Diguanylate Cyclases AdrA and STM1987 Regulate *Salmonella enterica* Exopolysaccharide Production during Plant Colonization in an Environment-Dependent Manner

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Increasing evidence indicates that despite exposure to harsh environmental stresses, *Salmonella enterica* successfully persists on plants, utilizing fresh produce as a vector to animal hosts. Among the important *S. enterica* plant colonization factors are those involved in biofilm formation. *S. enterica* biofilm formation is controlled by the signaling molecule cyclic di-GMP and represents a sessile lifestyle on surfaces that protects the bacterium from environmental factors. Thus, the transition from a motile, planktonic lifestyle to a sessile lifestyle may represent a vital step in bacterial success. This study examined the mechanisms of *S. enterica* plant colonization, including the role of diguanylate cyclases (DGCs) and phosphodiesterases (PDEs), the enzymes involved in cyclic di-GMP metabolism. We found that two biofilm components, cellulose and curli, are differentially required at distinct stages in root colonization and that the DGC STM1987 regulates cellulose production in this environment independent of AdrA, the DGC that controls the majority of *in vitro* cellulose production. In addition, we identified a new function for AdrA in the transcriptional regulation of colanic acid and demonstrated that *adrA* and colanic acid biosynthesis are associated with *S. enterica* desiccation tolerance on the leaf surface. Finally, two PDEs with known roles in motility, STM1344 and STM1697, had competitive defects in the phyllosphere, suggesting that regulation of motility is crucial for *S. enterica* survival in this niche. Our results indicate that specific conditions influence the contribution of individual DGCs and PDEs to bacterial success, perhaps reflective of differential responses to environmental stimuli.

Enteric human pathogens, such as *Salmonella enterica*, are frequently studied in the context of an animal host, but it has become increasingly apparent that a significant portion of their life cycle occurs on plants (for a review, see reference 1). *S. enterica* is exposed to numerous environmental stresses during colonization of animal or plant hosts; stresses common in plant niches include plant defense responses and fluctuations in temperature, humidity, and nutrient availability. Despite these obstacles, *S. enterica* is ubiquitous in the plant environment, is commonly isolated from water used for irrigation or pesticide application (2), and persists for months on roots and leaves during crop production (3, 4). These abilities have led to an increased incidence of human disease, particularly through the consumption of contaminated fresh produce (5, 6). We predict that the bacterium's ability to transition from a potentially motile lifestyle (in water) to a more sessile lifestyle (on plants) would impact its success on plants.

In support of this idea, several *S. enterica* attachment factors contribute to colonization of roots: proteinaceous curli (previously called thin, aggregative fimbriae), O-antigen capsule, and cellulose (a polymer of β -1,4 glucan chains) (7, 8). The transcription factor CsgD (previously referred to as AgfD) positively regulates these extracellular matrix components (9) and plays an important role in root colonization (7). For curli production, CsgD activates expression of the genes encoding the curli structural proteins (9). O-antigen capsule formation is positively regulated by CsgD and contributes to *S. enterica* desiccation tolerance on abiotic surfaces (10). Cellulose production is initiated through CsgD-activated transcription of *adrA* (11). AdrA is a diguanylate cyclase (DGC) that synthesizes the signaling molecule cyclic dimeric GMP (c-di-GMP) which binds the PilZ domain of the cellulose synthase BcsA, activating production of cellulose (12). AdrA is

also tied to curli production as an *adrA* mutant has decreased levels of the major curli subunit CsgA (13). Despite the reported requirement of AdrA for *in vitro* regulation of two root colonization factors, cellulose and curli (11, 13), an *adrA* mutant colonizes roots at wild-type levels (8).

In addition to AdrA, at least six other c-di-GMP metabolizing enzymes impact cellulose production (13, 14). In general, DGCs (GGDEF-containing proteins) synthesize c-di-GMP while phosphodiesterases (PDEs; proteins containing EAL or HD/GYP domains) degrade c-di-GMP (for a review, see reference 15). As with many other bacteria, *S. enterica* has multiple proteins that affect c-di-GMP production: five GGDEF-containing proteins, eight EAL-containing proteins, and seven proteins containing both GGDEF and EAL domains. The enzymatic activities of some *S. enterica* c-di-GMP proteins have been confirmed (13, 14, 16–18), while others are classified based on sequence homology alone. For cellulose and curli production in *S. enterica*, two DGCs (STM2123 and STM3388) activate CsgD, while four

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PDEs (STM1703, STM3611, STM4264, and STM1827) inhibit CsgD function (13, 14).

The presence of multiple proteins involved in the metabolism of c-di-GMP and in the regulation of the transcription factor, CsgD, suggests potential functional redundancy and/or environmental regulation of activity. For example, although both STM2123 and STM3388 are important for CsgD expression, STM2123 contributes early in *in vitro* biofilm formation, while STM3388 is involved later (13). Previous work from our lab suggests that c-di-GMP production may also be differentially regulated *in planta*. Promoter probe experiments and quantitative real-time PCR demonstrate that *adrA* is transcribed during root colonization (8). This result supports the argument that, even if transcribed, c-di-GMP metabolism proteins may require specific environmental cues for activity (19). The result demonstrating that an *adrA* mutant retains the ability to colonize roots could be explained by the presence of an additional DGC that activates BcsA during root colonization. However, this putative alternate mechanism for cellulose production does not appear to occur *in vitro* as an *adrA* mutant does not make cellulose in the typical biofilm formation assays (11). Thus, a different DGC may be involved in the activation of BcsA during root colonization and would demonstrate both environment-specific activity, as well as potential functional redundancy with *AdrA* in the rhizosphere.

In the present study, we hypothesized that a subset of c-di-GMP metabolizing proteins are functionally active on roots. Combined with our previous data (8), we demonstrate that cellulose production and bacterial colonization of roots proceeds in an *AdrA*-independent manner through a second DGC, STM1987. By identifying this new root colonization factor and characterizing the temporal importance of the known biofilm components cellulose and curli, our results have begun to detail the mechanisms by which *S. enterica* colonizes roots. In addition, the results of this study elucidate a new role for *AdrA* in the regulation of an additional extracellular matrix component, colanic acid, that is important for desiccation tolerance on the leaf surface, indicating that *AdrA* may play an even broader role in biofilm formation than was previously considered. Finally, this work continues to investigate the mechanisms that bacteria use to transition between motile and sessile lifestyles by demonstrating that misregulation of motility may also influence bacterial persistence on the leaf surface. This study adds to previous knowledge concerning the complex nature of biofilm regulation and highlights the versatility of *S. enterica* in the face of different environmental conditions, potentially impacting intervention strategies that could be used to improve food security practices.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions. Strains and plasmids used in this study are listed in Table 1 and see Table S1 in the supplemental material. All bacterial cultures were grown in lysogeny broth (LB) (20) at 37°C with shaking at 200 rpm. Antibiotics were used at the following concentrations: chloramphenicol (Cm), 30 µg ml⁻¹; gentamicin (Gm), 20 µg ml⁻¹; kanamycin (Kan), 50 µg ml⁻¹; and nalidixic acid (Nal), 20 µg ml⁻¹.

Molecular biological methods. Standard molecular biological methods were used in the present study (21). Restriction enzymes (Thermo Fisher Scientific, Waltham, MA), plasmid preparations (MidSci, St. Louis, MO), and gel extraction and PCR purification kits (Qiagen, Valencia, CA) were all used according to manufacturers' recommendations. Constructs were sequenced at the University of Wisconsin Biotechnology Center us-

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype ^a	Source or reference
Strains		
<i>S. enterica</i>		
JDB682	<i>S. enterica</i> serovar Typhimurium 14028s	ATCC
JDB1034	<i>S. enterica</i> serovar Typhimurium 14028s; Nal ^r	This study
JDB1023	Δ <i>adrA</i> ::Kan	This study
JDB1024	Δ <i>adrA</i> ::Kan <i>adrA</i> -Tn7	This study
JDB1094	Δ <i>bcsA</i> ::Kan	This study
JDB1092	Δ <i>csgB</i> ::Kan	This study
JDB1140	Δ <i>csgB</i> ::Cm	This study
JDB1090	Δ <i>csgB</i> ::Cm Δ <i>bcsA</i> ::Kan	This study
JDB1128	ΔSTM1283::Kan	This study
JDB1143	ΔSTM1344::Kan	This study
JDB1144	ΔSTM1697::Kan	This study
JDB1027	ΔSTM1703::Kan	This study
JDB0928	ΔSTM1987::Kan	This study
JDB1025	ΔSTM1987::Kan STM1987-Tn7	This study
JDB0929	ΔSTM2123::Kan	This study
JDB1129	ΔSTM2410::Kan	This study
JDB1137	ΔSTM2503::Kan	This study
JDB1138	ΔSTM2672::Kan	This study
JDB0920	ΔSTM3375::Kan	This study
JDB1026	ΔSTM3375::Kan STM3375-Tn7	This study
JDB0921	ΔSTM3388::Kan	This study
JDB0922	ΔSTM3615::Kan	This study
JDB1127	ΔSTM3615::Kan STM3615-Tn7	This study
JDB1149	ΔSTM4264::Kan	This study
JDB1139	ΔSTM4551::Kan	This study
JDB1210	Δ <i>wcaB</i> ::Kan	This study
JDB1211	Δ <i>wcaD</i> ::Kan	This study
JDB1213	Δ <i>wzc</i> ::Kan	This study
JDB0891	<i>S. enterica</i> Typhimurium KHLT2	Diana Downs
<i>E. coli</i>		
JDB0924	DB3.1 (CcdB-resistant <i>E. coli</i> strain)	Andrew Bent
Plasmids		
pRCK-GWY	<i>ccdB</i> ; Cm ^r Kan ^r	30
pRCG-GWY	<i>ccdB</i> ; Cm ^r Gm ^r	30
pRCKTn7B	<i>ccdB</i> ; Cm ^r Kan ^r ; Tn7B	This study
pRCKTn7AB	<i>ccdB</i> ; Cm ^r Kan ^r ; Tn7AB	This study
pRCGTn7AB	<i>ccdB</i> ; Cm ^r Gm ^r ; Tn7AB	This study

^a Cm^r, chloramphenicol resistance; Gm^r, gentamicin resistance; Kan^r, kanamycin resistance; Nal^r, nalidixic acid resistance.

ing ABI BigDye version 3.1 (Applied Biosystems, Foster City, CA). Unless otherwise specified, PCR amplification of DNA was performed using *Pfx* according to manufacturer's directions (Invitrogen Life Technologies, Grand Island, NY). The primers (0.2 µM) used in this study (Integrated DNA Technologies, Coralville, IA) are described in Table 2 and see Table S1 in the supplemental material.

Mutant construction. Mutations in STM1987, STM2123, STM3375, STM3388, STM3615, STM4264, and *bcsA* were generated using the primers listed in Table 2 and the *xRed* recombinase method (22). All other mutants were obtained from a mutant library collection (23). Deletion-insertion mutations were transduced into a fresh *S. enterica* serovar Typhimurium 14028s background using P22 (24) and confirmed by PCR (see Table 2 and Table S1 in the supplemental material for confirmation primers). The *bcsA csgB* double mutant was created by transducing *bcsA*::Kan into the mutant library *csgB*::Cm strain. Transductants were plated on LB Kan/Cm plates and confirmed by PCR.

TABLE 2 Primers

	Primer sequence (5'–3') ^c		
Function and primer	Forward	Reverse	Efficiency (%)
Mutant construction ^a			
<i>bcsA</i> deletion	AGCCTGCGATGAGCGCCCTTTCCCGGTGGCTGC TTATCCCGTGTAGGCTGGAGCTGCTTC	TTCATCGCATTATCATCATTGTTGAGCCTGAGC CATAACCCATATGAATATCCTCCTTAG	
STM1987 deletion	TCAGGGGGAGTTGTATCAATAACCAGGAGTTA CCAGGGTGGTGTAGGCTGGAGCTGCTTC	ACGAAGGGCCGGATGGCTGGCGCGAAGGAAT GGACTATTTTCATATGAAATCCTCCTTAG	
STM2123 deletion	ATTCCGCGACGTTACCGGCGATTTCGGATAGAG TGGTAATGGTGTAGGCTGGAGCTGCTTC	ACTGCATATCTGGTATACCCACGGCTGGCAAC CGTCAATGCATATGAATATCCTCCTTAG	
STM3375 deletion	TTTCATACTGTTTATAACCGTCGGAGTTAACT CAAGGATGGTGTAGGCTGGAGCTGCTTC	CGTTATTCTACGTGAAAACAAATTAACGGCA GGTTAAACCATATGAATATCCTCCTTA	
STM3388 deletion	CTGTTCTTTTCTCTGCATAACCAATGAGCTAAT GATACATGGTGTAGGCTGGAGCTGCTTC	TGCCAAATGAACAACAGGCGGAAGAAGCCTGA GATTATTTTCATATGAATATCCTCCTTAG	
STM3615 deletion	CAATTTGCGCGTCAGCCGCTCGTTAACAAATTA AACAGATGGTGTAGGCTGGAGCTGCTTC	AAGTTTGAGCTGGCTCGCACAAAGCGCGACCTT TTTTAACTCATATGAATATCCTCCTTAG	
STM4264 deletion	TGCAGGGAAATAGGCTGAAAATGAGTCAAAGC ACACGACGGTGTAGGCTGGAGCTGCTTC	CACTCCCAGCGATTACCGTCACCGCCTGTAGG GCGTCTGCCATATGAATATCCTCCTTAG	
Mutant confirmation ^b			
<i>adrA</i> confirm	CATACTTCCTCCATGCGC	CAGTAAATCCTGAAGCCC	
<i>bcsA</i> confirm	GGAGCCTGCGATGAGCGC	TAATCCTATTACCGCCGC	
<i>csdB</i> confirm	CTATGTACGACCAGGTCC	AACGGTATTAGCGTTGGG	
STM1283 confirm	GGTAAACTAAACGCATCCG	ATTTCTCACTCCTGGTGC	
STM1344 confirm	TACGTCTGTTTCCGTCCG	TAGACGGTTAATCACC GG	
STM1697 confirm	TAAGATCAAGTGGAGTGG	TTCTAACGCAACGGAAGG	
STM1703 confirm	TTCTTTTCGGGACACCTGG	TGCACAGGCTGTTCTCCC	
STM1987 confirm	TGGTATCCCTTGATGAGG	TTTGCCTAACGACGATCC	
STM2123 confirm	GCGATTCGGATAGAGTGG	AACCGTCAATGGATAGCG	
STM2410 confirm	GCATTAAAGCAATGCCGG	GTTTAATGCAGAAACCGG	
STM2503 confirm	AAAGTCAGGTGGGATGCC	TTGAGGAAGAAAGCTCGC	
STM2672 confirm	GTATTAATGCTTGCACGG	TTATGGGTTTGCTCCACG	
STM3375 confirm	GTATGCCTGCTTCATTGC	TTGCTGCAACAATCTGGC	
STM3388 confirm	CTCGTCTGTTCTTTTCC	CCAAATGAACAACAGGCG	
STM3615 confirm	ACAGCCTCTCTTAATGCC	AAGTTTGAGCTGGCTCGC	
STM4264 confirm	AATGAGTCAAAGCACACG	TATGTATCGCAGCAAACC	
STM4551 confirm	ATTCCGTGTATATCACCG	ATGGCAAGCGGTTTATGG	
<i>wcaB</i> confirm	ATGCTGGAAGACTTACGC	AATATTTCATGTGCTGACC	
<i>wcaD</i> confirm	GTCACAGATGCTGGAGG	CGAAAGGCGACGGTAACG	
<i>wzc</i> confirm	ACTGAATGCAGAGCAGGG	TATTTGGAATCTGACTGG	
Complementation ^c			
Tn7A fragment	NNN <u>GGG</u> CCCTTACATTACGCGGAAGC	NNNCTCGAGAAAGGCCGTCTGTCGACG	
Tn7B fragment	NNN <u>ACTAG</u> TTTTTGTGCGCTGTGACAG	NNN <u>AAGCT</u> TAAATTATGATGACTGGGCG	
Tn7compFor/Rev	CCGTAGAGTAAGTACAAATGTAGTACCAGGCA GAAGGCCG	TTCAGCCACCATAGAGAAAGAACAGCGCCTGT CACAGCGC	
Tn7compA/B	TTACATTACGCGGAAGC	AATTATGATGACTGGGCG	
<i>adrA</i> comp	NNN <u>CCTAG</u> GAAACAGTCAGTCAGCCCC	NNNTCTAGATGGAAGAACGTGGCTTCC	
STM1987 comp	NNN <u>CCTAG</u> GTGAGAATGAGCTCGTGCC	NNNTCTAGATTTGCGTAACGACGATCC	
STM3375 comp	NNN <u>CCTAG</u> GCTAAGATCAACGCCTGC	NNNTCTAGACGAGCAGACAAGCACACC	
STM3615 comp	NNN <u>CCTAG</u> GGTATGTTCTGTTATTCCGC	NNNTCTAGAACATGACAGAAATGCGCC	
Real-time PCR analysis ^d			
<i>rpoD</i>	GTGGATACCGTCTTATGG	GATAGCCTTGCTCCTTAC	91.4
<i>pykF</i>	GGAAGCCGTTTCTATCAT	TCGTTGTTGTAGTCCAGA	84.1
STM1987	TTTACCTCTCAACTCTCC	CTTTGCTCGATAAACCAT	115.6
<i>wcaB</i>	AAGAATGTGCTGAATAATCT	GATTTTCATACCCGAACAG	111.7
<i>wcaD</i>	AGGCTCATCTTCTTATTATCG	TCTGACTACACCATCAATATG	105.1
<i>wzc</i>	TTGGCATGATTAATAACC	CAGATAGTTACGGGTAAT	98.5

^a Mutant construction primers were designed and used following the λRed recombinase method (22).^b Mutant confirmation primers were used to amplify across potential deletion-insertion sites and compare the resulting product sizes to that observed when using wild-type genomic DNA as the template.^c Complementation primers were used to create the Tn7 complementation vector and amplify genes of interest (as described in Materials and Methods).^d Real-time PCR primers were used to confirm microarray analysis results (as described in Materials and Methods).^e Underlining in sequences indicates restriction sites utilized in vector construction.

Alfalfa root colonization assay. Alfalfa seeds (International Specialty Supply) were surface sanitized by placing 150 g in a 500-ml beaker, followed by two rinses in sterile water. The seeds were then incubated for 2 min in 70% ethanol (EtOH), followed by 7 min of incubation in 50% bleach (38,400 ppm) plus 3 drops of Tween 20. Finally, the seeds were rinsed six times in sterile water, incubated for 10 min in sterile water, and dried for 2 days in a laminar flow hood.

For colonization competition assays, bacterial cultures were grown overnight in LB, normalized to an optical density at 600 nm (OD_{600}) of 0.2 in sterile water and diluted 1:100 (to $\sim 10^6$ CFU/ml). One milliliter each of the wild type and a mutant strain were combined to create a 1:1 ratio of wild type to mutant as an inoculum solution. This inoculum was diluted 1:100 in sterile water and plated on LB-Nal and LB-Kan plates using a spiral plater (Autoplate 4000; Spiral Biotech, Norwood, MA) to differentiate wild-type (Nal^r) from mutant (Kan^r) colonies and to verify the 1:1 ratio. Then, 2 ml of inoculum and 18 ml of sterile water were added to a petri dish containing 0.3 g of surface-sanitized alfalfa seeds. The plates containing seeds, bacteria, and water were incubated with agitation on a Stovall Belly Dancer shaker at 24°C for 1 h under constant light. At that time, the water was removed and replaced with fresh, sterile water, and the plates were incubated for an additional 23 h (for a total time of 24 h). At 24 h postinoculation (hpi), samples were taken (as described below), and the water was removed and replaced with fresh, sterile water before the plates were returned to the Stovall Belly Dancer for a further 24 h of incubation. To monitor bacterial populations, four alfalfa seeds with (at 24 h) or without (at 48 h) seed coats were moved to conical tubes containing 35 ml of sterile water and vortexed for 30 s to remove loosely attached bacterial cells. Prior to completing these experiments, data from preliminary experiments with the wild type were analyzed to identify the source of variance in this assay. According to the results, the main source of variability can be attributed to experimental replication, while a sample size above four did not significantly contribute to variance. Thus, the sample size was set at four. After rinsing, individual seeds were placed in microcentrifuge tubes containing 500 μ l of sterile water and homogenized using a 4.8-V rotary tool (Dremel, Mt. Prospect, IL). Homogenates were diluted and plated on both LB-Nal and LB-Kan using a spiral plater, and the resulting colonies were counted after overnight incubation at 37°C to determine bacterial populations. Experiments were performed with three biological replicates.

Quantification of cellulose levels using calcofluor. Calcofluor fluorescence was used to estimate cellulose levels (16, 25, 26). For *in planta* samples, inoculum cultures were mixed with alfalfa seeds as described above for root colonization experiments, except that 2 ml of a single culture inoculum (either the wild type or a mutant strain) was added to seeds instead of a mixture of wild type and mutant. At 24 and 48 hpi, planktonic cells were collected by centrifuging 30 ml (10 ml from each of three petri dishes containing bacteria, water, and germinating alfalfa seeds) at 10,000 rpm at 4°C for 10 min in oakridge tubes. After the water was removed at 24 hpi, 20 ml of fresh, sterile water was added to each petri dish, and samples were returned to the Stovall Belly Dancer for continued incubation. Pelleted bacterial cells were resuspended in sterile water to a normalized OD_{600} reading of 0.5. One milliliter from each normalized sample was mixed with 2 μ l of calcofluor at 100 mg/ml in a microcentrifuge tube, followed by incubation in the dark at room temperature for 1 h. The cells were then washed twice with water to remove unbound calcofluor and resuspended in a final volume of 1 ml of sterile water. Relative fluorescence unit (RFU) readings were taken in a 96-well plate reader (Synergy HT; BioTek, Winooski, VT) at an excitation of 360/40 and an emission of 460/40. Calcofluor levels were measured for three biological replicates per strain at each time point, and statistical analyses were conducted using the Tukey's HSD function in the software package R.

RNA isolation. Bacteria were incubated with alfalfa seeds as described for the calcofluor assays above. Wild-type and *adra* mutant cultures were grown overnight in LB, normalized to an OD_{600} of 0.2 in sterile water, and diluted 1:100 (to $\sim 10^6$ CFU/ml). At 24 hpi, 10-ml samples containing

planktonic bacteria from three petri dishes were combined (for 30 ml total per treatment) in cold Oakridge tubes containing 1/5 volume ice-cold stop solution (5% phenol in EtOH) and pelleted by centrifugation at $10,000 \times g$ at 4°C. Pellets were frozen at -80°C for at least 30 min, and total RNA was isolated using the hot phenol method (27, 28). RNA was treated with Turbo DNA-free (Ambion, Austin, TX) for 1.5 h using the manufacturer's rigorous protocol and quantified by NanoDrop (Thermo Scientific). Real-time PCR was used to measure potential DNA contamination using 100 ng of RNA, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA), and STM1987 primers (Table 2). Samples with threshold cycles of ≥ 33 were considered DNA-free. RNA quality was confirmed using Bio-Rad Experion RNA StdSens or Agilent Bioanalyzer RNA 6000 Pico kit protocols. Total RNA was isolated from three biological replicates per strain for microarray analysis and from a second, independent set of three biological replicates per strain for real-time PCR confirmation of microarray results.

Microarray analysis. Microarrays and probes were designed using Agilent eArray software in an 8×60 grid format. Each microarray contained two 60-mer probes per open reading frame in the *S. enterica* Typhimurium 14028s genome (accession number NC_016856) replicated 5 to 10 times. cDNA synthesis, cRNA labeling, hybridization and washes were done according to the Agilent Low Input Quick Amp WT protocol (Agilent Technologies). The microarrays were scanned using a DNA microarray scanner, model G2565CA (Agilent, Palo Alto, CA), and data were extracted with Agilent Feature Extraction Software. All microarrays passed Agilent quality control standards, and gene expression patterns were analyzed using ArrayStar software (DNASTAR, Inc.).

cDNA synthesis and real-time PCR. cDNA synthesis was performed using the iScript cDNA synthesis kit according to the manufacturer's instructions (Bio-Rad) with 1 μ g of total RNA as input. Real-time PCR primers were designed with Beacon Designer software (Premier Biosoft International) avoiding template secondary structure (Table 2). Primer efficiencies (Table 2) were calculated using serial dilutions of *S. enterica* Typhimurium 14028s genomic DNA and CFX Manager 3.0 software (Bio-Rad). Five potential reference transcripts were chosen based on stable expression in all four treatments for the microarray experiment: *rpoD*, *pykF*, *sfcA*, *trkD*, and *ubiC*. Stable expression between the wild type and the *adra* mutant at 24 hpi was validated using the Best Keeper program and three independent RNA samples from each treatment. All transcripts were stably expressed except *ubiC*. We chose to use *rpoD* and *pykF* as reference genes because their mean *C_q* (quantification cycle) values were most similar to the values seen for the target genes. Real-time PCR experiments were performed as described previously (27). Briefly, RNA samples from three biological replicates per treatment were used to make cDNA for real-time PCR. SsoFast EvaGreen Supermix, 300 nM primers (Table 2), and a 1:10 dilution of cDNA were mixed and aliquoted in 20- μ l triplicates per sample. PCR conditions were 98°C for 2 min and 40 cycles of 98°C for 2 s and 55°C for 5 s, which was followed by a melting curve with 100 cycles of 70°C for 10 s with a 0.2°C increase per cycle. Experiments were performed using the CFX96 real-time system and analyzed with the CFX Manager 3.0 software (Bio-Rad). The mean *C_q* of each target transcript was normalized by the mean *C_q* of each reference gene using the formula: $2^{-(C_q \text{ target} - C_q \text{ reference})}$. As previously described (29), we determined the relative expression ratio (RER) of the target gene by dividing the normalized target RNA by a calibrator consisting of the average of the normalized values of the control samples (the expression in the wild type in this experiment). Statistical analysis of the RER values was performed with GraphPad Prism software using the unpaired two-tailed *t* test function (GraphPad Software, Inc.).

Complementation vector and strain construction. The complementation vector pRCGTn7AB was created as follows: sequence flanking the *S. enterica* attTn7 site (the intergenic region between *glmS* and STM3860) was PCR amplified using primers listed in Table 2. The first product (Tn7B; 456 bp) was digested with *SpeI* and *HindIII* and cloned into the corresponding sites of pRCK-GWY (30), replacing the *Ralstonia* sequence

found at that location on the vector. The resulting plasmid pRCKTn7B and the second PCR product (Tn7A; 403 bp) were digested with *ApaI* and *XhoI*, gel extracted to remove the second *Ralstonia* genome sequence, and ligated together (T4 ligase; Promega, Madison, WI) to create pRCKTn7AB. The final complementation vector pRCGTn7AB was generated by replacing the kanamycin resistance cassette in pRCKTnAB with the gentamicin resistance cassette from pRCG-GWY using *EcoRV* and *AvrII* restriction enzymes.

Complementation strains for the *adrA*, STM1987, STM3375, and STM3615 mutants were made by integrating the respective genes into the *attTn7* site on the *S. enterica* chromosome using λ Red recombination. Each gene and predicted promoter region (~300 bp upstream) were PCR amplified using *Pfx* and primers listed in Table 2. The resulting PCR product was gel extracted, digested with *AvrII* and *XbaI*, and cloned into the corresponding restriction sites on pRCGTn7AB. The full-length insert was confirmed by sequencing. To integrate the gene at the *attTn7* site, flanking primers Tn7compA and Tn7compB (Table 2) were used to PCR amplify the gentamicin resistance cassette and inserted gene (with promoter). The resulting PCR product was gel extracted and integrated into the *S. enterica* KHLT2 chromosome using the λ Red recombinase method (22). Integration was confirmed using primers flanking the *attTn7* site (Tn7compFor and Tn7compRev; Table 2). The gentamicin cassette and complementing gene product were transduced from the KHLT2 background into wild-type *S. enterica* 14028s and the corresponding mutant strain using P22 transduction (24). Integration was confirmed using Tn7compFor and Tn7compRev primers in combination with gene specific primers (Table 2).

Alfalfa phyllosphere colonization assay. Alfalfa plants were grown in a climate-controlled chamber maintained at 28°C. Approximately 50 seeds were planted per pot in Professional Growing Mix (Sunshine Redi-Earth). Two-week-old plants were dip inoculated as described below. Briefly, overnight bacterial cultures were normalized to an OD₆₀₀ of 0.125 in 200 ml of sterile water. For competition assays, equal volumes of wild-type and mutant suspensions were combined to generate an approximate 1:1 inoculum ratio, and inoculum populations (~10⁸ CFU/ml) were verified through dilution plating. Prior to inoculation, 0.025% Hi-Wett (Loveland Products, Inc.) was added to each bacterial mixture. Pots containing alfalfa plants were inverted in the inoculum suspension for 1 min with agitation to prevent bacterial cell settlement and then incubated for 2 days at high humidity in lidded, plastic bins under grow lights at 26°C. At 48 hpi, unifoliate leaves from six plants per pot (three pots per treatment) were transferred to microcentrifuge tubes and weighed to determine leaf mass. Two leaves and 500 μ l of sterile water were added to each microcentrifuge tube and homogenized as described above for root colonization assays. Homogenates were diluted 1:10 in sterile water and spiral plated on LB-Nal and LB-Kan plates. The resulting colonies were counted after overnight incubation at 37°C to determine bacterial populations. Experiments were performed with three biological replicates.

For alfalfa phyllosphere desiccation assays, plants were inoculated as described above. For “humid” conditions (relative humidity of 85%), inoculated plants were incubated in lidded, plastic bins under grow lights at 26°C as described above. For “dry” conditions (relative humidity of 15%), inoculated plants were incubated in a biosafety cabinet (SterilGARD; The Baker Company, Sanford, ME). Relative humidities were quantified with WatchDog data loggers (Spectrum Technologies, Inc., Aurora, IL) placed adjacent to plants during a representative experiment. At 24, 48, and 72 hpi, unifoliate leaves from two plants per pot (three pots per treatment) were transferred to microcentrifuge tubes, weighed to determine leaf mass, and homogenized in water as described above. Undiluted homogenates were plated on LB-Nal and LB-Kan plates, and the resulting colonies were counted after overnight incubation at 37°C to determine bacterial populations. Experiments were performed with three biological replicates.

Statistical analysis. Except for the real-time PCR analysis described above, all statistical analyses were performed using R software (version

2.14.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria [<http://www.R-project.org/>]) as described previously (31). Briefly, three biological replicates were performed for each experiment, and samples taken from one replicate were considered subsamples. The subsamples from each time point and each strain were averaged, and, for root colonization and leaf persistence competition experiments, the averaged percentage of the total population (compared to wild type) for each mutant was compared to the expected value of μ , 50, in one-sample *t* tests. A mutant with no competitive difference would be present at a 1:1 ratio with the wild type (50% of the total population). For the quantification of cellulose production using calcofluor, the averaged, logarithmic relative fluorescence units (log₁₀ RFU) were compared for each strain at each time point using Tukey’s HSD test. Results were considered statistically significant at a *P* of <0.05. To determine whether leaf persistence competition results differed between treatments in the desiccation experiments, analysis of covariance was used as previously described (32), with treatment and time as covariates.

Microarray data accession number. The array data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (62) and are accessible through GEO series accession number GSE75662 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75662>).

RESULTS

Cellulose and curli are required at different stages of root colonization. Previously, we had used single strain inoculation experiments to show that cellulose and curli are important attachment factors for alfalfa root colonization, whereas *AdrA* is dispensable (7, 8). To complement those studies and to determine whether the single strain inoculation method may have missed subtle phenotypes, we characterized the competitive fitness of mutant strains defective in *adrA*, cellulose (*bcsA*), curli (*csgB*), or both cellulose and curli (*bcsA csgB*) during colonization of roots. We found a significant competitive disadvantage in root colonization for the *bcsA* mutant at 24 hpi, whereas the *adrA* mutant was equally competitive with the wild type at 24 hpi and more competitive than the wild type at 48 hpi (Fig. 1A). We also observed a significant disadvantage for the *csgB* mutant at 48 hpi, while the *bcsA csgB* double mutant was significantly reduced at both 24 and 48 hpi compared to the wild type (Fig. 1A).

***AdrA* is dispensable for cellulose production during root colonization but influences transcription of the colanic acid biosynthetic genes.** To determine whether cellulose is produced during root colonization and to address the importance of *adrA* in that process, we monitored cellulose production using the fluorescent stain calcofluor (16, 25, 26). When bound to the β -1,4 carbohydrate linkages in cellulose polymers, calcofluor emits detectable levels of fluorescence that can be measured by spectrophotometry. Using this assay, we examined cellulose production in planktonic cells collected from root colonization assays at 24 and 48 hpi. Bacterial cells are found in multiple stages during the colonization assay: attached to, migrating toward, or migrating away from the alfalfa seeds and roots. Thus, the planktonic cell population is representative of cells that have been or will be attached to the growing alfalfa roots. Under these conditions, the cellulose synthase mutant *bcsA* bound significantly less calcofluor than the wild type, whereas the *adrA* mutant had the same amount of calcofluor fluorescence as the wild type (Fig. 1B).

To characterize the genome-wide transcriptional effects of an *adrA* mutant and potentially identify alternative regulators of cellulose production in this strain, we compared the expression profiles of wild type and an *adrA* mutant during root colonization using microarrays. Bacterial strains were mixed with alfalfa seeds,

FIG 1 Cellulose and curli are temporally important for root colonization, and cellulose production is independent of *adrA* in this environment. (A) Mutant strains were compared with the wild type for colonization of alfalfa roots after coinoculation with the wild type at a 1:1 ratio. Gray and white bars indicate the proportion of the population (% total population) that is composed of the mutant strain at 24 and 48 hpi, respectively. (B) Cellulose production was monitored by measuring calcofluor fluorescence and is presented as logarithmic relative fluorescence units ($\log_{10}$ RFU) for wild-type and mutant strains at 24 and 48 hpi. Each experiment was repeated three times, and the results of representative experiments are presented here. Error bars indicate standard deviations, while asterisks indicate significant differences either from 50% (A; dashed line), a percentage that represents equal proportions of the wild type and mutant, or from the wild type (B) ($P < 0.05$).

and planktonic cells were harvested for RNA extraction at 24 hpi. Of the 5,421 *S. enterica* genes, 68 genes had at least a 2-fold difference in expression (with a confidence interval of 95%) between the wild type and the *adrA* mutant (see Table S2 in the supplemental material). We found that 22 genes involved in the production of colanic acid, one component of the *S. enterica* extracellular matrix, had significantly lower transcript levels in the *adrA* mutant strain (Table 3). Expression results for representative genes from the colanic acid biosynthetic pathway were verified by quantitative real-time PCR (Table 3).

Multiple DGCs impact *S. enterica* root colonization and in planta cellulose production. To explore the possibility that other DGCs promote cellulose production during *S. enterica* root colonization in the place of AdrA, we created a single insertion-deletion mutation in each of the remaining 11 putative *S. enterica* Typhimurium DGC genes and monitored bacterial populations attached to roots at 24 and 48 hpi. We chose to investigate the competitive fitness of all DGC mutants regardless of GGDEF protein domain degeneracy to achieve the most thorough analysis. Two mutants had competitive disadvantages compared to the

wild type: the STM1987 mutant was outcompeted by the wild type at 24 hpi, whereas the STM3375 mutant was outcompeted by the wild type at both 24 and 48 hpi (Fig. 2A). Similar to the *adrA* mutant, an STM3615 mutant had a significant competitive advantage over the wild type at 48 hpi (Fig. 2A). The root colonization effects of the *adrA*, STM1987, STM3375, and STM3615 mutants were complemented by providing the respective gene in single copy at the *attTn7* site (Fig. 2B). In addition to the competition experiments highlighted in this work, we also examined the single strain inoculation root colonization phenotypes of the DGC mutants and found that, except for the STM2123 mutant, the DGC mutants behaved similarly in both assays (data not shown). For example, a mutant with a disadvantage compared with the wild type in the competition assay was found at reduced levels in the single strain inoculation assay. The STM2123 mutant was unique from the other DGC mutants in that it was equally competitive with the wild type in the competition experiments but was found at reduced levels (0.65 log lower) than the wild type at 48 hpi in the single strain inoculation assay (data not shown). Finally, to get a broader perspective of how c-di-GMP regulation impacts *S. enterica* root colonization, we created single insertion-deletion mutations in each of the eight putative *S. enterica* PDEs (Table 1) and quantified bacterial populations in the root colonization competition assay. All eight PDE mutants were equally competitive with the wild type for the colonization of roots except for the STM4264 mutant, which outcompeted the wild type at 48 hpi (data not shown).

TABLE 3 Colanic acid biosynthesis gene expression is reduced in the *adrA* mutant

Gene	Fold change ^a	Predicted function ^b
STM14_2599	8.9	Hypothetical protein
STM14_2614	5.2	Hypothetical protein
<i>gmd</i>	7.0	GDP-D-mannose dehydratase
<i>manC</i>	9.0	Mannose-1-phosphate guanylyltransferase
<i>rcsA</i>	12.4	Capsular biosynthesis activation protein A
<i>wcaA</i>	6.5	Glycosyltransferase family protein
<i>wcaB</i>	5.4 (6.1)	Putative colanic acid biosynthesis acetyltransferase
<i>wcaC</i>	5.8	Glycosyl transferase family protein
<i>wcaD</i>	7.2 (6.9)	Putative colanic acid biosynthesis protein
<i>wcaE</i>	9.5	Glycosyltransferase family protein
<i>wcaF</i>	11.5	Putative colanic acid biosynthesis acetyltransferase
<i>wcaG</i>	8.9	GDP-fucose synthetase
<i>wcaH</i>	5.4	GDP-mannose mannosyl hydrolase
<i>wcaI</i>	7.5	Glycosyltransferase family protein
<i>wcaJ</i>	3.6	UDP-glucose lipid carrier transferase
<i>wcaK</i>	5.4	Putative pyruvyltransferase
<i>wcaL</i>	4.0	Putative glycosyltransferase
<i>wcaM</i>	3.2	Putative colanic acid biosynthesis protein
<i>wza</i>	8.6	Putative outer membrane polysaccharide export protein
<i>wzb</i>	5.9	Tyrosine phosphatase
<i>wzc</i>	7.3 (10.7)	Tyrosine kinase
<i>wzx</i>	5.9	Colanic acid exporter

^a Numbers denote reduced transcript levels in the *adrA* mutant as measured by microarray analysis. Numbers in parentheses denote reduced transcript levels in the *adrA* mutant as measured by real-time quantitative PCR.

^b Predicted protein function information was obtained from the *S. enterica* serovar Typhimurium strain 14028s database on the BioCyc Database Collection website.

FIG 2 The DGC STM1987 contributes to root colonization and *in planta* cellulose production. (A) The 12 DGC mutants were compared to the wild type for colonization of alfalfa roots after coinoculation at a 1:1 ratio. (B) Complementation experiments for the *adrA*, STM1987, STM3375, and STM3615 mutants. Each respective gene was provided *in trans*, and competition experiments were performed as described. Gray and white bars indicate the proportion of the population (% total population) that is composed of the mutant strain at 24 and 48 hpi, respectively. (C) Cellulose production was monitored by measuring calcofluor fluorescence and is presented as logarithmic relative fluorescence units (log₁₀ RFU) for wild-type and mutant strains at 24 and 48 hpi. Each experiment was repeated three times, and the results of representative experiments are presented here. Error bars indicate standard deviations while asterisks indicate significant differences from 50% (dashed line), a percentage that represents equal proportions of wild type and mutant ($P < 0.05$), and letters indicate significant differences between strains within a single time point (uppercase letters for 24 hpi and lowercase letters for 48 hpi; $P < 0.01$).

To investigate a potential connection between the DGCs that displayed competitive differences from the wild type and *in planta* cellulose production, we examined calcofluor levels in the STM1987, STM3375, and STM3615 mutants as described above for the *adrA* mutant. At both 24 and 48 hpi, the STM1987 mutant produced intermediate levels of cellulose compared with wild type and the *bcsA* mutant (Fig. 2C). The STM3615 mutant produced wild-type levels of cellulose at both time points, whereas the STM3375 mutant had reduced levels of cellulose production at 48 hpi (Fig. 2C).

Environment determines the role of c-di-GMP metabolism and exopolysaccharides during *S. enterica* plant colonization. To determine the effect of environment on the role of DGCs and PDEs in *S. enterica*-plant interactions, we examined the importance of all 20 c-di-GMP metabolism genes in a different plant environment, the alfalfa phyllosphere. The phyllosphere is defined as all above-ground plant surfaces, and for the purposes of these experiments, we consider the alfalfa phyllosphere to be the leaves. Alfalfa plants were dip inoculated into a bacterial suspension containing an equal ratio of the wild-type and mutant strains. On average, the total recovered population size was 7×10^3 CFU/mg of leaf tissue at 48 hpi. After 48 hpi, we enumerated bacterial populations and found that the *adrA*, STM3375, and STM3615 DGC mutants had significant competitive disadvantages compared to the wild type (Fig. 3A). The STM1987 mutant, which had a rhizosphere colonization defect, and the remaining DGC mutants were not significantly different from the wild type in the phyllosphere (Fig. 3A and data not shown, respectively). With regard to the PDE mutants, we found that the STM1344, STM1697, and STM4264 mutants had significant defects in leaf persistence compared to the wild type (Fig. 3A), whereas the other PDEs were not different from the wild type (data not shown).

To determine the importance of known biofilm components for persistence on leaves, we performed leaf competition experiments for the *bcsA* and *csgB* mutants, as well as three colanic acid mutants (*wcaB*, *wcaD*, and *wzc*). After 48 hpi, we enumerated bacterial populations and found that the *bcsA* mutant and the three colanic acid mutants had significant competitive disadvantages compared with the wild type, while the curli mutant had no impact on leaf persistence (Fig. 3B).

To successfully colonize the leaf surface, bacteria must overcome the relative lack of water present in that environment. To examine desiccation tolerance as a potential mechanism involved in *S. enterica* survival on leaves, we monitored proportions of the wild type and the *adrA* or *wcaB* mutants on inoculated plants that were incubated under two different relative humidity conditions: 85% humidity ("humid") and 15% humidity ("dry"). We enumerated bacterial populations on leaves at 24, 48, and 72 hpi and found that the dry conditions further reduced the competitive abilities of the *adrA* (Fig. 4A) and *wcaB* (Fig. 4B) mutants. In addition, we found that the STM1987 mutant was equally competitive with the wild type under both humid and dry conditions (data not shown), indicating that the dry conditions do not cause a general loss of competitive fitness for this mutant.

DISCUSSION

Although *S. enterica* is often found in an animal host, it frequently contaminates agricultural crops, can persist on plants for the duration of crop production, and has become the most likely source of outbreaks and food borne illness in humans in recent years. The

FIG 3 c-di-GMP metabolism and exopolysaccharide production impact *S. enterica* persistence on leaves. (A) DGC (yellow) and PDE (blue) mutants and (B) cellulose, curli, and colanic acid mutants were compared to the wild type for colonization of alfalfa leaves after coinoculation at a 1:1 ratio. The results of representative experiments showing persistence of each mutant as a proportion of the total population (% total population) at 48 hpi are shown here. Experiments were performed three times, error bars indicate the standard deviations, and asterisks indicate significant differences from 50% (dashed line; $P < 0.05$).

phyllosphere, or above-ground parts of the plant, provides a harsh setting with a dearth of water and nutrients limited to few sites, leading to no net growth of *S. enterica* (3, 33–35). In contrast, the rhizosphere, such as that of the roots of germinating seeds used in this study, is bathed in the nutrients from root exudates, and *S. enterica* grows exponentially on or around the roots (36). Aggregation or biofilm formation can protect bacteria from environmental stresses (37–39). Accordingly, *S. enterica* uses attachment factors and biofilm formation to utilize plants as vectors to mammalian hosts (7, 8). In the present study, we have begun to detail the mechanisms involved in the regulation of cellulose production and biofilm composition in these environments and how that regulation leads to the successful colonization of plants.

By combining the data presented here with known regulatory connections from *in vitro* studies (14), we created a schematic model of *S. enterica* biofilm formation during plant colonization (Fig. 5). This figure summarizes our contributions to the new regulatory hierarchy that should be considered when discussing *S.*

FIG 4 AdrA and colanic acid production are important for desiccation tolerance on leaves. Leaf persistence competition assays were repeated under two relative humidity conditions: 85% humidity (black squares) and 15% humidity (red triangles). All data points from three independent experiments were combined and are presented as the proportion of the population (% total population) that is composed of the mutant strain (*adrA* [A] or *wcaB* [B]) at 24, 48, and 72 hpi. Lines (dashed black, humid conditions; solid red, dry conditions) correspond to a linear regression model, and shaded areas correspond to the respective 95% confidence interval.

enterica interactions with plants. A mutant in one of these factors, STM2123, displayed an intriguing phenotype; the root colonization defect of the STM2123 mutant was specific for single inoculation experiments. Therefore, we hypothesized that the wild-type

FIG 5 The regulation and importance of exopolysaccharide biofilm components in *S. enterica* plant colonization depends on the environment. The regulation presented here is modeled after data collected from *in vitro* studies (dashed lines) (14, 60, 61) but also highlights the results from plant experiments (solid lines). Colors indicate factors with plant colonization defects in the rhizosphere (tan) or phyllosphere (green). Putative DGCs and PDEs are shown as rectangles and ovals, respectively. The asterisk after STM2123 denotes that the root colonization defect observed in this mutant occurs specifically during single inoculation experiments and not competition assays.

cells present in the competition experiment may be providing some factor in *trans* that restored colonization to the STM2123 mutant. In *E. coli* and *S. enterica*, extracellular curli subunits can be shared between adjacent bacterial cells in interbacterial complementation (40, 41). However, we predict that curli is not the colonization factor provided by the wild type to the STM2123 mutant during competition assays because the interbacterial complementation of curli will not work for a strain like the STM2123 mutant that is missing both CsgA and CsgB. Although this study will contribute much knowledge in the field, we recognize that there are still questions that remain unanswered. For example, in our model, we show that CsgD is required for root colonization but not persistence on leaves (Fig. 5). As downstream factors, such as AdrA, cellulose, and colanic acid, impact leaf persistence, we had predicted that CsgD would also be involved. Our data do not resolve this issue, and future work should be performed to address potential alternate regulators of AdrA in this environment.

In this study, we continued a previous line of investigation (7, 8) and provide evidence that cellulose and curli are required at different stages of colonization. The different temporal requirements for cellulose and curli may reflect morphological differences in the roots themselves. At 24 hpi, the initially germinating seeds have a root radical emerging from the ruptured seed coat. The seedlings at 48 hpi have lost the seed coat and have a longer radical, developing cotyledons, and the preferred *S. enterica* root colonization site, root hairs (42). We hypothesize that cellulose may be needed prior to root hair development for its contribution to biofilm formation (26) on the emerging radical or for its role in the inhibition of flagellar motility (43) upon arrival to a preferred colonization site. After initial attachment to the germinating seed, *S. enterica* may use cellulose to remain in this niche. By 48 hpi, biofilms may have matured enough that the production of cellulose

is no longer needed to maintain this site, or the appearance of root hairs may provide a favorable structure for bacterial attachment that utilizes curli instead of cellulose. Alternatively, the temporal requirements for cellulose and curli may result from differential regulation of these factors by environmental cues. Solano et al. (26) demonstrated that the composition of the *S. enterica in vitro* biofilm changes in different media; in a rich medium, such as LB, biofilms are comprised of cellulose, curli, and other factors while in a nutrient-limiting medium (adherence test medium [ATM]) (44), cellulose is the major component of the biofilm (26). It is difficult to determine what natural environments either LB or ATM resemble and thus, what cues are important for biofilm regulation. The nutrients exuded by the germinating seeds change over time and impact *S. enterica* growth (31, 45). Thus, the nutrients that are available at 24 or 48 hpi may influence the requirement for different biofilm components. To thrive in this niche, the bacteria need to adapt and respond to these types of environmental changes.

Regulation of cellulose is a key example of the mechanisms necessary to successfully transition lifestyles from symbiont to pathogen. Thus, two additional goals of this work were to determine whether *S. enterica* produced cellulose during root colonization and, if so, to identify the regulatory factors responsible for cellulose production and root colonization. We demonstrated that *S. enterica* produces cellulose during root colonization and that *adrA* is not required for this activity (Fig. 1B). Although screening of all 20 putative c-di-GMP metabolism mutants for root colonization defects identified several genes with some role in this environment (Fig. 2A), we found that many mutants were equally competitive with the wild type. Mutants that displayed wild-type levels of root colonization may represent genes that have no function in this environment or, alternatively, have re-

dundant function with other enzymes that mask potential phenotypes in a single gene deletion mutant. Due to the large number of mutational combinations that would be required to fully address the latter possibility, we focused on the single gene deletion mutants that were significantly outcompeted by the wild type. The STM3375 mutant has a competitive defect at both 24 and 48 hpi and reduced cellulose production at 48 hpi (Fig. 2). Since STM3375 contains a degenerate GGDEF domain and, in *E. coli*, does not bind, synthesize, or degrade c-di-GMP (46), we chose to characterize STM1987 and not pursue STM3375 further. The intermediate levels of calcofluor binding by the STM1987 mutant indicated that STM1987 is only partially responsible for cellulose production and suggested the presence of yet another mechanism for cellulose production during root colonization. STM1987 was first identified as a DGC that complemented the biofilm formation defect of a *S. enterica* strain that did not make cellulose, SL1344 (17). When provided on a plasmid, STM1987 specifically restored cellulose production and biofilm formation in SL1344 under nutrient-deficient conditions at 37°C (17). Here, we have shown that STM1987 is responsible for cellulose production at early stages of root colonization through an AdrA-independent mechanism. Thus, these and other works corroborate our findings that bacteria use different regulatory mechanisms to control the production of cellulose in distinct environments.

Although AdrA does not affect cellulose production during root colonization, we found that AdrA affects transcription of the genes involved in colanic acid biosynthesis. Colanic acid is comprised of repeating units of D-glucose, L-fucose, D-galactose, and D-glucuronate. Although not required for biofilm formation, colanic acid contributes to the three dimensional structure of the *S. enterica* biofilm in cells grown in M63 glucose medium on a plastic coverslip and provides protection from environmental stresses, including acidity, osmotic shifts, and desiccation (10, 47, 48). Initially, we performed the microarray experiments to examine transcription of all DGCs in the *adrA* mutant; we hypothesized that changes in DGC transcription could explain the production of cellulose and the wild-type levels of root colonization observed in this strain. Although this hypothesis was disproven by the gene expression results (expression of other DGCs did not change in the *adrA* mutant), these data identified a new role for AdrA in the expression of colanic acid biosynthetic genes. Previously, we demonstrated that, similar to the *adrA* mutant, a colanic acid mutant (*wcaJ*) colonized alfalfa roots at wild-type levels in *S. enterica* serovar Enteritidis (8). Collectively, these data suggest that colanic acid is not important for alfalfa root colonization and that AdrA may regulate this exopolysaccharide for a different purpose.

In contrast to the root environment, on the leaf surface, *S. enterica* encounters a harsh environment with little protection from desiccation, UV stress, or nutrient starvation. Patterns of water and nutrient availability on a leaf surface are comparable to oases in a desert (49); thus, the arrival site on the leaf predisposes a subset of bacterial cells for survival or death (50). In addition, bacterial aggregation or formation of biofilms can provide a protective layer, trap nutrients, and generally aid in stress tolerance (37–39). *S. enterica* biofilm mutants are significantly impaired in persistence on produce leaves (51, 52). In support of the idea that biofilm formation is vital to *S. enterica* survival on leaves, we found that *adrA* and the production of exopolysaccharides, such as cellulose and colanic acid, contributed to persistence on alfalfa leaves (Fig. 3). Furthermore, we demonstrated that *adrA* and colanic

acid production are important for desiccation tolerance (Fig. 4), providing a potential mechanism for the impact of biofilm formation on *S. enterica* leaf persistence. These results parallel studies of aggregation and biofilm formation in the plant-pathogenic bacterium *Pseudomonas syringae* pv. *syringae*. *P. syringae* biofilm formation, in the form of alginate production, contributes to optimal fitness on the leaf surface (39). Furthermore, although dry conditions lead to smaller and less numerous bacterial aggregates (38), *P. syringae* survives better than the epiphytic bacterium *Pantoea agglomerans* specifically under those conditions (53), emphasizing the general importance of desiccation tolerance for bacterial survival on the leaf surface.

In addition to the importance of initial arrival site and biofilm formation for *S. enterica* survival, bacterial motility, such as movement toward more optimal sites like trichomes and cell junctions, positively impacts persistence (54–57). We found that two putative PDE mutants, STM1344 and STM1697, had competitive defects in the phyllosphere in comparison to wild type (Fig. 3A) but were not involved in root colonization (data not shown). *In vitro* assays showed that an STM1697 mutant is hypermotile at low temperatures and has enhanced swarming (58), which is the opposite result compared to strains with mutations in other PDEs with confirmed phosphodiesterase activity (14, 59). STM1697 can functionally replace STM1344 and vice versa, but neither protein degrades nor binds c-di-GMP (58). Both STM1344 and STM1697 suppress transcription of class 2 and class 3 flagellum regulon genes by binding to FlhD (58), and we posit that both genes play a role in leaf persistence via motility. Since STM1344 and STM1697 act additively *in vitro*, deletion of either may uncouple motility regulation *in planta* and could interfere with preferential niche establishment of *S. enterica* in the phyllosphere. Future studies will directly examine the role of motility in *S. enterica* colonization of both the phyllosphere and rhizosphere.

As described in the introduction, multiple c-di-GMP metabolizing enzymes affect cellulose production through the regulation of the transcription factor CsgD. The four PDEs that inhibit *in vitro* CsgD activity have been shown to have different levels of importance. During growth on low salt agar plates, STM1703 is the major inhibitor of CsgD, while the other three PDEs play more minor roles (14). Here, we show that only one of these four PDEs, STM4264, impacts leaf persistence (Fig. 3A). These data suggest that environmental factors may influence the contribution of the different PDEs. Thus, STM4264 may play a more significant role in CsgD regulation on the leaf surface than in other growth conditions. Alternatively, the leaf persistence competition defect of the STM4264 mutant may be unrelated to the regulation of CsgD since the *csgD* mutant had no defect in the phyllosphere. The contribution of individual PDEs to CsgD regulation and biofilm production may be environment dependent and the cues that affect PDE activities remain unidentified.

In this study, we elucidated additional details of the mechanisms of biofilm formation during plant colonization, provided further evidence that specific DGC regulatory functions are based on environmental cues, provided another example of AdrA-independent cellulose production, identified a new role for AdrA in colanic acid gene expression, and identified biofilm components that are important for *S. enterica* phyllosphere persistence and desiccation tolerance (Fig. 5). The complex nature of the regulatory network that impacts c-di-GMP levels, cellulose production, and biofilm formation further drives home the concept that *S.*

enterica excels at persisting in a wide variety of environments due to its ability to regulate downstream functions (i.e., biofilm formation) with overlapping and potentially redundant pathways. The robust regulatory flexibility is a hallmark of bacteria that thrive in radically distinct environments and lifestyles. The knowledge gained through a better understanding of the regulation of biofilm formation lays the groundwork for intervention strategies that will impact food security and human health.

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