectin binding (14), a marker for UPP formation. This result suggests that another DGC is involved in stimulating formation of the polar structure. Xu et al. (294) demonstrated that three other putative DGCs stimulate formation of the UPP, suggesting that multiple enzymes regulate polar attachment of the bacteria. Given that deleting avmA results in
increased polar attachment to glass slides, we considered the possibility that \textit{avmA} also regulates UPP production in \textit{A. tumefaciens}.

We examined the effects of overexpressing or deleting \textit{avmA} on UPP production by incubating strains with fluorescently-labeled WGA. Individual cells of NTL7 and NTL7\textit{Δcel} overexpressing \textit{avmA} showed increased binding of labeled lectin, as compared to the wild-type parents (Figures 4.2A-D; Table 4.2). Interestingly, a significant number of rosetting cells overexpressing \textit{avmA} bound the WGA label at the junction of where the poles of the cells interact (Table 4.2). These results suggest that UPP is responsible for rosetting, and that overexpressing \textit{avmA} affects rosette formation through increased UPP production. Deleting \textit{avmA} from NTL7 also resulted in increased WGA binding, as compared to the parent (Figures 4.2A, 4.2E and 4.2F; Table 4.2). These observations demonstrate that \textit{avmA} participates in regulating UPP formation, which may affect polar attachment to surfaces and to other bacteria.

\textbf{4.5.3 Modifying expression of \textit{avmA} significantly impacts biofilm formation on borosilicate glass.} That \textit{avmA} appears to regulate attachment in \textit{A. tumefaciens} opened the possibility that it may influence biofilm formation. To determine if the gene modulates establishment of biofilms, cultures were grown in MG/L medium in borosilicate glass tubes and adherent cells were stained with crystal violet as described in Materials and Methods. Strains NTL7 and NTL7\textit{Δcel} displayed similar levels of staining, forming rings of biofilm at the meniscus of the media (Figure 4.3). These results indicate that under the conditions tested cellulose does not contribute detectibly to biofilm formation on glass surfaces, in agreement with our previous findings (14). Overexpressing \textit{avmA} in NTL7 resulted in less staining as compared to its parent, while
overexpressing the gene in the cel- background did not affect staining (Figure 4.3). These results indicate that increased expression of avmA negatively affected biofilm formation, probably by stimulating cellulose synthesis. The deletion mutant NTL7ΔavmA exhibited significantly decreased levels of crystal violet staining at the meniscus as compared to wild-type (Figure 4.3). However, unlike its wild-type parent, the deletion mutant formed a thin film of cells bound to the glass tube (data not shown), suggesting increased individual cell attachment. This effect indicates that avmA is required for successful biofilm establishment on glass and negatively regulates attachment of individual cells to surfaces.

4.5.4 AvmA suppresses polar attachment of bacteria to leaf tissue. The effects of avmA on UPP formation suggest that the gene negatively regulates binding of cells to surfaces. To determine if the effects of the DGC also impact attachment of the bacteria to plant tissue, we submitted samples to Lois Banta (Williams College, MA) to examine interactions of the cells with leaf explants of Arabidopsis thaliana by SEM. In comparison to NTL7, fewer cells of the avmA mutant were attached to the plant tissue (Figures 4.4A and B). Additionally, as compared to the wild-type parent, a greater fraction of cells of the avmA mutant were attached in a polar orientation (Figure 4.4B). These results, which are consistent with our microscopy observations described above, show that deleting avmA has both qualitative and quantitative effects on attachment of the bacteria to plant cell surfaces.

4.5.5 Modifying avmA expression and structure significantly impacts virulence of A. tumefaciens. Strains NTL7 or NTL7Δcel overexpressing avmA are severely attenuated for virulence (14). Because the ΔcelR mutant is fully virulent (14), it
is possible that AvmA directly impacts tumorigenesis. Plants infected with the *avmA* mutant produced significantly larger tumors compared to those infected with NTL7, the wild-type parent (Figure 4.5A). As assessed quantitatively, the deletion mutant induced tumors at populations 10-fold lower than wild-type (Figure 4.5A), suggesting that deleting the gene lowers the number of cells needed to initiate tumorigenesis. To confirm that *avmA*, and not a random mutation, affects virulence, the wild-type gene under control of its native promoter was reintroduced into the mutant by Tn7 insertion. Complementing the mutant with *avmA* restored the dose dependence for tumor induction to wild-type levels (Figure 4.5A).

Given its domain structure, we considered the possibility that AvmA affects virulence by synthesizing c-di-GMP in response to some signal. To assess this, the two mutant alleles altered in the GGEEF or the CheY-like domain, under control of their native promoters, were tested for their ability to complement the Δ*avmA* mutant. Interestingly, both Tn7-pr*avmA*D59A and Tn7-pr*avmA*E338A restored dose-dependent virulence of the mutant to wild-type levels (Figure 4.5A). These observations suggest that AvmA does not affect virulence through c-di-GMP synthesis, and does not require activation through its CheY domain. However, while overexpressing wild-type *avmA* significantly attenuated virulence (Figure 4.5B; (14)), overexpressing either mutant allele in NTL7 failed to influence tumor induction as compared to the wild-type parent lacking the overexpression plasmid (Figure 4.5B). These results indicate that the overexpression phenotype of *avmA* on virulence is due to c-di-GMP production, and suggest that the DGC is crosstalking with another DGC, although likely not CelR.
4.5.6 Modifying avmA expression affects transcription of virB4. While our results suggest that AvmA affects virulence, the mechanism by which it does so is unknown. One possibility is that the product of the gene affects transcription of components of the vir regulon. To determine if expression of the vir operons is affected by AvmA, Lois Banta’s group at Williams College examined the transcript levels of virB4, a gene within the virB operon of the regulon, in strains modified for expression of avmA. In strain NTL7, virB4 was induced to increasingly higher levels after six and eighteen hours of induction in VIM supplemented with acetosyringone (Figure 4.6). The cel mutant exhibited a slight decrease in expression as compared to the wild-type parent (Figure 4.6), suggesting that failure to produce cellulose modestly affects induction of the virB operon. Overexpressing avmA in NTL7Δcel significantly lowered transcription of virB4, with no increase in expression even after eighteen hours as compared to NTL7 or the cel parent (Figure 4.6). Interestingly, deleting avmA in NTL7 slightly lowered expression of virB4 as compared to the wild-type, and to levels comparable to the cel mutant (Figure 4.6). Based on these observations, AvmA in its wild-type state likely does not affect tumor induction through regulation of transcription of the virB operon.

4.5.7 avmA is found only in biovar 1 agrobacteria. Given its roles in attachment and virulence, we examined the phylogenetic distribution of avmA in the family Rhizobiaceae. A BLAST search showed the gene to be present in only a subset of the available Agrobacterium genomes (NBCI). Strains with the gene all fell in the biovar 1 group, with some of the orthologs annotated as pleD (NBCI). Dr. Xavier Nesme, Lyon, France, has determined the complete genome sequence of one or more representatives of all nine genomovars of the biovar 1 group. His analysis showed that all members harbor
highly related orthologs of \textit{avmA}, and that the gene occupies the same syntenic location in all of these genomovars (Fig 4.7). Further, all of the other rhizobia examined have genes less related to \textit{avmA}, and are likely orthologs of \textit{celR}. We conclude from these analyses that \textit{avmA} is unique to the biovar 1 agrobacteria.

4.6 Discussion

4.6.1 \textit{AvmA directly regulates UPP production and attachment in \textit{A. tumefaciens}}. Previously, we demonstrated that overexpressing \textit{celR} stimulates aggregation and UPP formation, while deleting the gene does not affect these processes \cite{14}. These observations suggest that another DGC directly regulates attachment and UPP production. In this study, our genetic evidence indicates that \textit{atu1060}, we now call \textit{avmA}, negatively influences polar attachment by reducing deployment of the UPP. We also provide supporting evidence that the UPP is involved in attaching the bacteria to plant cells in a polar orientation, suggesting that this structure contributes to the initial attachment step necessary for the bacteria to establish an interaction with their plant hosts.

Several studies support the role of c-di-GMP in modulating UPP production and attachment. Xu \textit{et al.} \cite{294} reported that deployment of the polar adhesion structure is induced by c-di-GMP, resulting in increased polar lectin binding. These researchers also reported that three putative DGCs stimulate formation of the polar structure \cite{294}. Increased c-di-GMP levels also drive holdfast production in \textit{C. crescentus} \cite{94}, suggesting that DGC-mediated regulation of UPP synthesis is a theme that is conserved among the alphaproteobacteria.
Here we show that *avmA*, a fourth DGC, influences UPP production. Interestingly, either overexpressing or deleting *avmA* results in increased UPP production and polar attachment in *A. tumefaciens* (Table 4.2). This is a surprising result, given that in many cases, overexpressing and deleting a gene result in opposite phenotypes. These observations raise the question as to which modification of *avmA* best represents its function in UPP production. Similar to these effects, either overexpressing or deleting *wspR*, a DGC of *P. fluorescens*, stimulates attachment of the bacteria to surfaces (57, 152). Further research demonstrated that overproduction of WspR results in crosstalk with other DGCs involved in modulating attachment (152), suggesting that deleting *wspR* is a more accurate assessment for the role of the gene. This observation indicates that overexpressing *avmA* increases levels of c-di-GMP and induces UPP production, probably by crosstalk with one or more of the other three putative DGCs in this system (294). Based on the phenotypes of the Δ*avmA* mutant, it is likely that AvmA negatively regulates UPP deployment. We propose that the c-di-GMP signal produced by AvmA targets some unknown factor that modulates UPP assembly or disassembly.

Our studies using lectin binding indicate that the UPP also is responsible for rosetting and that the cells are held together by the UPP at their poles. A number of alphaproteobacteria form rosettes, including biovar 1 strains of *Agrobacterium* (22, 96, 289), some species of *Rhizobium* (88), uncharacterized species of oceanic alphaproteobacteria (213), *Caulobacter* (185) and *Roseobacter* (28). In *Caulobacter*, the rosettes are formed by the bacteria interacting with each other at their adhesive holdfasts at the tip of the stalks (168, 185). In *Agrobacterium*, these arrangements form at the surface of unshaken medium, and only after several hours of incubation (22). Our results
demonstrate that the production of the UPP is linked to the formation of rosettes, and that AvmA prevents the cells from forming into these arrangements. Combined, these observations strongly suggest that UPP-like structures are responsible for rosette formation among other species of alphaproteobacteria.

The function of the UPP in attachment to nonbiological surfaces is well known (143, 267), yet its role in interactions with plant tissue has not been described. Our studies of avmA confirm that overproduction of the UPP increases individual cell attachment to glass surfaces in a polar orientation (Figure 4.3). Importantly, we demonstrate that the avmA deletion mutant exhibits increased polar attachment of individual bacteria not only to glass surfaces, but also to leaf tissue (Figure 4.4). While we did not directly confirm that the pole containing the UPP contacts the plant surface, our evidence suggests that the structure is necessary for polar attachment to plant tissue.

Our work on the function of avmA in attachment and UPP formation provides evidence for a role of the polar structure in the initial attachment of A. tumefaciens to plant tissue. We propose a model in which production of the UPP is stimulated by surface contact (143), suggesting that a rapid signaling mechanism, perhaps mediated by the three DGCs described by Xu et al. (294), is necessary to form the polar attachment structure. Deployment of the UPP is responsible for the initial polar interaction between the bacterium and the plant cell. At some later time in the process of attachment, in response to a signal, AvmA is activated, resulting in inactivation or disassembly of the UPP and release of the polar-bound bacteria. Presumably, the bacteria then reestablish a second, stable binding to form microcolonies. Interestingly, lon mutants of strain C58, which are strongly attenuated (252), display increased and very strong polar attachment
(Su, unpublished), suggesting that the protease plays a role in transitioning from polar binding to some other type of interaction. It is possible that AvmA recruits or activates Lon in some manner, resulting in removal of the UPP. Further analysis of AvmA and polar attachment will be necessary to explore these possibilities.

### 4.6.2 AvmA is the first DGC directly linked to virulence in *A. tumefaciens.*

In earlier studies we reported that overexpressing either *celR* or *avmA* attenuates virulence in *A. tumefaciens* (14). Here, we demonstrate that AvmA exerts a modulatory effect on tumorigenesis. Deleting *avmA* significantly lowers the population size of bacteria necessary to initiate tumors (Figure 4.5), suggesting that the DGC affects virulence efficiency in some manner. Surprisingly, even though more efficient at inducing tumors, the *avmA* mutant displayed a statistically lower level of transcription of *virB4* (Figure 4.6), demonstrating that tumor induction does not require high levels of expression of the *virB* operon. That other supervirulent strains of *A. tumefaciens* display high levels of expression for both the *virB* operon and *virG* (109) suggests that the effects of *avmA* on tumor induction involve a previously undescribed mechanism.

The role of *avmA* in virulence is clouded by complementation studies with the two mutant alleles of the gene. Both *avmA*D59A and *avmA*E338A complemented the deletion mutant for tumor induction (Figure 4.5A), indicating that AvmA does not require activation or enzymatic activity to impact virulence. However, in contrast to the attenuated phenotype of cells overexpressing wild-type *avmA*, overexpressing either altered allele did not reduce virulence (Figure 4.5B). From these observations, we hypothesize that overexpressing *avmA* affects virulence through c-di-GMP synthesis and
crosstalk with other DGCs, while deleting the gene affects tumor induction through a separate mechanism.

Based on these combined studies, it is likely that AvmA negatively modulates deployment of the UPP, indirectly affecting virulence in biovar 1 species of \textit{Agrobacterium}. Surface contact with the plant cell results in production of the polar structure (143), likely due to signaling through specific DGCs (294). An increased number of bacteria producing the UPP results in more cells associated with the plant, keeping the bacteria within the nutrient-rich rhizosphere. AvmA most likely is activated in response to some unknown signal, likely resulting in phosphorylation at its CheY-like domain. Activation of AvmA results in removal or inactivation of the UPP. In this manner, AvmA plays a role in transitioning the cells from UPP-dependent polar binding for initial interactions with the plant to later stages of attachment, including microcolony formation through cellulose.

### 4.6.3 AvmA is unique to biovar 1 genomovars of agrobacteria.

The high degree of relatedness and organization of \textit{avmA} among the biovar 1 isolates suggests that the DGC plays a specific role in attachment within these genomovars. No other member of the family Rhizobiaceae contains \textit{avmA} (NBCI), suggesting that the species evolved this mode of regulation of initial attachment separately from the rest of the family. Interestingly, another set of genes involved in attachment, annotated as the \textit{rap} genes, are only present in select isolates on the genus \textit{Rhizobium} (171), suggesting that different species of the Rhizobiaceae utilize variations in the attachment mechanism to interact with plants. These observations strongly suggest that AvmA was established as a specific
regulator for transitioning biovar 1 genomovars of agrobacteria from polar attachment to later stages of interaction with the plant.
### 4.7 Tables and Figures

#### Table 4.1. Strains and plasmids used in this study.

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<tr>
<th>Strain or Plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<td>DH5α</td>
<td>supE44ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>(219)</td>
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<td>S17-1 λpir</td>
<td>Pro⁻ Res⁻ Mod⁺ recA; integrated RP4- Tc⁻::Mu-Kan::Tn7, Mob⁺;Sm⁻ λ::pir</td>
<td>(62)</td>
</tr>
<tr>
<td><strong>A. tumefaciens</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTL4</td>
<td>A derivative of C58, ΔtetRS, lacks pTiC58</td>
<td>(150)</td>
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<td>NTL7</td>
<td>NTL4 with pTiC58 re-introduced</td>
<td>(150)</td>
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<tr>
<td>NTL7Δcel</td>
<td>NTL7 with celC and celDE deleted, Tc⁻</td>
<td>(14)</td>
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<td>(14)</td>
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*aAbbreviations: Ap', resistance to ampicillin; Cm', resistance to chloramphenicol; Gm, resistance to gentamicin; Km', resistance to kanamycin; Sm', resistance to streptomycin; Tc', resistance to tetracycline.*
Table 4.2. Altering \( avmA \) expression affects lectin binding, polar binding and rosette formation.

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<th>Total Cells</th>
<th>% Labeled(^a)</th>
<th>% Polar(^b)</th>
<th>% Rosette(^c)</th>
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<td>0</td>
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<td>NTL7(Δcel)</td>
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<td>1</td>
<td>0</td>
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<td>12*</td>
<td>2*</td>
<td>2*</td>
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<td>478</td>
<td>9*</td>
<td>4*</td>
<td>3*</td>
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<tr>
<td>NTL7(ΔavmA::Km)</td>
<td>209</td>
<td>23*</td>
<td>18*</td>
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</table>

\(^a\) Percentage of cells examined that displayed polar lectin binding.

\(^b\) Percentage of cells examined that were bound to the glass slide in a polar orientation.

\(^c\) Percentage of cells examined that were observed in rosettes.

\( * \) \( p \leq 0.05 \) compared to NTL7 using Chi-squared analysis.
Figure 4.1. Overexpressing *avmA* affects aggregation and rosetting. Cultures of NTL7 or NTL7Δ*cel* and their derivatives were grown in MG/L and samples were viewed by DIC microscopy as described in Materials and Methods. A) NTL7. B) NTL7(pZLQ*avmA*). C) NTL7Δ*cel*. D) NTL7Δ*cel*(pZLQ*avmA*). E) Enlargement of rosette arrangement from NTL7Δ*cel*(pZLQ*avmA*). F) NTL7Δ*avmA*::Km. G) NTL7(pZLQ*avmA*E338A). H) NTL7(pZLQ*avmA*D59A). Circles mark representative rosette formations. Scale bars represent 5 µm or 20 µm.
Figure 4.2. Cells modified in avmA expression display increased polar binding of WGA at their poles. Cells grown in MG/L with the appropriate antibiotics were collected, incubated with WGA-Alexafluor 633 and observed by fluorescence microscopy as described in Materials and Methods.  

A) NTL7. B) NTL7(pZLQavmA). C) NTL7Δcel(pZLQavmA). D) NTL7Δcel. E) NTL7ΔavmA::Km. F) Enlargement of cells of NTL7ΔavmA::Km. Circled cells represent examples of polar-bound lectin, with polar attached cells marked with arrows. Scale bars represent distance as depicted.
Figure 4.3. AvmA affects biofilm formation on glass surfaces. Cultures were grown in MG/L with the appropriate antibiotics in borosilicate tubes and assayed for adherence to the glass surface by crystal violet staining as described in Materials and Methods. Each strain was grown in triplicate, and each experiment was repeated three times. The values represent the average of the nine total samples, with error bars representing the standard error of the experiments.
Figure 4.4. Cells altered in the expression of avmA are affected in plant attachment.

Strains were cultured and inoculated onto Arabidopsis thaliana leaves, and the interactions were visualized by SEM as described in Materials and Methods by the laboratory of Dr. Lois Banta, Williams College, MA. A) NTL7. B) NTL7ΔavmA::Km.
Figure 4.5. Altering expression of *avmA* affects virulence. 

**A)** Derivatives of NTL7 expressing altered alleles of *avmA* at wild-type levels were inoculated onto tomato stems and assessed for tumor induction as described in Materials and Methods. Each experiment was repeated three times, with five samples per experiment. The data indicate the averages of samples for each strain tested, with error bars representing standard error.

**B)** Derivatives of NTL7 overexpressing alleles of *avmA* were inoculated onto tomato stems as described in Materials and Methods. Each experiment was repeated three times, with five samples per experiment. The data indicate the averages of samples for each strain tested, with error bars representing standard error.
Figure 4.6. Modifying expression of \textit{avmA} affects transcription of \textit{virB4}. Strains of NTL7 and NTL7\textit{Δcel} were grown in Vir Induction Medium with acetylsyringone as described in Materials and Methods by the laboratory of Dr. Lois Banta, Williams College, MA. Total RNA was isolated from cells sampled at six and 18 hours and amplified by qPCR as described in the Materials and Methods. Each experiment was performed in triplicate, and was repeated four times. The values represent the average of the twelve total samples, with error bars representing the standard error of the experiments.
Figure 4.7. Phylogenetic tree of AvmA in biovar 1 isolates of *A. tumefaciens*, with representative genomes from other members of *Rhizobiaceae*. Analysis of isolates was prepared by Dr. Xavier Nesme, Lyon, France.
Chapter 5

Conclusions

5.1 CelR is a direct regulator of cellulose synthesis through production of c-di-GMP

In this study, I proposed to determine factors involved in regulating attachment and cellulose synthesis in *Agrobacterium tumefaciens*. Cellulose is a component of the matrix used to establish microcolonies and biofilms (158), although little is known concerning regulation of production of this polymer in *A. tumefaciens*. Early research demonstrated that cellulose synthesis is stimulated by increased levels of c-di-GMP (6). Here, I show that one diguanylate cyclase, CelR, is involved in stimulating production of the polymer in *A. tumefaciens*. Overexpressing *celR* results in increased cellulose synthesis, while this phenotype is lost when the *cel* operons are disrupted (Chapter 2). Deleting *celR* results in decreased production of the polymer to levels equivalent to the *cel* mutant (Chapter 2). These results identify a specific DGC, CelR, as being required for cellulose synthesis in *A. tumefaciens*.

While the phenotypes associated with deleting *celR* indicate that the gene is necessary for stimulating cellulose production, the data do not establish whether the encoded protein synthesizes c-di-GMP. Overexpressing an allele of *celR* altered at the GGEEF motif did not stimulate cellulose synthesis (Chapter 3). Further, the altered allele failed to complement the *celR* deletion mutant (Chapter 3). These observations confirm that CelR is a *bona fide* DGC, and suggest that CelR stimulates cellulose production through synthesis of c-di-GMP.
This study compellingly links a functional DGC, CelR, to cellulose production in *A. tumefaciens*, complementing previous work in other laboratories. Amikam and Benziman (6) connected c-di-GMP to cellulose synthesis in *A. tumefaciens* through biochemical analysis. The ortholog of CelR in *R. leguminosarum*, CelR2, was implicated in stimulating cellulose synthesis (11), suggesting that c-di-GMP regulates production of the polymer in other species of the Rhizobiaceae. However, no specific active DGC had been identified in *A. tumefaciens*. My research describes CelR as the enzyme stimulating cellulose synthesis.

Stimulation is necessary for full production of the cellulose fibrils in *A. tumefaciens*. The amount of cellulose synthesized by strain C58 in culture is very low (Chapter 2), and the *cel* genes are not transcribed at a high level (Chapter 3). Production of the polymer also is likely repressed, as *celG* and *celI* mutants of C58 display increased synthesis of cellulose (158). That CelI has a helix-turn-helix domain suggests that the expression of the *cel* regulon is controlled, in part, at the level of transcription (158). My results demonstrate that CelR stimulates cellulose synthesis above basal levels of production through a second, signal-dependent level of regulation, likely in response to some upstream signal.

### 5.2 CelR requires activation, probably by phosphorylation, to stimulate cellulose synthesis

The protein structure of CelR consists of the catalytic domain for synthesizing c-di-GMP and two CheY-like receiver domains, with one containing a conserved aspartate residue (Chapter 2). The presence of a conserved CheY domain with a putative active site
residue suggests that enzymatic activity of the DGC requires activation. Indeed, an allele of \textit{celR} in which Asp53 was changed to an alanine is unable to stimulate cellulose synthesis (Chapter 3). This observation demonstrates that the conserved aspartate is required for CelR to stimulate production of the polymer. In other proteins with CheY-like receiver domains, converting the aspartate residue to glutamate results in a constitutively-active form of the protein (190, 220). It is generally accepted that this alteration to glutamate mimics phosphorylation (190, 220). Expressing CelR with an Asp53 to glutamate mutation increased the level of stimulation of cellulose synthesis as compared to expressing wild-type \textit{celR} (Chapter 3). Combined, these effects of altering Asp53 suggest that CelR is an activation-dependent DGC that requires phosphorylation to stimulate synthesis of c-di-GMP.

The requirement for an active CheY domain suggests that CelR is an intermediate component of a larger signaling pathway, similar to other two-component response regulators (63, 190). That CelR is a bona fide diguanylate cyclase suggests that the DGC synthesizes c-di-GMP that then is sensed by another component of the cellulose synthesis pathway. Through this mechanism, CelR channels the upstream signal from some unidentified environmental cue to a downstream receptor.

5.3 CelA is likely stimulated by c-di-GMP synthesized by CelR

That CelR produces c-di-GMP (Chapter 3) points to an enzymatic component of cellulose synthesis that is activated by the nucleotide signal. In \textit{A. tumefaciens}, the cellulose synthase subunit CelA contains a C-terminal PilZ-type motif that could be a c-di-GMP-binding region (7). This observation led me to hypothesize that the subunit
responds to the signal produced by CelR. This hypothesis is consistent with the biochemical analysis demonstrating that addition of c-di-GMP to cell free extracts of *A. tumefaciens* stimulates cellulose synthesis (6). My studies demonstrate that CelA requires an intact PilZ domain to fully stimulate cellulose synthesis (Chapter 3). Further, overexpressing *celR* in NTL7ΔcelA::Km complemented with *celA* altered at the PilZ domain resulted in only a slight increase in production of the polymer (Chapter 3). These observations strongly suggest that CelA responds to c-di-GMP to stimulate cellulose synthesis, with the signal likely produced by CelR.

It was possible that c-di-GMP produced by CelR affects cellulose synthesis by regulating transcription of the *cel* regulon. Examining the transcription rate of *celABCG* revealed that expression of the operon is generally low (Chapter 3). Further, deleting *celR* did not affect expression of *celA*, while overexpressing the DGC resulted in a slight drop in transcription of *celA* (Chapter 3). These results confirm that CelR does not stimulate cellulose synthesis by regulating transcription of *celA*.

In a number of other bacteria c-di-GMP stimulates cellulose synthesis by activating the cellulose synthase complex. In *G. xylinus*, *in vitro* synthesis of cellulose by the purified complex of BcsA and BcsB is stimulated by c-di-GMP (166, 210). In *S. enterica*, biochemical, crystallographic and molecular studies of BcsA, the catalytic component, demonstrate that the subunit directly binds c-di-GMP, and that this binding stimulates cellulose synthesis (173, 184, 306, 307). The critical residues of the PilZ domain, the c-di-GMP binding region, are highly conserved among the cellulose synthases in bacteria (7). These observations suggest that c-di-GMP is used to regulate cellulose synthesis in most if not all bacteria that produce the polymer.
While the use of c-di-GMP as an inducing signal for cellulose synthesis is conserved among bacteria, the cyclases and pathways resulting in the signal are varied. In the Alphaproteobacterium *G. xylinus*, stimulation of cellulose synthase is driven by the diguanylate cyclases Cdg1, Cdg2 and Cdg3 (Figure 5.1; (257)). These three DGCs each contain a PAS oxygen-sensing domain, the conserved GGDEF domain and a disrupted EAL domain (Figure 5.1), and all three cyclases are activated by high levels of oxygen (257). *Salmonella enterica* activates cellulose production through the global regulator CgsD and the diguanylate cyclase AdrA (207, 307), with AdrA containing a MASE2-type membrane-associated signal-sensing domain and the GGDEF domain (Figure 5.1). These cyclases are structurally distinct from CelR of *A. tumefaciens* and *R. leguminosarum* (Figure 5.1). Combined, these observations indicate that regulation of cellulose synthesis is adapted for different conditions among cellulose-producing bacteria, and even between members of the alphaproteobacteria.

Interestingly, cellulose synthesis in species of *Pseudomonas* is stimulated by surface contact through a modified chemosensory system encoded by the *wsp* operon. Signal perception results in activation of the DGC WspR (87, 102, 152, 182). Although only 30% identical to CelR, WspR contains a single CheY-like receiver domain and the GGDEF domain (Figure 5.1). In *Pseudomonas* spp., the Wsp signal transduction complex recognizes surface contact, eventually resulting in phosphotransfer from a specific kinase, WspE, to an aspartate in the CheY domain of WspR (97, 102, 152, 182). The gene context of *wspR* and *celR* also differ. While the gene encoding WspR is part of the operon encoding most or all of the Wsp system (97), *celR* is part of a two-gene operon with *divK* in *A. tumefaciens* (15), and there are no highly conserved orthologs of the *wsp*
operon in *A. tumefaciens* (NCBI). These observations suggest that although the activities of the two DGCs are modulated by phosphorylation cascades, the systems differ in how they sense signals and transfer the information to downstream components.

### 5.4 Modifying expression of *celR* affects attachment and cell-cell interactions

Cellulose synthesis is an important component of the stable attachment to and colonization of plant surfaces by *A. tumefaciens* (158). Therefore, modulating production of this polymer by CelR and its c-di-GMP signal likely will affect attachment and establishment of microcolonies. In support of this hypothesis, overexpressing the gene results in increased UPP production and rosette formation, decreased attachment to plant tissue and reduced biofilm formation (Chapter 2). Deletion mutants of *celR* attach as individual cells at higher frequencies to plant tissue, as compared to the wild-type (Chapter 2). Further, the *celR* mutant does not efficiently establish multicellular groups of cells on solid surfaces (Chapter 2). These observations suggest that c-di-GMP is used to regulate the nature of the attachment mechanism and associated phenotypes. Consistent with my results, mutants of strain C58 displaying increased cellulose synthesis exhibit increased cellular aggregation and weaker attachment to surfaces as compared to the wild-type strain, with both traits affecting establishment of biofilms (158). These observations suggest that cellulose production must be regulated to optimize attachment of the bacteria to surfaces.

Overexpressing *celR* affected UPP deployment and binding of the bacteria to plant tissue (Chapter 2), suggesting that c-di-GMP is involved in regulating initial attachment events. Studies in other laboratories confirm that the nucleotide signal induces
UPP deployment (294). Of five putative DGCs examined in my study, only overexpression of \textit{celR} or \textit{avmA} induced deployment of the polar structure (Chapter 2). However, deleting \textit{celR} did not affect UPP deployment (Chapter 2), suggesting that another DGC, perhaps AvmA, is involved in regulating this process.

These studies point to a regulatory role for CelR in determining the stability of the interaction of the bacteria with plant tissue. CelR likely induces cellulose synthesis to aid in establishing microcolonies on plant surfaces. I propose that under specific conditions, CelR stimulates production of the polymer, likely to levels of cellulose synthesized by the \textit{celR} mutant expressing the constitutively-active form of the cyclase. Cellulose fibrils allow the cells to aggregate, while interacting with the plant surface. This contact between bacteria and the plant results in stable aggregates of tethered cells that are anchored to the colonized surface. Over time, these aggregates develop into established microcolonies and biofilms.

5.5 Cyclic-di-GMP is targeted to the appropriate signal pathway by variations in the structure of diguanylate cyclases

Overexpressing \textit{celR} leads to a number of altered phenotypes (Chapter 2), suggesting that the c-di-GMP signal is involved in several if not many regulatory pathways in \textit{A. tumefaciens}. Alternatively, overexpression may lead to overall higher cellular concentrations of signal, which could result in activating non-cognate systems. To prevent crosstalk between systems utilizing c-di-GMP, the signaling pathways must be separated, either by containment of the signal within a compartment of the cell or by variations in the sensitivity of the receptors to c-di-GMP. Examining the effects of
overexpressing different DGCs in *A. tumefaciens* could shed light on how c-di-GMP-dependent regulatory pathways are insulated from each other.

Similar to the effects of *celR*, overexpressing *avmA* results in increased cellulose synthesis, UPP deployment and attenuated virulence (Chapter 2, Chapter 4). These two DGCs are structurally related, each with two CheY-like receiver domains and the GGEEF domain (Chapter 2). These observations suggest that the two DGCs are similar enough to crosstalk between each other. In support of this hypothesis, overexpressing *atu2228*, which could code for an unrelated DGC, affects cell morphology and colony size but no other notable phenotypes (Chapter 2). Taken together, these results suggest that under normal levels of expression structurally distinct DGCs may not interact significantly between their respective target pathways. These variations in protein structure in other systems could allow the DGCs to partition to different components, localize with specific components, interact with the appropriate receptor, or activate by different mechanisms (129, 154). It is tempting to speculate that such alterations in domain organization provide a means to segregate each cyclase from the others, preventing excessive crosstalk among all the signaling systems utilizing c-di-GMP (154, 205).

My results demonstrate that CelR and AvmA can affect similar processes. However, deleting *avmA* did not affect cellulose synthesis (Chapter 2), confirming that only CelR directly regulates this polymer. Further, the *celR* deletion mutant displays normal levels of UPP deployment and virulence (Chapter 2), demonstrating that the DGC does not directly impact these processes. These observations suggest that despite their structural similarities, CelR and AvmA modulate different systems in *A. tumefaciens*.  

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5.6 Mutating avmA affects UPP production and virulence

Although modifying expression of celR affected several phenotypes, my further analysis indicated that not all of these traits are directly regulated by the DGC. Overexpressing celR results in increased UPP formation and polar attachment, and severely attenuated virulence (Chapter 2). However, deleting celR did not affect any of these phenotypes (Chapter 2). Given that overexpressing avmA resulted in phenotypes similar to those displayed by cells overexpressing celR (Chapter 2), these observations suggest that avmA may regulate one or more of these processes.

Deleting avmA affects UPP deployment and polar binding to surfaces, indicating that the product of this gene participates in regulating initial attachment events. Deletion mutants of this DGC display increased polar-oriented attachment and increased UPP production (Chapter 4). From these observations I conclude that AvmA negatively regulates UPP deployment, which in turn influences polar attachment of the bacteria to surfaces. However, overexpressing the DGC also results in increased UPP production (Chapter 4), suggesting that c-di-GMP stimulates deployment of the structure. Work from Fuqua’s laboratory showing that deleting three putative DGCs results in decreased UPP formation (294) strongly supports the hypothesis that c-di-GMP regulates this process. It is possible that UPP deployment is affected by different DGCs depending on the upstream signal. In this model, contact of the bacteria with a surface stimulates the three DGCs. Increased synthesis of c-di-GMP activates their respective receptors, resulting in deployment of the UPP and tight polar binding. Upon sensing some different signal, or at some subsequent time, AvmA is activated and synthesizes c-di-GMP. The signal from
AvmA is bound by an unidentified receptor, which activates a system that disassembles the UPP. This attendant release from polar attachment likely allows the bacteria to interact with the plant tissue by other attachment mechanisms. In this manner, AvmA would act as a switch from UPP-mediated attachment to other forms of interaction, with one involving cellulose synthesis. Interestingly, \textit{lon} mutants of \textit{A. tumefaciens} bind tightly to surfaces, and in a polar orientation (Su, unpublished data). Further, on the cellular level the \textit{lon} mutant displays a significant increase in polar lectin binding and rosette formation (unpublished data), suggesting that the protease is necessary to release the bacteria from UPP-mediated attachment. It is possible that AvmA recruits Lon to the pole, thereby signaling the degradation or disassembly of the UPP.

In addition to affecting polar attachment, modifying expression of \textit{avmA} results in increased rosette formation (Chapter 4). These aggregates of cells are held together by interactions between the UPP-containing poles of the bacteria (Chapter 4). Further, AvmA prevents the cells from forming these arrangements. Interestingly, rosette formation by wild-type \textit{A. tumefaciens} was first described in cultures grown in a broth prepared from steamed carrots (22). Moreover, the rosettes apparently were confined to the pellicle of static cultures (22). It is possible that some component from the plant stimulates deployment of the UPP, increasing rosette formation. Other alphaproteobacteria form rosettes, and in some species, UPP-like structures were identified in these arrangements (22, 28, 88, 185, 213). Based on these observations, it is possible that other DGCs modulate deployment of UPP-like structures, and that these adhesive structures are responsible for forming rosettes in select alphaproteobacteria.
AvmA greatly impacts tumorigenesis, connecting c-di-GMP and a specific DGC directly to regulation of this process in *A. tumefaciens*. As with *celR*, cells overexpressing *avmA* are strongly attenuated (Chapter 2). Unlike *celR*, *avmA* deletion mutants induce tumors at population sizes considerably lower than wild-type (Chapter 4). These results suggest that high levels of c-di-GMP from the two DGCs negatively impact virulence, and that AvmA, expressed at normal levels, directly affects this process. Interestingly, alleles of *avmA* altered at either the GGEEF motif or the conserved aspartate residue of the CheY-like domain complemented the deletion mutant (Chapter 4). These results clearly show that AvmA-mediated regulation of virulence does not involve c-di-GMP synthesis or an upstream signal, and must operate through some different mechanism.

Based on my observations, it is likely that AvmA participates in reversing UPP deployment in biovar 1 strains of *A. tumefaciens*. I suggest a model in which at the onset of attachment to normal tissue, such as roots, the bacteria physically contact the plant cell, resulting in deployment of the polar structure (143). This rapid response is likely mediated by c-di-GMP, synthesized by three DGCs (294). Once the bacteria are associated with the plant by polar attachment, the cells recognize signals from the plant and the environment. If the environment is conducive to growth, a signal activates AvmA, initiating removal of the UPP, possibly through disassembly or degradation of components by Lon. Once the cells are released from polar attachment through disassembly of the UPP, CelR stimulates cellulose synthesis, anchoring the bacteria to each other and to the surface of the plant, and thereby transitioning the cells from polar attachment to microcolony formation. This model is entirely consistent with both light
and SEM microscopy studies by the Matthysse laboratory showing that wild-type cells form plant-associated aggregates held together by cellulose fibrils (156, 157).

A similar model of attachment could account for the interactions that occur when the bacteria encounter an infection site, such as a wound on the plant. Polar attachment via the UPP is likely stimulated by surface contact, similar to attachment to normal tissue. Once associated with the wounded plant, the bacteria initiate other forms of attachment, while signaling from AvmA releases the UPP. These associations involve cellulose synthesis, since cel Δ mutants, but not wild-type cells, can be washed out of infection sites (156). Disassembly of the UPP may be directed by Lon, although it is important to note that lon mutants are severely attenuated (252). Given that deleting lon results in a number of altered phenotypes, including altered cell morphologies, increased polar attachment and attenuated virulence ((252); Su, unpublished data), it is likely that the protease is involved in several distinct processes, any or all of which could affect tumorigenesis. In this model, Lon assists in releasing the UPP, while also modulating virulence. In addition, cel Δ mutants are unable to induce tumors when the bacteria are washed out of wound sites up to eight hours after inoculation (156, 256), suggesting that cellulose fibrils, but not UPP, stabilize attachment of the bacteria at the infection site during tumor induction. This observation supports a model in which the UPP is removed early during infection, perhaps by a pathway dependent on AvmA, and suggests that the cellulose fibrils are necessary to maximize subsequent host cell binding of the bacteria at the infection site.
5.7 The response regulator DivK impacts cellulose synthesis through CelR

Our studies of CelR and AvmA suggest that they are likely part of regulatory pathways governing cellulose synthesis and attachment processes, respectively. *celR* is the distal gene in a two-gene operon that begins with *divK*. This gene encodes a response regulator consisting of a single CheY-like receiver domain (Chapter 3). Homologs of DivK in the alphaproteobacteria are involved in cell cycle regulation, and in some species holdfast production (89, 93, 124, 136, 189). These observations suggest that DivK may regulate attachment systems in *A. tumefaciens*. My studies not only confirm the role of *divK* in cell cycle regulation (Chapter 3; (124)), but demonstrate that the regulator stimulates cellulose synthesis through CelR. Compared to wild-type, *divK* mutants produce lower levels of the polymer (Chapter 3). Further, a constitutively-active form of DivK increases stimulation of cellulose synthesis, but only when *celR* is present (Chapter 3). These results show that DivK regulates both cell cycle progression and cellulose synthesis.

The dual roles of DivK in *A. tumefaciens* mirror the regulation network of cell cycle progression and polar attachment in *C. crescentus*. The ortholog of DivK in *C. crescentus* acts as a critical localization factor for both cell cycle and polar morphogenesis pathways, linking the two systems (1, 30). DivK is activated by phosphorylation through the kinase DivJ (9, 200). The activated regulator then binds and localizes DivL, another kinase, initiating a cascade resulting in polar localization and dephosphorylation of the master regulator CtrA (1, 189). CtrA is then degraded by ClpXP, allowing the cell to progress past the G1-to-S phase transition checkpoint (1, 189, 268). In this manner, DivK drives cell cycle progression in *C. crescentus*.
DivK also regulates pole morphogenesis by stimulating activation of PleD, the ortholog of CelR in *C. crescentus*. In swarmer cells, PleD is inhibited by the hybrid kinase/phosphatase PleC (190, 240, 242). Upon stimulation of DivJ and the kinase activity of PleC by DivK, the two proteins phosphorylate PleD (1, 189). The phosphorylated DGC dimerizes and localizes to the flagellated pole of the swarmer cell, resulting in detachment of the flagella and formation of the holdfast stalk (4, 35, 188, 190). By connecting the activation of PleD and degradation of CtrA, DivK links cell cycle progression to holdfast attachment in *C. crescentus*.

While my studies demonstrate that DivK in *A. tumefaciens* maintains regulatory control over both cellulose synthesis and cell cycle progression, it is likely that these processes are not directly linked. A constitutively-active form of the CheY-like regulator stimulates production of the polymer, but does not affect cell division (Chapter 3). Further, DivK is essential in *C. crescentus* (93), but not in *A. tumefaciens* or *R. leguminosarum* (11, 15, 124). *Agrobacterium tumefaciens* also contains two orthologs each of PleC and DivJ, as well as other orthologous components of the division signaling pathway of *C. crescentus* (124). PleC is not essential in *A. tumefaciens*, while it is required in *C. crescentus* (124). These observations suggest that DivK-mediated regulation of cell cycle progression in *A. tumefaciens* differs from that of *C. crescentus*. Interestingly, DivK is essential in *Sinorhizobium meliloti*, and components of the cell checkpoint pathway with *divK* are important for the symbiotic interaction of the bacteria with alfalfa (195). This observation suggests that among alphaproteobacteria, and even among members of *Rhizobiales*, there are variations in the regulatory systems controlled
by DivK. In the case of *A. tumefaciens*, the adaption of DivK may be due to the switch from regulating holdfast formation to cellulose synthesis.

DivK functions as a localization factor in *C. crescentus* (1), and this mechanism may be conserved in *A. tumefaciens*. Indeed, there is evidence that DivK of *A. tumefaciens* is required for proper localization of the FtsZ ring during cell division (124). While DivK and CelR do not physically interact (Chapter 3), it is possible that DivK functions by localizing another factor, such as the kinase of the DGC, resulting in activation of CelR and stimulation of cellulose synthesis. Further, localization studies of the cellulose synthase complex in some species of Rhizobiaceae suggest that production of the polymer occurs at the pole (140). These observations lead to the hypothesis that DivK may serve to localize many proteins to the pole of the cell, including the cellulose synthase complex. In this manner, DivK could influence the site of cellulose synthesis, but not the enzymatic activity of the synthase complex itself.

### 5.8 DivK may connect CelR and AvmA in determining the type of attachment in *A. tumefaciens*

There is a possibility that DivK modulates AvmA as well as CelR, although evidence for this hypothesis remains circumstantial. Because DivK likely localizes proteins (1, 124), the response regulator may direct other factors to the pole at which the UPP structure assembles. In *C. crescentus*, DivK localizes factors that target proteases such as ClpXP to one pole of the cell (1, 268). It is possible that DivK in *A. tumefaciens* also activates localization factors that target proteases to the pole, affecting UPP deployment or assisting in removing initial attachment factors to begin long-term binding.
through cellulose synthesis. Given that lon mutants attach extremely tightly in a polar orientation to plant tissue (Su, unpublished data), it is likely that the protease has some function in controlling UPP deployment. Based on these observations, activated AvmA may be targeted to the pole by localization through DivK. Once localized to the pole, AvmA may recruit proteases, including Lon, to the UPP. This process could then initiate reversal or prevention of UPP deployment though targeted proteolysis of attachment components.

Examining the functions of AvmA and CelR in A. tumefaciens suggests that c-di-GMP regulates different attachment mechanisms, and raises the possibility that DivK contributes to determining which attachment system is activated. It is possible, for example, that in response to some signal, DivK is activated by phosphorylation, resulting in localization of the kinases of CelR and AvmA. These kinases would phosphorylate both DGCs, which could target both CelR and AvmA to the pole. In this manner, DivK could influence the switch from initial polar attachment to cellulose production.

The site of attachment to the plant likely affects the transition between initial attachment and subsequent events. At many sites of interaction, such as with roots, the transition from polar attachment to microcolony formation may depend on cues associated with environmental conditions such as nutrient availability. After initial attachment, and if the bacteria sense an appropriate environment, they can initiate a signal to switch from polar attachment to establishment of microcolonies. This conversion explains why cel⁻ mutants are loosely associated with carrot disks (156), since signals from the plant likely stimulate the transition from UPP-mediated attachment to an intrinsically less stable form of binding. Once the bacteria are released from polar
attachment, the failure to synthesize cellulose could limit colonization by preventing aggregation of the cells with each other and also lessens the stability of the interaction between the bacteria and the plant.

The functions of AvmA and CelR in switching *A. tumefaciens* from polar attachment to other forms of interaction with surfaces provide insight into similar attachment processes in other alphaproteobacteria. Although AvmA is present only in biovar 1 isolates of *A. tumefaciens* (Chapter 4), many members of the family Rhizobiaceae associate with plants in a polar orientation (10, 139), and eventually colonize the tissue (140). My research demonstrates that a molecular switch in *A. tumefaciens* involving both CelR and AvmA transitions the bacteria from one form of attachment to another, and a similar system may function in other colonizing bacteria. In these other species, the regulatory control mediated by AvmA must be replaced by a different factor, adapted to specialized processes, such as nodulation, unique to those isolates. Given the number of identified alphaproteobacteria that both attach in a polar orientation and colonize surfaces (3, 28, 140), it is likely that similar molecular controls exist in these systems.

**5.9 Future Directions**

A significant amount of research is necessary to fully understand the molecular switch between polar attachment and microcolony formation. While both AvmA and CelR are known regulators in these systems, my research demonstrates that both DGCs require activation, likely by phosphorylation, for full stimulation of their activity. Therefore, determining the activating kinases for both proteins will help determine when
the DGCs are activated, as well as provide insight into the upstream signals determining their activation. Further, the environmental signals stimulating the activation of AvmA and CelR remain unknown.

An increasing body of evidence suggests that localization of AvmA and CelR is necessary for the successful delivery of c-di-GMP to its target receptor. Therefore, subcellular localization studies are necessary to determine if the activated DGCs target to specific regions of the cell. Similarly, determining whether CelR colocalizes with the cellulose synthase will shed light on how this DGC targets its c-di-GMP signal to its prospective target.

While my research identified AvmA as a modulator of UPP deployment, it remains unknown how the DGC affects this process. Determining the downstream receptor of AvmA is critical to understanding how the UPP is regulated. While it is likely that production of c-di-GMP by AvmA reduces deployment of the polar structure, there are only three PilZ-containing proteins in *A. tumefaciens* (7), of which one is the cellulose synthase subunit CelA. It is possible that the signal synthesized by AvmA is bound by a different receptor, such as degenerate DGC and PDEA domains, or riboswitches.

My analysis of DivK demonstrated that the regulator is involved in modulating cellulose synthesis, and its function in other systems suggests that DivK is involved in organizing regulatory systems. Therefore, it is critical to determine what DivK targets during the switch from polar attachment to microcolony formation. The factors which interact with DivK will not only determine the role the regulator plays in attachment, but also reveal why the interaction with plant tissue is loosely associated with cell cycle progression through DivK.
Figure 5.1. Domain structure of GGDEF-containing proteins involved in regulating cellulose synthesis. The domains as derived by analysis of amino acid sequences in the NCBI databases. CheY, CheY-like receiver domain; GG(D/E)EF, diguanylate cyclase domain; EAL, phosphodiesterase A domain; PAS, oxygen-sensing heme-bound domain; MASE2, membrane-associated oxygen-sensing domain.
Chapter 6

References


