CelR, an Ortholog of the Diguanylate Cyclase PleD of *Caulobacter*, Regulates Cellulose Synthesis in *Agrobacterium tumefaciens*

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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 di-GMP (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 cyclase genes, *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 Δcel mutants allowed differentiation between phenotypes associated with cellulose production, such as biofilm formation, 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 *celA*, which encodes the catalytic subunit of cellulose synthase, 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.

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. In particular, the interaction of the plant pathogen *Agrobacterium tumefaciens* with its plant host is dependent on such attachment phenomena (1, 2). 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 β 1,4-linked glucose polymer. The cellulose fibrils apparently serve to anchor bacteria to each other as well as to plants (3). Mutants deficient in the production of cellulose bind less tightly to plant cell surfaces (4, 5) and do not efficiently establish biofilms (6).

The components for production of cellulose by *A. tumefaciens* strain C58 are encoded by two closely linked operons, *celABCG* and *celDE*, located on the linear chromosome (7). 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 (8–10). CelC is similar to outer membrane porin-like proteins and may serve as the complex for secreting the polymer into the extracellular environment (11). The functions of CelD and CelE in the synthesis and secretion of cellulose are unknown (8, 11), while CelG, although of unknown function, apparently contributes to the regulation of cellulose synthesis (6). Based on evidence in other bacteria, the cellulose fibrils are believed to be extruded into the extracellular milieu from the synthase complex, which is embedded within the

membrane of the cells (12, 13). While much is known about the synthesis of the polymer, little is known concerning the mechanisms that control cellulose production in *A. tumefaciens*.

In some bacteria, production of cellulose is activated in response to the intracellular signal cyclic di-GMP (c-di-GMP) (for reviews, see references 14 to 17). Synthesis of c-di-GMP is catalyzed by a family of enzymes called diguanylate cyclases (DGCs), most of which are characterized by a conserved GG(D/E)EF motif (18). 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 (18) or by the HD-GYP domain (19, 20). 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 (21–26). 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 references 17 and 27 to 31).

The production of c-di-GMP regulates at least two attachment

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description	Reference/source
Strains		
<i>E. coli</i> strains		
DH5 α	<i>supE44</i> ϕ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	72
S17-1 λ <i>pir</i>	Pro [−] Res [−] Mod ⁺ <i>recA</i> ; integrated RP4-Tc ^r ::Mu-Kan::Tn7; Mob ⁺ Sm ^r λ :: <i>pir</i>	73
<i>A. tumefaciens</i> strains		
NTL4	Derivative of C58, Δ <i>tetAR</i> , lacks pTiC58	74
NTL4(pTiC58)	Derivative of C58, Δ <i>tetAR</i> , with pTiC58 reintroduced	74
NTL4 Δ <i>cel</i> (pTiC58)	NTL4 with <i>celC</i> and <i>celDE</i> deleted; Tc ^r	This study
ATCC 31749	Curdlan-overproducing strain	52
NTL4 Δ <i>celR</i> ::Gm(pTiC58)	NTL4 <i>celR</i> ::Gm ^r	This study
NTL4 Δ <i>atu1060</i> ::Km(pTiC58)	NTL4 <i>atu1060</i> ::Km ^r	This study
Plasmids		
pUC18	Cloning vector; Ap ^r	Invitrogen
pUC <i>celR</i>	<i>celR</i> gene cloned into pUC18	This study
pUC <i>atu1060</i>	<i>atu1060</i> gene cloned into pUC18	This study
pUC <i>atu0826</i>	<i>atu0826</i> gene cloned into pUC18	This study
pUC <i>atu2228</i>	<i>atu2228</i> gene cloned into pUC18	This study
pUC <i>atu4490</i>	<i>atu4490</i> gene cloned into pUC18	This study
pUC <i>celRdel</i>	Gm cassette between DNA sections flanking the <i>celR</i> gene; Gm ^r Ap ^r	This study
pUC <i>atu1060</i> region	Section of chromosome surrounding <i>atu1060</i> locus cloned into pUC18	This study
pUCpr	Promoter of <i>celR</i> cloned into pUC18	This study
pUCpr <i>celR</i>	pUCpr with <i>celR</i> cloned into constructed NdeI site	This study
pMGm	Vector source of gentamicin cassette; Ap ^r Gm ^r	75
pZLQ	pBBR1MCS-2-based expression vector; Km ^r	76
pZLQ <i>celR</i>	<i>celR</i> gene from pUC <i>celR</i> cloned into NdeI/BamHI sites of pZLQ	This study
pZLQ <i>atu1060</i>	<i>atu1060</i> gene from pUC <i>atu1060</i> cloned into NdeI/BamHI sites of pZLQ	This study
pZLQ <i>atu0826</i>	<i>atu0826</i> gene from pUC <i>atu0826</i> cloned into NdeI/BamHI sites of pZLQ	This study
pZLQ <i>atu2228</i>	<i>atu2228</i> gene from pUC <i>atu2228</i> cloned into NdeI/BamHI sites of pZLQ	This study
pZLQ <i>atu4490</i>	<i>atu4490</i> gene from pUC <i>atu4490</i> cloned into NdeI/BamHI sites of pZLQ	This study
pUC18mini-Tn7T-Km	Tn7 carrier vector containing Km cassette; Ap ^r Km ^r	42
pTNS2	Tn7 helper plasmid encoding the TnsABC+D-specific transposition pathway; Ap ^r	42
pUCTn7-Km-pr <i>celR</i>	pr <i>celR</i> fragment cloned into BamHI site of pUC18mini-Tn7T-Km	This study
pRK415	InP1 α broad-host-range cloning vector; Tc ^r	77
pRK <i>atu1060</i> region	<i>atu1060</i> region inserted into pRK415	This study
pRK <i>atu1060</i> kan	Allelic replacement of <i>atu1060</i> with a kanamycin cassette on pRK <i>atu1060</i> region	This study
pWM91	λ <i>pir</i> -dependent cloning vector; Ap ^r	78
pWM <i>celRdel</i>	<i>celRdel</i> fragment cloned into BamHI site of pWM91	This study
pWM <i>atu1060</i> kan	<i>atu1060</i> kan fragment cloned into BamHI site of pWM91	This study
pKD46	Lambda Red recombinase helper plasmid; Ap ^r	40
pKD4	Template plasmid of kanamycin cassette for chromosomal exchange; Ap ^r Km ^r	40
pBBR1MCS-3	Mobilizable broad-host-range cloning vector with extended multiple-cloning site; Tc ^r	79
pSR47s	Suicide vector; Ap ^r	80

processes in alphaproteobacteria. 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 (32, 33). *Rhizobium leguminosarum* bv. Trifoli uses an ortholog of PleD, called CelR2, to regulate production of cellulose (34). There is also evidence that c-di-GMP plays a role in exopolysaccharide production in *A. tumefaciens*; addition of the signal to cell lysates resulted in an increased rate of cellulose synthesis (35). Consistent with this observation, the CelA component of the cellulose synthase of *A. tumefaciens* contains a PilZ domain (22).

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 and the formation of attachment structures, and the behavioral consequences of these changes. Our studies

indicate that overexpressing two putative DGCs, encoded by *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.

MATERIALS AND METHODS

Strains, cultures, and growth conditions. The strains used in this study are listed in Table 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 (36) 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 (37) 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 (37) supplemented with 0.2% glucose and 200 µg/ml acetosyringone (ABIM). 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* and 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 to assess production of exopolysaccharides.

Strain construction. (i) Production of overexpression strains. Genomic DNA was prepared from an overnight culture of *A. tumefaciens* NTL4(pTiC58) as described previously (38). Genes to be cloned were amplified by PCR using *Pfu* polymerase (Stratagene) and the following primer sets: *atu1297-f* (5'-GCGGATCCCATATGACGGCGAGAGTTC T-3') and *atu1297-r* (5'-CGGATCCTCAGCCGCGGCGGCCACGAC GCG-3'), *atu1060-f* (5'-CGGATCCCATATGCAGGATAAAATCCTTC TG-3') and *atu1060-r* (5'-CGGATCCTCAGCCGTTAGCCCGAT-3'), *atu0826-f* (5'-GCGGATCCCATATGCAGGCGCTCGCGCTA-3') and *atu0826-r* (5'-GCGGATCCTCAATTTGCTCGCGCAATAC-3'), *atu2228-f* (5'-CGGATCCCATATGGCTCATTCGTCGAAAGC-3') and *atu2228-r* (5'-CGGATCCTCAGCCTTGTCGCGCCGC-3'), and *atu4490-f* (5'-CGCG GATCCCATATGCGGATTGCGCCGCGC-3') and *atu4490-r* (5'-CGCG GATCCTCAGCCCCCGCCGAAG-3'). The PCR products were digested with BamHI and ligated into BamHI-digested pUC18. The resulting ligation products were introduced into *E. coli* DH5α by CaCl₂ transformation, with selection on LB plates containing ampicillin. Resistant colonies were selected, and the plasmids were purified and digested with NdeI and BamHI. The resulting fragments were ligated into the expression vector pZLQ (Table 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 and analyzed, and the correct constructs were electroporated into the appropriate strains of *A. tumefaciens*.

(ii) Deletion of the *A. tumefaciens* chromosomal *cel* locus. 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: the *celD*/Bm (5'-C GGGATCCATGCGCATCGATATC-3') and *celD*/Hind (5'-CCCCAGCT TTCGCGGAACCAAGC-3') primers and the *celC*/Hind (5'-CCCCAAG CTACCGGATTGACCAACCG-3') and *celB*/Sp (5'-GCTCTAGAAGT TGGATGAAGCGGAAT-3') primers, 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 1). The resulting ligation products were transformed into S17-1 λ pir. A ligation product, pSR Δ cel, in which the *tetA* gene was flanked on one side by the first 500 bp of *celC* and on the other side by the last 800 bp of *celD* (see Fig. S1 in the supplemental material), was identified and mated with NTL4(pTiC58) as previously described (39). NTL4(pTiC58) 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.

(iii) Mutation of *celR* by allelic exchange. Two flanking regions of *celR* were amplified from genomic DNA of strain NTL4(pTiC58) by using *Pfu* DNA polymerase and two pairs of primers: the *celRdel1-f* (5'-GCTC TAGAGGGCCCCACGTAGCCAACTACTCCG-3') and *celRdel1-r* (5'-GCGCCCGGGCTCGCGCTCATCAACAGTTCC-3') primers and the *celRdel2-f* (5'-CGCGGCATGCTTACGAGGCGAAACATGC-3') and *celRdel2-r* (5'-CGGGATCCACTAGTCGTGAAATAAAGGCAGAGC-

3') primers. The fragments were digested using XbaI and SmaI for the *celRdel1* fragment and SphI and BamHI for the *celRdel2* fragment, and the fragments were then inserted separately into pUC18. The resulting ligation products were transformed into DH5α. The plasmids were identified by the correct insertions and digested again with XbaI and SmaI for the *celRdel1* fragment and SphI and BamHI for the *celRdel2* fragment. A gentamicin resistance cassette from pMGm (Table 1) was digested using SmaI and SphI, and the fragment was ligated between the two flanking regions of *celR*, forming pUC*celRdel* (see Fig. S2 in the supplemental material). The new construct was digested with XbaI and BamHI, ligated into pWM91 (Table 1), a suicide vector containing *sacB*, and transformed into S17-1 λ pir by electroporation. Successful constructs were selected 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.

(iv) Indel mutation of *atu1060*. The *atu1060* gene was replaced with a kanamycin resistance cassette by using a protocol modified from that of Datsenko and Wanner (40). Briefly, a set of primers, *atu1060ftr-f* (5'-GC GTTTTTGTGCCTAGAGACTAGAGCTGAGCGTTGCCGCGCCTG TGTAGGCTGGAGCTGCTTC-3') and *atu1060ftr-r* (5'-GAGGAAA GACTGGGGAGACGGGCCAGGGGGCTTGGGACGGCCCATATGA ATATCCTCCTTA-3'), was used to amplify the kanamycin cassette from pKD4 (Table 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 NTL4(pTiC58) genomic DNA by 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 pRK*atu1060region*. The construct was introduced into DH5α by CaCl₂ transformation, and the plasmid was purified and analyzed by restriction digestion and sequencing. The kanamycin fragment was then electroporated into an *E. coli* strain harboring both the *red* recombinase plasmid pKD46 (Table 1) and pRK*atu1060region*. The transformants were selected by resistance to kanamycin, and plasmids were purified and examined for replacement of *atu1060* by restriction digestion and PCR. The correct plasmids containing the replaced gene were digested with KpnI, the modified *atu1060* gene was cloned into pWM91 (Table 1), producing the construct pWM*atu1060kan*, and this plasmid was transformed into S17-1 λ pir. Successful constructs were selected by resistance to ampicillin and kanamycin, and a verified plasmid was electroporated into *A. tumefaciens*. Initial transformants were selected by resistance to kanamycin and 5% sucrose, followed by screening for sensitivity to carbenicillin. Potential mutants were confirmed using PCR and Southern analysis.

(v) Complementation of NTL4 Δ *celR*::Gm. Wild-type *celR* is the second gene in an operon with *atu1296*, an ortholog of *divK* in *Caulobacter crescentus* (41) (see Fig. S2 in the supplemental material). 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'-GCGGATCCTG GCCGGCATTGCCTTTGTTT-3') and *prcelR-r* (5'-GCGGATCCCATATG TGGTGGGACAGTCCCCGTTTC-3'), digested with BamHI, and cloned into pUC18. The promoter fragment and the cloned *celR* gene were digested with NdeI and BamHI and ligated together to form pUC*prcelR* (see Fig. S2). The clone was purified and confirmed by sequence analysis. In this construct, *divK* is deleted and *celR* is driven directly by the *divK-celR* promoter. The correct clone was digested with BamHI, and the *prcelR* fragment was inserted into pUC18-miniTn7T-Km (42) to form pUC Δ Tn7-*prcelR*. The new construct and the transposase plasmid pTNS2 (Table 1) (42) were electroporated into NTL4 Δ *celR*::Gm(pTiC58), with selection for resistance to kanamycin. Potential mini-Tn7 integrants were confirmed by PCR analysis.

Cellulose extraction assays. Cellulose was quantified following extraction by use of a modified Updegraff protocol (43). Briefly, cells were grown in 12 ml of MG/L medium with appropriate antibiotics overnight at 28°C with shaking. From this culture, 10 ml was centrifuged at $3,000 \times g$ at 4°C for 10 min, while the remaining 2 ml was reserved for protein concentration determinations. The cell pellets were resuspended in 3 ml of 85% acetic acid–5% nitric acid, and the suspension was boiled for 30 min. The resulting suspension was centrifuged for 30 min, and the pellet was washed with 5 ml of double-distilled water (ddH₂O) and collected by centrifugation for 30 min. The resulting pellet, which represented the remaining acid-stable carbohydrate polymers, was resuspended in 5 ml 67% H₂SO₄ and incubated for 1 h at room temperature. The acid-digested samples were diluted 1:5 in ddH₂O, mixed with 3 volumes of anthrone reagent (50 mg of anthrone [Sigma-Aldrich] per 1 ml H₂SO₄), boiled for 15 min, and chilled to room temperature. The absorbance of the solution at 620 nm was determined using a Bio-Rad SmartSpec Plus spectrophotometer. Absorbance values were compared to a standard curve created from a stock solution of pure cellulose (Sigma-Aldrich) dissolved in 67% H₂SO₄.

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 μ l 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 the absorbance of Coomassie-bound protein was measured at 595 nm. Statistical analysis was performed with the Student *t* test, using a one-sided distribution model.

Cellulase treatment. Cells from cultures grown as described above were collected by centrifugation at room temperature for 10 min at $3,000 \times g$ 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 μ g/ml, and the suspension was incubated for 1 h 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.

Microscopy and lectin-binding assays. Cells grown in liquid culture for 2 days at 28°C were collected by centrifugation for 5 min before being resuspended in 0.9% NaCl to a final optical density at 600 nm (OD₆₀₀) of 0.4. For lectin staining, the resuspended cells were incubated with 100 μ g per ml of Alexa Fluor 633-WGA (Thermo Scientific) for 15 min and then 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 200 M microscope equipped with an Apo-tome structured-illumination optical sectioning system set at a $63\times/1.40$ objective, and images were captured using a Zeiss MRc 5 camera. For cells 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 randomly chosen images containing cells were compiled, and the number of cells in each image and their arrangement and lectin labeling were determined. The data were analyzed for statistical significance by using the chi-square test.

Microscopic analysis of bacterial attachment to *Arabidopsis* surfaces. 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 mg/liter ticarcillin (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 μ M acetosyringone and grown on a rotary shaker at 22°C to mid-exponential phase (OD₆₀₀ $\approx$ 0.5). Sterile forceps were used to wound leaves excised from seedlings before cocultivating them with bacterial cells for 2 days at 21°C. Cocultivated leaf pieces were

rinsed three times in ABIM with gentle vortexing (20 s/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 0.1 M HEPES (pH 7.1) before being postfixed in 1% OsO₄ for 1 to 2 h. 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 by use of a Polaron SEM auto-coating unit, and viewed on an FEI Quanta 400 series scanning electron microscope (SEM). 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 were analyzed for statistical significance by using the Student *t* test.

Biofilm assays. Cells were grown overnight with shaking in MG/L at 28°C with appropriate antibiotics and diluted 1/1,000 into 2 ml of MG/L with antibiotics. The diluted samples were incubated for 5 days at room temperature without shaking in 13- by 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 min. 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 Bio-Rad SmartSpec Plus spectrophotometer.

Virulence assays. Two different assays were utilized on different host plants. For *Kalanchoe 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 OD₆₀₀ of 1.0, and the suspensions were then diluted 1:10 and 1:100 in 1 ml of 0.9% NaCl. *Kalanchoe* leaves were wounded using a thin syringe needle, and 2- μ l samples of cell suspension 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 10-fold increments from 10^{-1} to 10^{-5} . Twenty-millimeter-long wounds between the primary leaves and the first set of secondary leaves were produced using a razor blade. As described above, 2- μ l samples of bacterial suspension 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. The 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 for 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.

RESULTS

Overexpressing different putative diguanylate cyclases in *Agrobacterium tumefaciens* has various effects on exopolysaccharide production. In *A. tumefaciens*, the stimulation of cellulose production in cell extracts by the addition of exogenous c-di-GMP (35) 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 (44, 45). Of these candidates, five

FIG 1 Overexpressing GGDEF domain proteins results in varied phenotypes on solid and liquid media. Strain NTL4(pTiC58) with constructs expressing genes coding for GGDEF domain proteins was grown for 2 days at 28°C on ABM plates containing Congo red (A) and in MG/L (B), with shaking. Both media contained the appropriate antibiotics.

genes—*atu0826*, *atu1060*, *atu1297*, *atu2228*, and *atu4490*—were chosen for testing based on the association of the GGDEF motif with a signaling domain (see Fig. S3 in the supplemental material). Of particular interest was *atu1297*, annotated *pleD*, the product of which synthesizes c-di-GMP in *C. crescentus* (46, 47). 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 open reading frames (ORFs) were cloned into the overexpression vector pZLQ (Table 1) and introduced into strain NTL4(pTiC58). Overexpression of *atu4490* had no effect on colony morphology on solid medium or growth in liquid medium (Fig. 1A and B). Strains overexpressing either *atu0826* or *atu2228* formed smaller colonies on solid medium (Fig. 1A), although the strains were unaffected in growth in liquid culture (Fig. 1B). However, overexpression of *atu1060* and *atu1297* resulted in the formation of small, hard colonies on agar surfaces (Fig. 1A). When tested on solid medium containing Congo red, colonies of these strains, but not those of strains expressing the other three genes, incorporated more dye than did the strain lacking an overexpression construct (Fig. 1A). Unlike the parent, cells of both NTL4(pTiC58, pZLQatu1297) and NTL4(pTiC58, pZLQatu1060) grown in liquid medium formed large aggregates (Fig. 1B), 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.

Increasing the expression of *atu1060* and *atu1297* affects the production of cellulose. Congo red binding is indicative of exopolysaccharides and some amyloid proteins (48, 49) and suggests that *atu1060* and *atu1297* influence the production of such products. To test if the exopolysaccharide induced by overexpression of *atu1297* or *atu1060* was cellulose, the effects

of pZLQatu1297 and pZLQatu1060 on NTL4(pTiC58) were examined by genetic manipulation and by quantification of total anthrone-positive material. First, the constructs were introduced into NTL4Δ*cel*(pTiC58) (Table 1), a mutant in which components of the *cel* locus have been deleted. Overexpressing either of the two genes in NTL4Δ*cel*(pTiC58) 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 medium (Fig. 2A 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, produces at least two polyglucose-type exopolysaccharides: β1,4-linked cellulose and β1,3-linked curdlan (50). To determine if curdlan biosynthesis was 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 (51). Colonies overexpressing either of the two genes were no more intensely blue than those of wild-type NTL4(pTiC58), while a curdlan-overproducing strain, *Agrobacterium* sp. ATCC 31749 (52) (Table 1), grew as dark blue colonies (see Fig. S4 in the supplemental material). Moreover, NTL4Δ*cel*(pTiC58) yielded colonies that bound amounts of aniline blue similar to those with NTL4(pTiC58) (see Fig. S4). Notably, colonies of ATCC 31749 were darker red on Congo red plates than those of NTL4(pTiC58) (see Fig. S4), 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 by using the Updegraff protocol (43) (see Materials and Methods). Wild-type NTL4(pTiC58) produced 998 μg, on average, while the Δ*cel* mu-

FIG 2 Overexpressing *atu1297* and *atu1060* does not affect exopolysaccharide production in NTL4Δ*cel*. Strains NTL4(pTiC58) and NTL4Δ*cel*(pTiC58), with or without constructs overexpressing either *atu1297* or *atu1060*, were grown for 2 days at 28°C on ABM plates containing Congo red (A) and in MG/L (B), with shaking. Both media contained the appropriate antibiotics.

tant produced 545 μg of anthrone-positive material per milligram of soluble protein (Fig. 3). Strains overexpressing either *atu1060* or *atu1297* produced two or three times as much anthrone-positive material, respectively, as that produced by NTL4(pTiC58) (Fig. 3 and Table 2). NTL4Δ*cel*(pTiC58) overexpressing either *atu1060* or *atu1297* produced amounts of anthrone-positive material comparable to the levels produced by the parent *cel* mutant (Fig. 3 and Table 2).

To confirm that the anthrone-reacting material recovered by

FIG 3 Overexpressing *atu1297* and *atu1060* results in increased production of anthrone-reacting material. Strains NTL4(pTiC58) and NTL4Δ*cel*(pTiC58) with constructs overexpressing either *atu1297* or *atu1060* were grown in MG/L with the appropriate antibiotics for 2 days at 28°C with shaking. The cells were harvested and assessed for production of anthrone-reacting material as described in Materials and Methods. Each experiment was repeated four times. The values represent the averages for the four samples of each strain, and the error bars represent the standard errors of the experiments.

the Updegraff protocol was cellulose, the cultures were pretreated with purified cellulase prior to analysis, as described in Materials and Methods. In cells of NTL4(pTiC58) pretreated with the enzyme, the amount of anthrone-positive material recovered dropped to levels comparable to those seen in NTL4Δ*cel*(pTiC58) (Table 2). The decrease in the amount of material collected from wild-type cells suggests that the difference between NTL4(pTiC58) and NTL4Δ*cel*(pTiC58) cultures represents the amount of cellulose produced by the wild-type bacteria (Table 2). The strain overexpressing *atu1297* also showed smaller amounts of anthrone-reacting material after cellulase treatment (Table 2). The results taken as a whole suggest that overexpression of *atu1297* and *atu1060* causes increased production of cellulose by NTL4(pTiC58) 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

TABLE 2 The increase in anthrone-reacting material resulting from *atu1297* overexpression is due to cellulose

Strain	Plasmid	Concn (μg/mg of protein) of extractable anthrone-positive material ^a	
		Without cellulase	With cellulase
NTL4(pTiC58)		782 ± 76	585 ± 66
NTL4(pTiC58)	pZLQatu1297	1,831 ± 199	728 ± 59
NTL4Δ <i>cel</i> (pTiC58)		542 ± 46	590 ± 45
NTL4Δ <i>cel</i> (pTiC58)	pZLQatu1297	620 ± 68	544 ± 58

^a Data are average values and standard errors for four experiments.

NTL4 Δ cel(pTiC58) is curdlan and perhaps other, still unidentified glucose-containing exopolysaccharides. In subsequent experiments, we expressed levels of anthrone-positive material in relative units normalized against the amount detected from NTL4 Δ cel(pTiC58), which was set at a value of zero.

***atu1297*, but not *atu1060*, is a positive regulator of cellulose production.** Overexpressing DGCs in bacteria often results in pleiotropic phenotypes (24, 33, 53, 54). This effect suggests not only that multiple regulatory systems are dependent on c-di-GMP but also that the signal produced by overexpression of any active DGC can cross talk 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 gentamicin resistance cassette. The resulting mutants, NTL4 Δ *atu1297*::Gm(pTiC58) and NTL4 Δ *atu1060*::Km(pTiC58), 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 NTL4(pTiC58) (Fig. 4A). When assessed quantitatively, the *atu1297* mutant produced significantly less cellulose than NTL4(pTiC58), its wild-type parent (Fig. 4B). The *atu1060* mutant, however, showed no significant difference in the amounts of cellulose produced compared to NTL4(pTiC58) (Fig. 4B), 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, NTL4 Δ *atu1297*::Gm(pTiC58) 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 (42, 55). The complemented *atu1297* mutant produced levels of cellulose comparable to that of wild-type NTL4(pTiC58) (Fig. 4B). 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).

Overexpression of *celR* affects the aggregation phenotype of individual cells. Cellulose produced by *A. tumefaciens* is involved in stabilizing colonization of plant surfaces (3–6). In addition, production of cellulose may affect 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 NTL4(pTiC58) overexpressing *celR* formed dense masses at a much higher frequency than that for wild-type NTL4(pTiC58) (compare Fig. 5A and B). Interestingly, cells of NTL4(pTiC58, 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 (Fig. 5B and Table 3). NTL4 Δ cel(pTiC58, pZLQ*celR*) produced fewer and smaller aggregates (Fig. 5C). However, a number of cells were still associated with rosettes (Fig. 5D and Table 3). We concluded from these results that the aggregation phenotype, but not rosette formation, is due to overproduction of cellulose resulting from overexpression of *celR*.

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 (32, 33, 56). A similar but stalkless lectin-binding holdfast structure was recently described for *A. tumefaciens*, with the structure forming at one pole of the cell (47, 57, 58). This

FIG 4 The *atu1297* gene, but not the *atu1060* gene, positively regulates cellulose production. Derivatives of NTL4(pTiC58) with mutations in *atu1297* or *atu1060* were grown on ABM plates containing Congo red for 2 days at 28°C (A) and in MG/L (B), harvested, and assayed for extractable cellulose as described in Materials and Methods. The total amount of anthrone-reactive material from each strain was normalized by comparison to the amount of material extracted from NTL4 Δ cel(pTiC58), which was set to zero. Each strain was tested four times, and the data were averaged, with the error bars representing the standard errors of the experiments.

unipolar polysaccharide (UPP) structure may also play a role in initial attachment of bacteria to plant cells (58). 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 (57), when strain NTL4(pTiC58) was incubated with WGA-Alexa Fluor 633, a small subset of the cells showed polar binding of the lectin label (Fig. 6A and Table 3). In contrast, cells of NTL4(pTiC58) overexpressing *celR* were labeled over the entirety of the aggregates (Fig. 6B), making it difficult to identify the location of the lectin on individual cells. When cells of NTL4(pTiC58, pZLQ*celR*) were separated from the aggregate, about three times as many exhibited polar lectin binding (Table 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

FIG 5 Overexpression of *celR* affects aggregation and rosetting. Cultures of NTL4(pTiC58) or NTL4Δ*cel*(pTiC58) and their derivatives were grown in MG/L, and samples were viewed by DIC microscopy as described in Materials and Methods. (A) NTL4(pTiC58). (B) NTL4(pTiC58, pZLQ*celR*). (C) NTL4Δ*cel*(pTiC58, pZLQ*celR*). (D) NTL4Δ*cel*(pTiC58, pZLQ*celR*) in rosettes.

TABLE 3 Overexpressing CelR affects lectin binding and rosetting

Strain	Total no. of cells	% labeled ^a	% rosettes ^b
NTL4(pTiC58)	272	3	1
NTL4Δ <i>cel</i> (pTiC58)	272	3	1
NTL4(pTiC58, pZLQ <i>celR</i>)	584	11 ^c	1
NTL4Δ <i>cel</i> (pTiC58, pZLQ <i>celR</i>)	890	16 ^c	3 ^c
NTL4Δ <i>celR</i> (pTiC58)	228	5	1

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

^b Percentage of cells examined that were observed in rosettes.

^c $P \leq 0.005$ compared to NTL4(pTiC58), by chi-square analysis.

Δ*cel* background. Such cells continued to show an increase in polar lectin binding (Fig. 6C and Table 3). Additionally, the rosettes produced by the Δ*cel* strain overexpressing *celR* showed polar labeling at the center of the clustered cells. NTL4Δ*celR*::Gm (pTiC58), on the other hand, did not display any alteration in lectin binding or cellular aggregation compared to wild-type cells (Fig. 6D and Table 3). Consistent with the report by Xu et al. (47), 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.

Modification of *celR* expression affects the attachment of cells to plant tissue. While cellulose helps to stabilize the attachment of 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, wild-type and mutant strains were incubated with *Arabidopsis* leaves, and binding was visualized using SEM. In comparison to NTL4(pTiC58), the cells of the *celR* mutant exhibited a statistically significant increase in attachment to plant tissue (Fig. 7A and B; see Table S1 in the supplemental material). In addition, compared to the wild-type parent, a significantly larger number of Δ*celR* cells attached to the surface in a polar orientation (Fig. 7B; see Table S1). These results suggest that deletion of *celR* and its resultant negative effect on cellulose production affect the initial attachment of bacteria to plant cell surfaces.

To determine if CelR affected initial cellulose-independent attachment, the *celR* gene was overexpressed in the Δ*cel* background. NTL4Δ*cel*(pTiC58) bound to the plant tissue at numbers comparable to those of NTL4(pTiC58) (Fig. 7A and C). On the other hand, in comparison to the *cel* mutant parent, NTL4Δ*cel*(pTiC58) overexpressing *celR* was more sparsely bound to the plant tissue (Fig. 7C and D). Interestingly, when we viewed the cultured material by light microscopy before fixation for SEM, NTL4Δ*cel*(pTiC58, pZLQ*celR*) formed large aggregation patches on the surfaces of the plant tissue (data not shown). These patches were 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 (Fig. 7E and F). These effects on cell morphology suggest that increasing expression of *celR* can alter cell division programming in the cells.

Overexpression of *celR* affects production of biofilms. Both the production of the UPP and cellulose production influence attachment of *A. tumefaciens* to surfaces (6, 57, 58). To test the influence of *celR* on the ability of 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 NTL4(pTiC58,

FIG 6 Cells overexpressing *celR* display increased polar binding of lectins. Cells grown in MG/L with the appropriate antibiotics were collected, incubated with WGA-Alexa Fluor 633, and observed by fluorescence microscopy as described in Materials and Methods. (A) NTL4(pTiC58). (B) NTL4(pTiC58, pZLQ*celR*). (C) NTL4 Δ *cel*(pTiC58, pZLQ*celR*). (D) NTL4 Δ *celR*::Gm(pTiC58). Circled cells represent examples of polarly bound lectin.

pZLQ*celR*) exhibited a significant decrease in crystal violet staining compared to its parent (Fig. 8), indicating that overexpressing *celR* negatively affects biofilm formation. The amount of bound crystal violet was increased in the Δ *cel* strain, regardless of whether or not *celR* was overexpressed (Fig. 8). This increase in biofilm production by the Δ *cel* 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, in which 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 (6). Deleting *celR* resulted in increased crystal violet staining compared to that of the wild-type parent (Fig. 8), with the Δ *celR* mutant binding to glass at the same level as the Δ *cel* mutant of NTL4(pTiC58) (Fig. 8).

CelR overexpression severely attenuates virulence in *Agrobacterium tumefaciens*. Pathogenic isolates of *A. tumefaciens* induce tumors on wounded plants, with tumor induction requiring attachment of bacteria to plant cells (1, 2). 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 *Kalanchoe daigremontiana* and onto wounded tomato stems, and virulence was quantified as described in Materials and Methods. Overexpressing *atu0826*, *atu2228*, or *atu4490* had no detectable effect on virulence on *Kalanchoe* leaves (Fig. 9A). However, NTL4(pTiC58) overexpressing either *celR* or *atu1060* was strongly attenuated on both host plants (Fig. 9A and B). Overexpressing *celR* or *atu1060* in the cellulose-deficient background led to the same attenuated phenotype (Fig. 9B), while the parent Δ *cel* strain remained fully virulent (Fig. 9B). These results suggest that cellulose production is not a contributing factor to the loss of tumorigenicity in the overexpressing strains. Interestingly, NTL4 Δ *celR*::Gm(pTiC58) produced slightly larger tumors on tomato stems than those produced by NTL4(pTiC58), although this difference was not statistically significant (Fig. 9B). 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*.

DISCUSSION

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 (Fig. 4). 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 stimulating synthesis of the polymer (Fig. 4).

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* (35). 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 *Glucoacetobacter xylinum* (59, 60). Second, Xu et al. (47) reported that extracts of *E. coli* overexpressing *A. tumefaciens* CelR, which they called PleD, contained a 46-fold larger amount of c-di-GMP than that in 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 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.

FIG 7 Cells altered in the expression of *celR* 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. (A) NTL4 (pTiC58). (B) NTL4 Δ *celR*::Gm(pTiC58). (C) NTL4 Δ *cel*(pTiC58). (D) NTL4 Δ *cel*(pTiC58, pZLQ*celR*). (E and F) Higher-magnification images of NTL4 Δ *cel*(pTiC58, pZLQ*celR*).

Agrobacterium tumefaciens also synthesizes curdlan, another polyglucose polymer (50, 51), and it is conceivable that production of this polysaccharide is affected by c-di-GMP. Curdlan binds both aniline blue and Congo red (51), which 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 ATCC 31749 bound both aniline blue and Congo red (see Fig. S4 in the supplemental material). However, overexpressing either *celR* or *atu1060* in strain NTL4(pTiC58) resulted in increased binding of Congo red

only, not aniline blue (see Fig. S4), suggesting that neither protein product stimulates curdlan production. Alignments of CelsA, the cellulose synthase of *A. tumefaciens*, and the curdlan synthase, CrdS (Atu3056), show that while both share similar catalytic sites for polymer elongation, only CelsA has the conserved PilZ c-di-GMP binding domain (22; data not shown). 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 Δ *cel* mutant (Fig. 3).

FIG 8 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 averages for the nine total samples, with error bars representing the standard errors of the experiments.

Additionally, these results demonstrate that of the two reagents, Congo red is the better for use in *in situ* assays of cellulose production.

CelR, but not Atu1060, directly controls production of cellulose. Overexpression of a second gene, *atu1060*, also resulted in increased cellulose production. Only overexpression of *atu1060* and *celR*, not that of the other three putative DGCs studied, affected these phenotypes. *Atu1060* has a domain structure similar to that of CelR (see Fig. S3 in the supplemental material), which when combined with the similar phenotypes observed when either gene is overexpressed, suggests that these two potential synthases can cross talk to their respective target pathways. However, while deleting *celR* resulted in decreased levels of cellulose, deleting *atu1060* had no such effect (Fig. 4), suggesting that at native levels of expression, *celR*, but not *atu1060*, is a regulator in the pathway. Consistent with this interpretation, *celR* is conserved in all members of the *Rhizobiaceae* that produce cellulose, while *atu1060* is found only in the genomes of biovar 1 agrobacteria.

Assuming that CelR is an active DGC and that overexpressing *celR*, but not three of the other potential DGCs studied, affects cellulose production suggests that these enzymes or their signal product can be compartmentalized. Overexpression of *atu0826* and *atu2228*, while having no effect on Congo red binding, did result in greatly reduced colony sizes (Fig. 1A). The observation that overexpressing other putative DGCs affects different phenotypes suggests that the role of a particular DGC may not affect processes outside that specific system. Of the genes examined, only *atu1060* appears to cross talk with *celR*. These observations suggest that signal compartmentalization, as well as some level of specificity, is held in common by the two proteins.

The impact of *celR* overexpression on other phenotypes suggests that 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 NTL4(pTiC58) resulted in a greatly reduced colony size, similar to the effect of overexpress-

FIG 9 Overexpression of *celR* and *atu1060* severely attenuates virulence. (A) Derivatives of NTL4(pTiC58) containing constructs overexpressing one of the GGDEF-containing proteins were inoculated onto leaves of *Kalanchoe daigremontiana* as described in Materials and Methods. (B) NTL4(pTiC58) altered in expression of either *celR* or *atu1060* was 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 for samples of each strain tested, with error bars representing standard errors.

ing *atu0826* and *atu2228* (Fig. 1A). However, overexpression of *celR* or *atu1060* in NTL4Δcel(pTiC58) resulted in colonies of a size comparable to that of either wild-type NTL4(pTiC58) or NTL4Δcel(pTiC58) (Fig. 2A). 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 are a result of cellulose production and that *celR* and *atu1060* do not cross talk to the cellular processes affected by *atu0826* and *atu2228*.

Overexpressing *celR* affected the morphology of wild-type NTL4, 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, then 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 c-di-GMP is a critical intracellular signaling component in cell cycle regulation for *A. tumefaciens* (61). However, as with several other phenotypes, the signal produced by overexpressing *celR* may be cross talking to some noncognate system.

The influence of *celR* on rosetting, a unipolar attachment phenomenon first described by Braun and Elrod (62), is interesting. Overexpressing the gene in both the wild type and the Δcel mutant resulted in increased frequencies 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 the CelR protein in the process. The overexpression of *celR* also increased UPP production in both backgrounds, a phenotype seen in other studies (47, 61). However, as with rosetting, deleting *celR* did not negatively affect the number of cells expressing UPP compared to the wild type (Fig. 6), 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 NTL4 Δcel (pTiC58) resulted in the same attenuated phenotype as that observed in wild-type NTL4(pTiC58) (Fig. 9B). Furthermore, overexpression of the two putative DGC genes that affected colony size, i.e., *atu0826* and *atu2228*, had no impact on virulence (Fig. 9A). Deleting *celR* did not influence virulence (Fig. 9B), suggesting that this protein does not contribute to regulating this process. This result is consistent with the observation that *cel* mutants of *A. tumefaciens* are fully virulent (6), 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.

Altering the expression of *celR* affected biofilm formation and attachment to plants, exclusive of cellulose production. Overexpressing *celR* in wild-type NTL4 dramatically decreased biofilm formation (Fig. 8) and attachment to leaf surfaces (Fig. 7). This result is in contrast to the results reported by Xu et al. (47) and may be due to differences in the methodology of the experiments. NTL4 Δcel (pTiC58) overexpressing *celR* yielded wild-type levels of crystal violet staining (Fig. 8), 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 (6). 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 NTL4 Δcel (pTiC58) overexpressing *celR* to plant tissue, with the cells aggregating through some means other than through cellulose production.

Deleting *celR* resulted in increased polar attachment of individual bacteria to plant tissue (Fig. 7), 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* (34). Furthermore, strains of *Rhizobium* produce aggregate caps at root hair tips, with the formation of these caps being dependent on cellulose (63). 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

TABLE 4 Genes present in members of the alphaproteobacteria^a

Family	Contains the <i>divK-celR</i> operon	Contains CelA subunit with a PilZ domain	Uses CelR to regulate:	
			Cell differentiation	Cellulose synthesis
<i>Acetobacteraceae</i>	N	Y	U	N
<i>Bradyrhizobiaceae</i>	Y	Y ^b	U	U
<i>Brucellaceae</i>	Y	N	U	N
<i>Caulobacteraceae</i>	Y	N	Y	N
<i>Methylobacteriaceae</i>	N	Y	U	U
<i>Phyllobacteriaceae</i>	Y	Y	U	U
<i>Rhizobiaceae</i>	Y	Y	N	Y
<i>Rhodobacteraceae</i>	N	Y	U	N

^a N, no members of the family contain the genes or enzymes; Y, at least a significant number of members of the family contain the genes or enzymes; U, no available literature.

^b Only *Bradyrhizobium* species contain *celA*; all other members of the *Bradyrhizobiaceae* lack the gene.

as wild-type cells, resulting in increased single-cell attachment to the plant tissue.

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 (22, 24). In both *Gluconacetobacter xylinum* 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 (24, 60, 64). Additionally, the genomes of a number of other bacterial species encode a putative cellulose synthase with a PilZ domain orthologous to CelA (22). 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 (10, 65). In *Gluconacetobacter xylinum*, a member of the *Acetobacteraceae* of the alphaproteobacteria, three DGCs, annotated Dgc, are involved in controlling cellulose production (18). Moreover, other families of the alphaproteobacteria, including the *Rhodobacteraceae*, carry a *celA* gene and contain the *dgc* operons (Table 4). These three enzymes, which are related to each other, are not orthologous to *AdrA/YaiC*. In both *A. tumefaciens* and *R. leguminosarum*, the probable DGC CelR and its ortholog CelR2 regulate cellulose production (34). However, apart from shared GGDEF domains, CelR and its orthologs are structurally distinct from the Dgc proteins of *G. xylinum* 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 that of CelR are found throughout many families of the alphaproteobacteria (NCBI) and have been identified in at least two members of the gammaproteobacteria: *Pseudomonas aeruginosa* (66, 67) and *Pseudomonas fluorescens* (53, 68). Within the alphaproteobacteria, these CelR gene orthologs are usually organized as the second gene of a two-gene operon, with the first, generally annotated *divK*, encoding a small CheY-like receiver protein (Table 4;

see Fig. S5 in the supplemental material). Studies of *divK* in the alphaproteobacteria, including *Caulobacter*, *Brucella*, and *Agrobacterium*, link this gene to the polar localization of cell division proteins (33, 61, 69, 70). 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 (32, 33). Interestingly, this group of bacteria apparently does not produce cellulose; genome analyses indicate that the members of the *Caulobacteraceae*, as well as several other families of the alphaproteobacteria that contain the *divK-celR* (*pleD*) gene set, do not carry a *celA* gene or any other genes associated with cellulose biosynthesis (Table 4) (71). 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 encodes a PilZ-containing CelA subunit (Table 4). Furthermore, *celR* is not a component of cell cycle regulation in *A. tumefaciens* (61). 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 tracks: controlling polar localization and adhesion, as in the case of the *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*.

Cellulose biosynthesis in *A. tumefaciens* is regulated at several levels. Our work and previous studies (6) suggest that in *A. tumefaciens*, cellulose production is regulated in at least two levels. CelR contains a pair of CheY-like domains (see Fig. S3 in the supplemental material), 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 *cell*, result in overproduction of the polymer (6). While CelG has no predictable structure, Cell 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 *cell* 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 *cell*, 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 (56). 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 remain 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.

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