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RESEARCH ARTICLE

Shigella flexneri Diguanylate Cyclases Regulate Virulence

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ABSTRACT *Shigella flexneri* is an intracellular human pathogen that invades colonic cells and causes bloody diarrhea. *S. flexneri* evolved from commensal *Escherichia coli*, and genome comparisons reveal that *S. flexneri* has lost approximately 20% of its genes through the process of pathoadaptation, including a disproportionate number of genes associated with the turnover of the nucleotide-based second messenger cyclic di-GMP (c-di-GMP); however, the remaining c-di-GMP turnover enzymes are highly conserved. c-di-GMP regulates many behavioral changes in other bacteria in response to changing environmental conditions, including biofilm formation, but this signaling system has not been examined in *S. flexneri*. In this study, we expressed VCA0956, a constitutively active c-di-GMP synthesizing diguanylate cyclase (DGC) from *Vibrio cholerae*, in *S. flexneri* to determine if virulence phenotypes were regulated by c-di-GMP. We found that expressing VCA0956 in *S. flexneri* increased c-di-GMP levels, and this corresponds with increased biofilm formation and reduced acid resistance, host cell invasion, and plaque size. We examined the impact of VCA0956 expression on the *S. flexneri* transcriptome and found that genes related to acid resistance were repressed, and this corresponded with decreased survival to acid shock. We also found that individual *S. flexneri* DGC mutants exhibit reduced biofilm formation and reduced host cell invasion and plaque size, as well as increased resistance to acid shock. This study highlights the importance of c-di-GMP signaling in regulating *S. flexneri* virulence phenotypes.

IMPORTANCE The intracellular human pathogen *Shigella* causes dysentery, resulting in as many as one million deaths per year. Currently, there is no approved vaccine for the prevention of shigellosis, and the incidence of antimicrobial resistance among *Shigella* species is on the rise. Here, we explored how the widely conserved c-di-GMP bacterial signaling system alters *Shigella* behaviors associated with pathogenesis. We found that expressing or removing enzymes associated with c-di-GMP synthesis results in changes in *Shigella*'s ability to form biofilms, invade host cells, form lesions in host cell monolayers, and resist acid stress.

KEYWORDS *Shigella*, c-di-GMP, biofilm, invasion, cell-cell spread, diguanylate cyclase, intracellular pathogen

Shigella, a member of the *Enterobacteriaceae* family, diverged from commensal *Escherichia coli* to become a human pathogen and the causative agent of bacillary dysentery (1, 2). *Shigella* is transmitted through inadvertent ingestion and impacts millions of people worldwide, particularly children below the age of 5 years (3). The rise in multidrug-resistant *Shigella* strains and the lack of effective vaccines complicate shigellosis treatment and prevention strategies (3–5).

Shigella flexneri is one among the four subgroups of the genus *Shigella*, and its pathogenesis has been well characterized (4), predominantly using *in vitro* tissue culture models (6–14). In the intestinal lumen, bile and glucose act as signals to initiate biofilm formation and activate *S. flexneri* virulence (8, 15). When *S. flexneri* reaches the colon epithelium, it interacts with microfold cells (M-cells) that endocytose the bacteria

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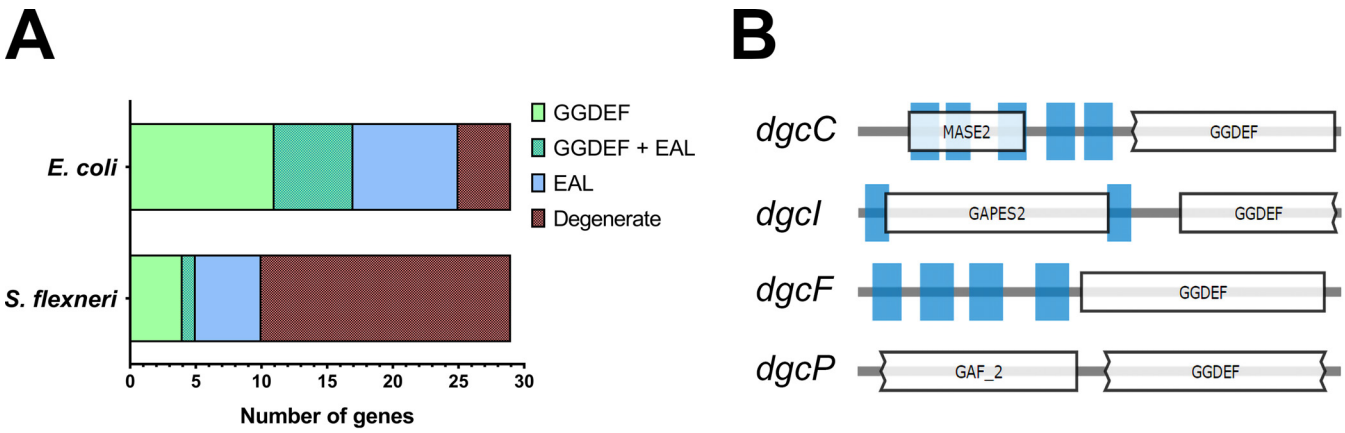

FIG 1 Comparison of *E. coli* and *S. flexneri* c-di-GMP-metabolizing genes. (A) c-di-GMP turnover genes were identified as encoding GGDEF, EAL, and GGDEF plus EAL based on conserved C-terminal domains. Genes were classified as degenerate if they contained mutations that disrupted the conserved C-terminal c-di-GMP turnover domain. *S. flexneri* has presumably lost functionality of ~64% of GGDEF domain enzymes, ~37% of EAL domain enzymes, ~83% of both GGDEF and EAL domains enzymes, and cumulatively 60% of the total c-di-GMP turnover enzymes. (B) Conserved domains of *S. flexneri* DGCs. Blue boxes indicate predicted transmembrane domains. Determination of DGC domain features was performed using MiSTDB (87).

(16). From inside an M-cell, *S. flexneri* can then spread laterally to adjacent epithelial cells (17) or is transcytosed to the basolateral face of the epithelium, where it invades colonic epithelial cells (18, 19). *S. flexneri* entry into the epithelial cells is mediated through a type III secretion system (T3SS) encoded on a large virulence plasmid (20–23). Once inside the host cytosol, *S. flexneri* escapes the endocytic vacuole (24) and replicates to a high cell density (25). *S. flexneri* has lost flagellar motility, likely a consequence of pathoadaptation (26–28), and instead exploits host actin synthesis for intercellular spread using the bacterial outer membrane protein IcsA (also known as VirG) (29–32).

Intestinal pathogens like *S. flexneri* are exposed to many different environmental conditions during the course of an infection, requiring them to rapidly adapt their physiology to effectively cause disease. Other bacteria have evolved diverse mechanisms of sensing and responding to environmental changes; one such mechanism is signaling via the second messenger cyclic di-GMP (c-di-GMP) (33–35). c-di-GMP signaling is widely conserved among bacteria (36, 37). This signaling system is controlled by opposing activity of two enzyme classes: diguanylate cyclases (DGCs), which synthesize c-di-GMP with an intact GGDEF domain, and c-di-GMP-specific phosphodiesterases (PDEs), which hydrolyze c-di-GMP with an EAL or HD-GYP domain (33–37). Many of these enzymes in various bacteria contain input sensory domains which interact with environmental ligands to activate or repress c-di-GMP synthesis or hydrolysis (34, 38–40); changes in c-di-GMP levels can then elicit changes in gene expression or directly interact with various proteins to change downstream bacterial phenotypes (35–37, 41–48).

The number of DGCs and PDEs encoded by different bacteria is variable. Because *Shigella* evolved from commensal *E. coli*, the genomes are highly similar. The genomes of the two organisms are greater than 90% homologous, as determined by DNA hybridization studies (49, 50), and the pathoadaptation of *Shigella* can be defined primarily by its loss of chromosomal genes and its acquisition of a virulence plasmid (51, 52). In *E. coli* strain K-12 MG1655, there are 29 DGCs and PDEs (37, 44, 45, 48) that regulate different functions, including virulence in pathogenic *E. coli* (45). Although *Shigella* evolved from commensal *E. coli*, *S. flexneri* encodes only 4 DGCs with intact GGDEF domains, 7 PDEs with intact EAL domains, and no known PDEs with HD-GYP domains (Fig. 1). Thus, it appears that *S. flexneri* has undergone an extensive reduction in its c-di-GMP signaling network through the process of pathoadaptation, and yet the remaining DGCs and PDEs are highly conserved from *E. coli* (>98% nucleotide identity). Despite its known role in regulating various phenotypes in *E. coli*, the role of c-di-GMP in regulating gene expression in *S. flexneri* has not yet been examined. In this study, we sought to determine role

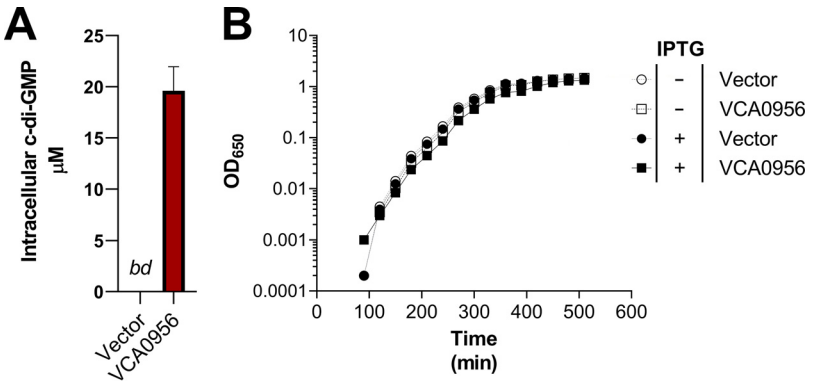

FIG 2 VCA0956 expression increases *S. flexneri* c-di-GMP. (A) *S. flexneri* c-di-GMP levels of strains containing pVCA0956 or the empty vector control (pEVS143) were measured using UPLC-MS/MS. VCA0956 expression corresponds to a significant increase in c-di-GMP levels compared to that of the vector control, which was below the detection threshold of 0.2 μM (*bd*). Results are an average from three replicates, and error bars indicate standard deviation. (B) Growth curves (OD_{650}) were determined for *S. flexneri* carrying pVCA0956 or pEVS143, grown in LB. Strains were grown with IPTG (indicated by "+") and without IPTG (indicated by "−") (100 μM). VCA0956 expression had no notable impact on *S. flexneri* growth rate.

of c-di-GMP signaling in *S. flexneri* pathogenesis. We artificially increased *S. flexneri* c-di-GMP levels by ectopically expressing a constitutively active DGC from *V. cholerae* and found that this increases *S. flexneri* biofilm formation, downregulates acid resistance genes, and reduces the ability of *S. flexneri* to invade and form plaques in the human Henle-407 cell line. We then proceeded to determine the role of *S. flexneri* DGCs in regulating virulence phenotypes by individually deleting each *S. flexneri* DGC gene. We found that all four individual DGC deletion mutants show significant decreases in biofilm formation and that *dgcF* and *dgcC* deletion decreases invasion of Henle-407 cells. The Δ*dgcF* mutant also exhibits a defect in forming plaques in Henle-407 cell monolayers. These findings demonstrate that *S. flexneri* uses c-di-GMP signaling to regulate phenotypes associated with human infection.

RESULTS

VCA0956 expression increases *S. flexneri* cyclic di-GMP levels and biofilm formation. c-di-GMP signaling regulates many phenotypes in both nonpathogenic and pathogenic *E. coli*, including biofilm formation, adherence, motility, and virulence gene expression (44, 45, 53–55). Because of the genetic similarity between *E. coli* and *Shigella*, we hypothesized that c-di-GMP also regulates similar phenotypes in *S. flexneri*. To test this, we decided to express a foreign DGC in *S. flexneri*; this strategy has been used in other bacteria to examine how c-di-GMP alters various phenotypes (56, 57). We expressed the DGC VCA0956 from *V. cholerae* in *S. flexneri* by using the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible plasmid pVCA0956 (47). VCA0956 has been shown to synthesize c-di-GMP in a variety of different organisms, including *Vibrio vulnificus* (41), *Shewanella oneidensis*, *E. coli* (58), HeLa cells (59), and mice (59, 60). We confirmed that VCA0956 was expressed in *S. flexneri* by Western blotting, using an identical plasmid containing a FLAG-tagged VCA0956 allele (see Fig. S1 in the supplemental material).

To confirm that VCA0956 expression increases c-di-GMP levels in *S. flexneri*, we quantified the c-di-GMP of *S. flexneri* expressing VCA0956 using ultraperformance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS). The c-di-GMP levels of *S. flexneri* harboring the empty vector plasmid (pEVS143) were below our detection threshold, suggesting that physiological c-di-GMP levels may be relatively lower in *S. flexneri* than in other organisms (61). We found that VCA0956 expression in *S. flexneri* leads to significantly elevated c-di-GMP levels in *S. flexneri* cells, likely a considerable increase from the normal physiological range (Fig. 2A). In *E. coli*, elevated c-di-GMP levels are associated with a decrease in growth rate; this is attributed to the DGC YfiN directly interacting with FtsZ

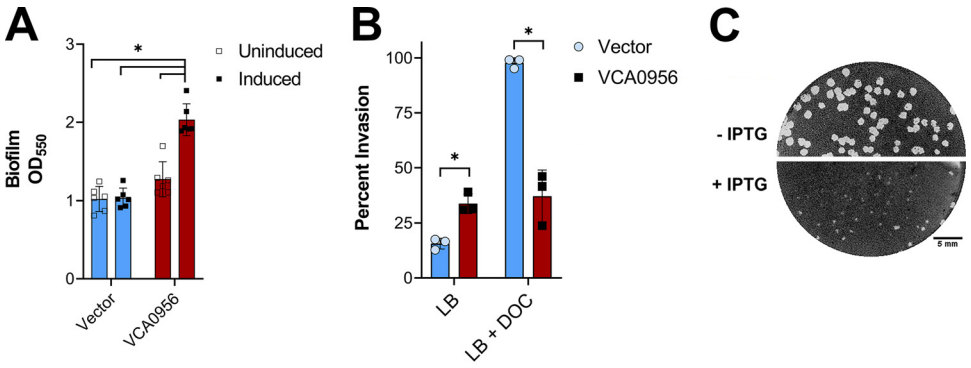

FIG 3 Overexpressing VCA0956 increases *S. flexneri* biofilm formation and alters *S. flexneri* host invasion frequency and plaque formation. (A) *S. flexneri* containing pVCA0956 or the empty vector control (pEVS143) was grown statically in glass tubes containing TSB supplemented with DOC and glucose. The resulting biofilms were stained and quantified at OD_{550}. Supplementation with IPTG led to significantly increased biofilm formation of the *S. flexneri* carrying pVCA0956 but not the vector control. Bars indicate the mean from three independent replicates, and error bars indicate standard deviation. Statistical significance was determined using two-way analysis of variance (ANOVA) with Sidak's multiple-comparison posttest (*, $P < 0.05$). (B) *S. flexneri* carrying pVCA0956 or the empty vector control (pEVS143) was grown with IPTG in LB or LB plus DOC, and then the ability of the bacteria to invade Henle-407 cells was assessed. When *S. flexneri* was precultured in LB, we observed that VCA0956 expression led to a significant increase in host invasion, whereas when *S. flexneri* was precultured in LB plus DOC, we observed that VCA0956 expression led to a significant decrease in host invasion. Bars indicate the mean from three replicates, and error bars indicate standard deviation. Statistical significance was determined using two-way ANOVA with Sidak's multiple-comparison posttest (*, $P < 0.05$). (C) Henle-407 cell monolayers were infected with *S. flexneri* carrying pVCA0956; after infection, IPTG was added to the tissue culture medium. We observed that VCA0956 expression corresponds to a significant reduction in the resulting plaque size in Henle-407 monolayers.

and ZipA to block septal peptidoglycan synthesis (62). Notably, *S. flexneri yfiN* has a single nucleotide deletion after 263 bp, leading to a frameshift mutation and a premature stop codon 120 residues from the *yfiN* start codon, which results in truncation of 288 amino acids. To determine if increasing intracellular c-di-GMP levels alter the *S. flexneri* growth rate, *S. flexneri* expressing VCA0956 was grown in Luria-Bertani (LB) broth, and the bacterial growth rate was quantified by measuring the optical density at 650 nm (OD_{650}) over time. We found that VCA0956 expression had no notable impact on the growth dynamics of *S. flexneri* (Fig. 2B). Because increased c-di-GMP leads to increased biofilm formation in *E. coli*, we hypothesized that expression of the DGC VCA0956 would increase *S. flexneri* biofilm formation. We grew *S. flexneri* carrying either the empty vector plasmid or the plasmid pVCA0956 in tryptic soy broth (TSB) supplemented with glucose and the bile acid deoxycholate (DOC), which are required to stimulate biofilm formation in *S. flexneri* (15), and measured the biofilm formation of strains grown with or without IPTG. A previous study has demonstrated that *S. flexneri* grown in a 1:1 ratio of cholate to DOC has no growth defect in the human physiological range (0.2% to 2%) (15, 63). After 24 h, we found that induction of VCA0956 expression with IPTG increased *S. flexneri* biofilm formation, resulting in a 2.0-fold increase compared to the empty vector control, consistent with c-di-GMP positively regulating biofilm formation (Fig. 3A). *S. flexneri* containing the empty vector plasmid shows no difference in biofilm formation, with or without IPTG.

VCA0956 expression reduces *S. flexneri* plaque formation and alters invasion. We next wanted to assess the effect of elevating c-di-GMP on virulence phenotypes, as c-di-GMP signaling has been implicated in controlling virulence-related phenotypes in other intracellular pathogens; for example, an increase in c-di-GMP levels in *Salmonella* and *Listeria* results in significant reduction in host cell invasion (64–66). A previous study has shown that when LB broth is supplemented with the bile acid DOC during growth prior to infection, *S. flexneri* invades host cell monolayers at a higher frequency (8); therefore, we first studied the effect of VCA0956 expression on *S. flexneri* invasion frequency when the organism was grown with DOC. We found that when *S. flexneri* was supplemented with DOC prior to invasion, VCA0956 expression significantly decreased host cell

invasion frequency, compared to *S. flexneri* carrying the empty vector plasmid grown under the same condition (Fig. 3B). We also measured the invasion frequency without DOC in *S. flexneri* expressing VCA0956 and found that VCA0956 expression increased *S. flexneri* host cell invasion compared to *S. flexneri* carrying the empty vector (Fig. 3B). Interestingly, there was no significant difference in invasion frequency between *S. flexneri* cells expressing VCA0956 grown with or without DOC.

Another method of assessing the virulence of *S. flexneri* strains is by measuring its ability to form plaques in epithelial cells grown in tissue culture (67). Plaque assays simultaneously assess the ability of the bacteria to invade, replicate, and spread from a host cell and can be quantitative by measuring the clear area of lysed host cells after staining. Previous studies have shown a correlation between reduced plaque size and decreased virulence in animal models (6, 67–70). We sought to determine if VCA0956 expression alters the ability of *S. flexneri* to form plaques in Henle-407 cell monolayers. *S. flexneri* containing pVCA0956 was used to infect Henle-407 cell monolayers. Because VCA0956 expression alters host cell invasion, IPTG was added to the tissue culture medium after invasion, to observe how VCA0956 expression alters *S. flexneri* growth and spread among host cells. We found a significant reduction in plaque size for the IPTG-induced VCA0956 strain in *S. flexneri* compared to the uninduced *S. flexneri* strain carrying VCA0956 (Fig. 3C). This suggests that increasing *S. flexneri* c-di-GMP levels inhibits plaque formation and regulates invasion of host cells.

VCA0956 expression alters the *S. flexneri* transcriptome. In many bacteria, one way that c-di-GMP exerts changes in physiology and behavior is by interacting with transcriptional regulators to alter gene expression (38, 71, 72). To see whether increasing c-di-GMP levels alters *S. flexneri* gene expression, we examined how VCA0956 expression alters the *S. flexneri* transcriptome by using RNA sequencing (RNA-seq). *S. flexneri* carrying pVCA0956 was subcultured in LB broth (containing DOC and glucose) with and without IPTG, and RNA was extracted at mid-log growth in three independent replicates. RNA-seq was performed with paired-end reads at 150-bp length, and we were able to map an average of 35.4 million reads per sample to the *S. flexneri* genome (Table S1). We identified significantly regulated *S. flexneri* genes by using a binomial test with a P value cutoff of $\leq$0.05. Principal-component analysis showed distinct groupings between the transcriptomes of cells supplemented with IPTG and cells without (Fig. S2). After comparing *S. flexneri* carrying pVCA0956 grown with and without IPTG, we found that 803 of the 4,084 *S. flexneri* genes were differentially expressed, with VCA0956 expression corresponding to 333 genes upregulated and 470 repressed.

Interestingly, the range of expression changes resulting from VCA0956 expression was modest, from -1.1 to 1.1 $\log_2$ fold change (Fig. 4A). We speculate that this could be due to leaky expression of VCA0956 from the pTac promoter of pVCA0956 in the uninduced condition, which would indicate that this list does not comprehensively identify all *S. flexneri* c-di-GMP-regulated genes. Although we found that IPTG induction of pVCA0956 in *S. flexneri* resulted in a 19.4-fold increase in VCA0956 RNA levels as expected (Table S1), we observed that the fragments per kilobase per million (FPKM) of VCA0956 in the uninduced samples was still relatively high (mean FPKM of 1,570.5).

We next used gene set enrichment analysis (GSEA) to identify significantly altered pathways. GSEA is a more robust statistical approach that groups genes by biological function and then identifies groups containing significantly more up- or downregulated genes than a random distribution (73). This analysis incorporates all the genes in a gene set and their respective fold changes to identify significantly regulated pathways and can distinguish between upregulated and downregulated gene sets. We chose to focus on this analysis to identify c-di-GMP-regulated processes rather than individual significantly regulated genes, as we observed such a high number of differentially regulated genes with a modest range of fold changes (Fig. 4A). We categorized genes into functional bins based on the KEGG pathway list and analyzed significantly regulated pathways using GSEA (Fig. 4B) (73, 74). The degree to which each gene set was significantly represented was determined using enrichment scores and analyzed

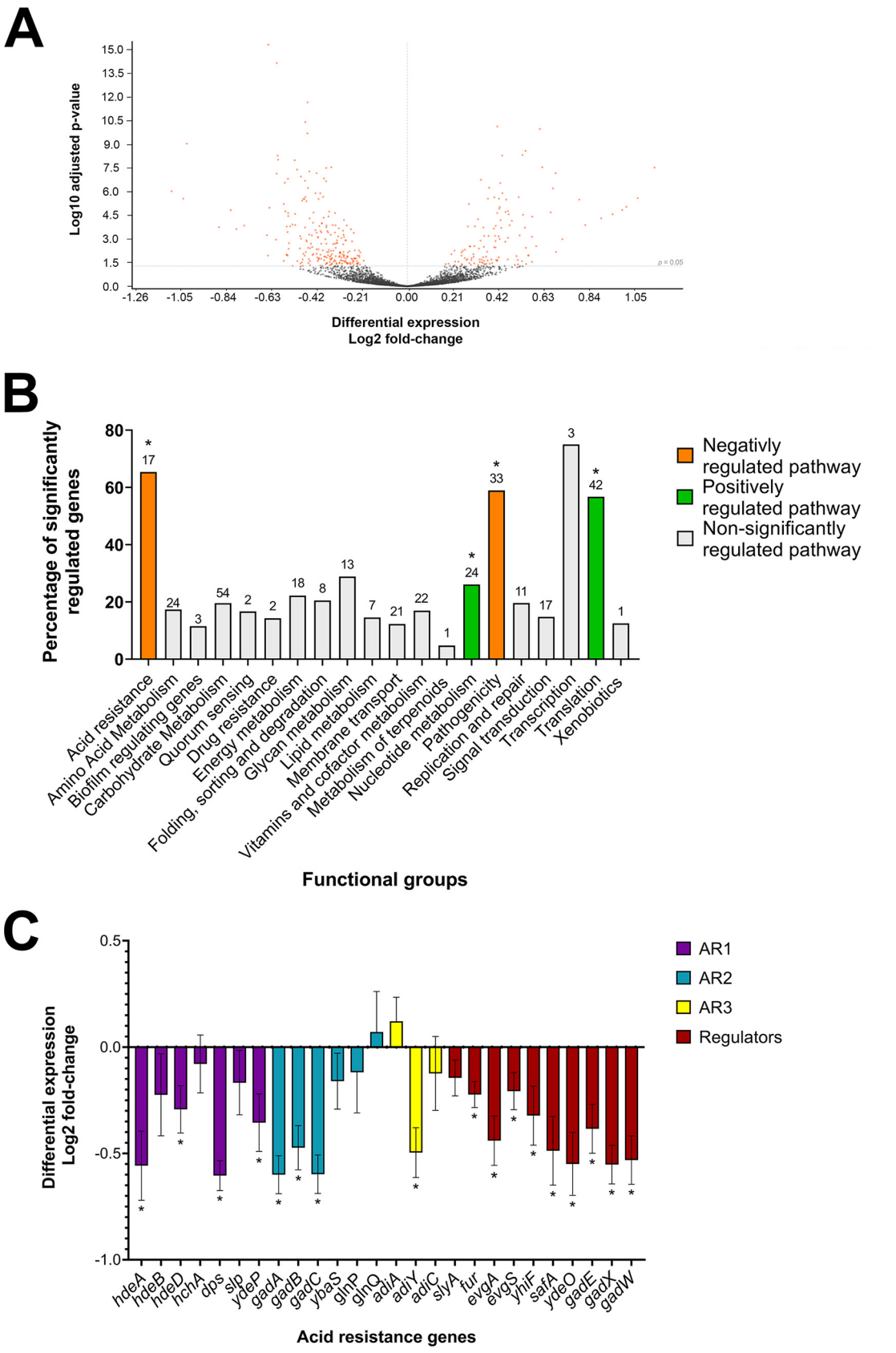

FIG 4 VCA0956 expression in *S. flexneri* results in the differential expression of gene functional groups. (A) Volcano plot showing differentially regulated genes due to VCA0956 expression. We found that the range of differential gene expression was between

(Continued on next page)

using a *P* value and false discovery rate (FDR) cutoff (<0.05). The enrichment scores were normalized to avoid gene set size differences among different gene sets and measure the global distribution of genes in a gene set, irrespective of the gene set size. Surprisingly, we did not observe differential expression of *S. flexneri* genes known to be related to biofilm formation, suggesting that c-di-GMP-mediated regulation of biofilm formation could be posttranscriptional. Among all the gene sets, acid resistance and pathogenicity pathways were significantly downregulated (enriched in the negative phenotype), while nucleotide metabolism and transcription pathways were significantly upregulated (enriched in the positive phenotype) (Table S3).

***S. flexneri* Ipa protein expression is modestly decreased by VCA0956.** Because VCA0956 expression decreases *S. flexneri* plaque size and invasion frequency in the presence of DOC, we focused on the genes associated with pathogenicity that were differentially regulated by VCA0956 expression. The genes *ipaABCD* were among those downregulated by VCA0956 expression (Fig. S3A); these genes are present in an operon, and the associated proteins are essential components of the T3SS which is required for host cell invasion and spread (21, 75). To confirm that VCA0956 expression inhibits *ipa* expression, we assessed Ipa protein levels of *S. flexneri* carrying pVCA0956 grown with and without IPTG using Western blot analyses. After cell lysates were run on an SDS-PAGE gel, a Coomassie blue stain showed a band at 37 kDa in lysates derived from *S. flexneri* grown with IPTG, matching the size of VCA0956 (Fig. S3B and C). Western blots of cell lysates were probed with monkey convalescent-phase antiserum, a monoclonal antibody against the T3SS protein IpaC, or a polyclonal antibody against the actin motility protein IcsA (76). We found that Ipa protein levels were modestly lower in the *S. flexneri* lysates in which VCA0956 was induced, consistent with RNA levels observed from the transcriptome (Fig. S3B and C). IcsA protein levels did not significantly change when probed with either monkey convalescent-phase antiserum or with a polyclonal IcsA antibody, which is also consistent with *icsA* transcript levels observed using RNA-seq between the two conditions.

VCA0956 expression increases *S. flexneri* acid sensitivity. Before causing infection, enteric pathogens like *Shigella* must pass through the stomach, a highly acidic environment. Acid tolerance is also critical when *S. flexneri* is in acidic phagocytic compartments, such as macrophages (77, 78). Unlike closely related *V. cholerae,* which removes H^+ ions by inducing the lysine decarboxylase gene *cadA* (79), *E. coli* and *S. flexneri* primarily use glutamate metabolism to persist through acidic environments (80). *S. flexneri* acid resistance genes can be grouped into three categories: glutamate-independent acid resistance (AR1), glutamate-dependent acid resistance (AR2), and arginine-dependent acid resistance (AR3) (81–83). Of note, *S. flexneri* 2457T differs from other *Shigella* or *E. coli* strains in that it has a mutation in the general stress response sigma factor *rpoS*, which regulates *E. coli* AR1 and AR2 (26, 84). *S. flexneri* also has a deletion encompassing the *cadC* gene, which regulates AR3 in *Shigella* and enteroinvasive *E. coli* (EIEC) (26, 85). In other *S. flexneri* strains and EIEC, arginine offers limited protection from acid shock, and a complementation study shows that *cadC* negatively impacts AR3 system activation (85).

Interestingly, our RNA-seq results showed that VCA0956 expression correlates with the repression of known *S. flexneri* acid resistance genes, particularly genes associated with AR1 and AR2 (Fig. 4C). Therefore, we hypothesized that VCA0956 expression makes *S. flexneri* more sensitive to acid shock. To test this hypothesis, we grew *S. flexneri* carrying

FIG 4 Legend (Continued)
1.1 and -1.1 $\log_2$ fold change. (B) Genes were grouped into pathway bins using KEGG annotations (Table S2), and the percentage of statistically significant differentially regulated genes was determined for each pathway. The number above each bar represents the total number of differentially regulated genes. Green bars indicate a pathway was significantly upregulated, and orange bars indicate a pathway was significantly downregulated. Significantly regulated pathways (*) were determined using GSEA in a preranked mode; *P* value and FDR cutoffs were <0.05. (C) VCA0956 expression corresponds to differential expression of acid resistance genes. Genes within the acid resistance functional group were further categorized into AR1, AR2, AR3, or regulatory genes, and the $\log_2$ differential expression was plotted. Bars indicate the mean differential expression of three replicates, and error bars represent $\log_2$ standard error. Asterisks indicate statistical significance ($P < 0.05$).

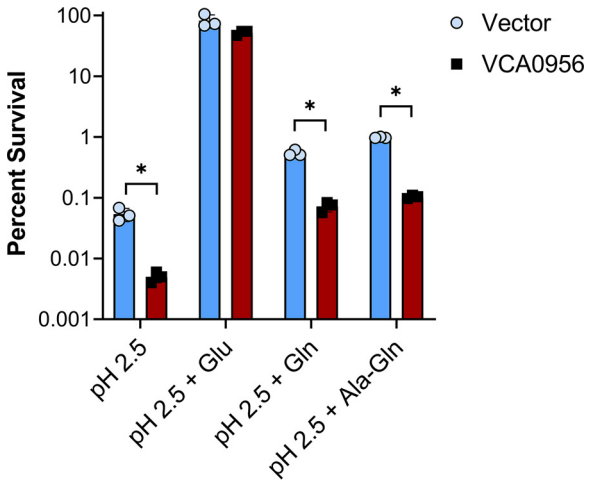

FIG 5 VCA0956 expression reduces *S. flexneri* survival after acid shock. *S. flexneri* carrying pVCA0956 or the empty vector control (pEVS143) was exposed to LB medium at pH 2.5 for 1 h, and then viable CFU were determined. Each strain was also exposed to LB at pH 7.0 to determine the percent survival. *S. flexneri* expressing VCA0956 showed a significant reduction in survival compared to *S. flexneri* carrying the empty vector. Supplementation of 50 mM glutamate substantially increased the survival of *S. flexneri* carrying either pVCA0956 or the empty vector, and there was no significant difference between the two strains, whereas supplementation with 50 mM glutamine or Ala-Gln dipeptide had a minimal effect. Each bar represents the mean from three replicates, and error bars indicate standard deviation. Asterisks indicate statistical significance, determined by one-way ANOVA ($P < 0.05$).

pVCA0956 or the vector control in LB broth with IPTG to mid-log phase and then exposed cells to LB broth at pH 2.5 for an hour and determined the percent survival. Consistent with our hypothesis, *S. flexneri* expressing VCA0956 was more sensitive to acid shock (Fig. 5). Because we observed that VCA0956 repressed the expression of the glutamate-dependent acid resistance system (AR2), we also measured the survival of *S. flexneri* exposed to LB at pH 2.5 in the presence of glutamate. The *E. coli* acid resistance system is glutamate dependent, where the glutamate decarboxylase (GAD) genes *gadA* and *gadB* regulate glutamate conversion to GABA. GadC, another gene which gets transcribed with *gadB* and encodes an antiporter, facilitates the import of glutamate, thus assisting in acid resistance (80–83). Glutamate supplementation greatly increased the survival of *S. flexneri* during acid shock, and VCA0956 expression did not significantly alter *S. flexneri* survival in the presence of glutamate, suggesting that c-di-GMP regulation is dispensable for AR2-mediated acid resistance. In contrast, VCA0956 expression decreased *S. flexneri* survival in LB at pH 2.5 when glutamine or Ala-Gln dipeptide was added.

***S. flexneri* has four DGC genes encoding intact GGDEF domains.** Because VCA0956 expression increases *S. flexneri* c-di-GMP levels far above physiological levels (Fig. 2A), we cannot ascertain from prior experiments whether these phenotypes bear any physiological relevance. Additionally, many DGCs interact directly with protein effectors to elicit effects, termed signaling specificity (34, 47); these types of c-di-GMP-mediated effects may not be observed upon VCA0956 expression in *S. flexneri*. Therefore, we next sought to determine the role of the native *S. flexneri* DGCs in mediating changes in phenotypes we observe. Genomic studies have shown that *Shigella* spp. diverged directly from nonpathogenic *E. coli*, primarily through gene loss and the acquisition of a large virulence plasmid (86).

We compared the DGCs and PDEs of *S. flexneri* 2457T to *E. coli* K-12, as these organisms are highly similar (26). The *E. coli* K-12 strain MG1655 has 11 genes encoding GGDEF domains, 8 genes encoding EAL domains, and 6 genes encoding both GGDEF and EAL domains; additionally, *E. coli* MG1655 has 4 genes encoding degenerate GGDEF or EAL domains (predicted to be incapable of synthesizing or hydrolyzing c-di-GMP) (44) (Fig. 1; Table S4). Of note, we use the term "degenerate" to describe genes

encoding proteins predicted to be incapable of synthesizing or hydrolyzing c-di-GMP based on sequence, but we have not verified the catalytic function of these proteins. We compared each of these genes to its *S. flexneri* ortholog and found that *S. flexneri* has 4 genes encoding intact GGDEF domains, 5 genes encoding EAL domains, and 1 gene encoding both GGDEF and EAL domains (although the GGDEF domain is degenerate) (37), and we classified the remaining 19 c-di-GMP turnover enzyme genes as degenerate; notably, the conversion of *S. flexneri* c-di-GMP turnover domains to degenerate occurs through a mixture of deletions, insertions, nonsense mutations, and frameshift mutations (Table S4). Therefore, *S. flexneri* has presumably lost the functionality of 60.0% of its DGC and PDE enzymes compared to *E. coli* MG1655; this is a much higher rate of disruption than the overall frequency of gene disruption between *E. coli* strain K-12 and *S. flexneri*, which is 20.5% (26). This indicates a disproportionate selection against c-di-GMP turnover genes, but the remaining intact c-di-GMP turnover enzymes are highly conserved, with $>$98% sequence similarity, suggesting that they could retain a regulatory role.

For the scope of this study, we focused on DGC genes encoding intact GGDEF domains. We identified conserved protein domains and putative membrane topology using MiSTDB (Fig. 1B) (87). The *S. flexneri* gene *pdeK*, which encodes both a GGDEF and EAL domain, was excluded because the GGDEF domain is predicted to be degenerate (37). *S. flexneri dgcC* is predicted to encode five transmembrane proteins with a MASE2 input domain in the N-terminal region (88). *E. coli dgcC* interacts with the cellulose synthase enzyme *bcsB* to regulate a localized c-di-GMP pool near the cellulose synthase complex (89), and an *E. coli* Δ*dgcC* mutant exhibits defects in cellulose biosynthesis (37, 90). The N-terminal domain of *S. flexneri dgcI* contains the periplasmic sensor domain GAPES2, flanked by two putative transmembrane domains (91). *S. flexneri dgcF* is predicted to encode four transmembrane regions and no other characterized N-terminal domain features. And *S. flexneri dgcP* encodes a GAF domain (92) in the N-terminal region and is the only *S. flexneri* DGC without predicted transmembrane domains.

DGC mutation reduces *S. flexneri* biofilm formation. Increasing *S. flexneri* c-di-GMP levels by expressing VCA0956 promotes biofilm formation, decreases host cell invasion in the presence of DOC, and reduces plaque formation. To determine the role of *S. flexneri* DGCs in regulating c-di-GMP-associated phenotypes, we generated *S. flexneri* genomic deletion strains of each of the 4 DGCs. We determined that the *S. flexneri* wild type (WT) and individual DGC mutant strains were indistinguishable in growth dynamics, either with or without DOC (Fig. S4). Notably, the *S. flexneri* WT and DGC strains with DOC show higher absorbance than those without DOC, presumably indicative of higher biofilm formation. Because VCA0956 expression increases *S. flexneri* biofilm formation, we hypothesized that deleting *S. flexneri* DGCs would reduce *S. flexneri* biofilm formation. A biofilm assay was set up in a 96-well plate by following a previously published protocol (15). As a negative control, we included an *S. flexneri* Δ*bcsE* mutant, presumably deficient in cellulose production (93, 94). To induce biofilm formation, DOC and glucose were added to TSB growth medium, and biofilm formation was quantified after 24 h of static incubation at 37°C using crystal violet staining. Interestingly, we observed that all four DGC deletion strains show significant reduction in biofilm formation compared to the WT strain, with the Δ*dgcF* strain showing the greatest reduction in biofilm levels compared to the WT strain (Fig. 6A). The Δ*bcsE* strain showed a significant reduction in biofilm formation, indicating that cellulose synthesis is required for *S. flexneri* biofilm formation. These findings suggest that all four *S. flexneri* DGCs contribute to *S. flexneri* biofilm formation.

To compare how DGCs impact biofilm formation between *S. flexneri* and *E. coli* K-12, we assessed the biofilm formation of *E. coli* mutants of the orthologous DGC genes, using the same assay and growth conditions. Similar to *S. flexneri*, *E. coli* Δ*dgcC*, Δ*dgcI*, and Δ*dgcF* mutants showed significantly less biofilm formation than the *E. coli* WT strain. The *E. coli* Δ*dgcP* mutant did not show a significant change in biofilm formation compared to the *E. coli* WT strain (Fig. S5).

***S. flexneri* Δ*dgcC* and Δ*dgcF* mutants are defective in host cell invasion.** The pathogenesis of *S. flexneri* is contingent on its ability to invade and multiply within a

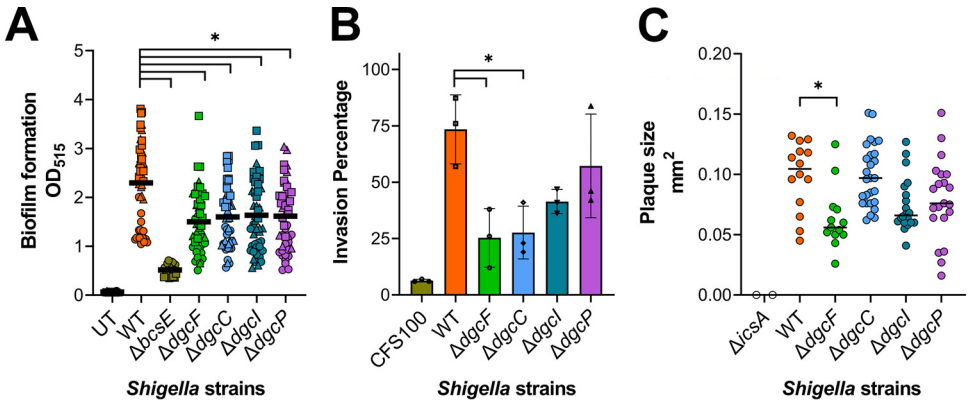

FIG 6 *S. flexneri* individual DGC mutant strains exhibit reduced biofilm formation and reductions in invasion frequency and plaque formation. (A) The *S. flexneri* WT and DGC mutant strains were grown statically in microplates containing TSB plus DOC plus glucose, and the resulting biofilms were quantified by crystal violet staining (OD_{515}) after ~20 h. UT, uninoculated wells. All individual DGC mutant strains show a significant reduction in biofilm formation compared to WT *S. flexneri*. Each point represents a replicate; shapes indicate different days, lines indicate the mean, and error bars indicate standard deviation. An asterisk indicates statistical significance, determined by one-way ANOVA ($P < 0.05$). (B) The *S. flexneri* WT and DGC mutant strain invasion frequency of Henle-407 cells was determined by microscopy. For a negative control, we included the *S. flexneri* CFS100 strain, which lacks the virulence plasmid and cannot invade. Bars indicate the mean percentage of invaded cells for three replicates, and error bars indicate standard deviation. (C) The plaque formation of *S. flexneri* WT and DGC mutant strains was measured in Henle-407 cell monolayers. As a negative control, we included the *S. flexneri* Δ*icsA* strain that cannot spread. Each point is an individual plaque in a 6-well plate. Lines indicate the mean plaque size, and error bars indicate standard deviation. An asterisk indicates statistical significance, determined by one-way ANOVA ($P < 0.05$).

host cell (29, 95, 96). Because we observed that VCA0956 expression in *S. flexneri* decreases host cell invasion, we hypothesized that *S. flexneri* DGC mutants would have increased host cell invasion, relative to the WT strain. To test this, we assessed the invasion frequency of each of the four *S. flexneri* DGC mutants. As controls, we included the *S. flexneri* WT and CFS100 strains, the latter of which lacks the virulence plasmid and is therefore incapable of invading host cells.

In contrast to our hypothesis, we found that the *S. flexneri* Δ*dgcC* and Δ*dgcF* strains show a significant reduction in host cell invasion compared to the WT strain (Fig. 6B). The invasion frequency of the Δ*dgcI* mutant was modestly lower than that of the wild type but not statistically significant. In contrast, there was no significant difference in the number of invaded tissue culture cells between the WT strain and the Δ*dgcP* mutant strain. The CFS100 strain showed less than 6% invasion, indicating a low background detection of external, adherent bacteria. These findings suggest that both *dgcC* and *dgcF* contribute to *S. flexneri* invasion of host cells.

***S. flexneri* Δ*dgcF* is deficient at plaque formation in host cell monolayers.** VCA0956 expression in *S. flexneri* also significantly decreases *S. flexneri* plaque formation. Therefore, we hypothesized that the plaque size would increase for the *S. flexneri* DGC mutants. Henle-407 cell monolayers were infected with the four *S. flexneri* DGC mutants, as well as the *S. flexneri* WT strain; we also included the *S. flexneri* Δ*icsA* strain, which has an outer membrane protein which facilitates intercellular spread (29–32), as a negative control (6). Interestingly, we found that the *S. flexneri* Δ*dgcF* mutant showed a significant reduction in plaque size compared to the WT strain, while the plaque sizes for Δ*dgcC*, Δ*dgcP*, and Δ*dgcI* mutants showed no significant differences (Fig. 6C). No plaques were observed in Henle-407 cell monolayers infected with the Δ*icsA* strain.

An *S. flexneri* Δ*dgcF* mutant has increased acid tolerance. We found that increasing c-di-GMP through VCA0956 expression in *S. flexneri* corresponds to the decreased levels of AR1 and AR2 RNA levels. Based on this, we hypothesized that individual DGC mutants would exhibit increased survival when exposed to acidic pH. We performed the same acid shock assay as described above, comparing the survival of individual *S. flexneri* DGC mutants to the WT strain in response to LB at pH 2.5. We recovered

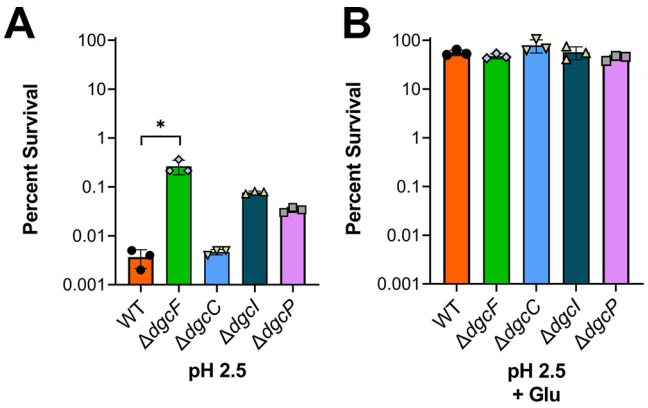

FIG 7 The *S. flexneri* Δ*dgcF* mutant strain has increased resistance to acid shock. (A) DGC mutants were exposed to LB medium at pH 2.5 for 1 h, and then viable CFU were determined. Each strain was also exposed to LB at pH 7.0 to determine the percent survival. (B) When glutamate was added to LB at pH 2.5, all strains showed a significant increase in survival, with no differences between strains. Results are representative of three independent trials, indicated with different symbols. An asterisk indicates statistical significance, determined by one-way ANOVA ($P < 0.05$).

significantly higher *S. flexneri* Δ*dgcF* mutant CFU following acid shock than of the WT strain (Fig. 7A). The *S. flexneri* Δ*dgcI* and Δ*dgcP* mutants also exhibited modest but statistically insignificant increases in acid shock survival compared to the WT strain, while the Δ*dgcC* mutant showed no significant difference. We also found that glutamate supplementation increased *S. flexneri* survival for both the *S. flexneri* WT and DGC mutant strains, resulting in no significant differences between the *S. flexneri* WT strain and any of the DGC mutants (Fig. 7B). This was consistent with glutamate supplementation negating VCA0956-mediated differences in *S. flexneri* acid shock survival (Fig. 5).

DISCUSSION

Here, we report that expression of VCA0956 in *S. flexneri* increases c-di-GMP levels and alters *S. flexneri* virulence phenotypes and gene expression. By expressing a nonnative DGC from *V. cholerae*, we were able to study the effect of raising c-di-GMP levels in *S. flexneri*, avoiding likely endogenous regulatory feedback from expression of native DGCs. Consistent with its role in *V. cholerae*, expression of VCA0956 also increased biofilm formation of *S. flexneri*, though the precise mechanisms underlying this regulatory activity have yet to be revealed. While c-di-GMP positively regulates *E. coli* and *S. flexneri* biofilm formation, there are differences in the way these two organisms form biofilms. *E. coli* c-di-GMP-mediated biofilm formation is regulated primarily by the transcriptional regulator CsgD (37, 44, 48, 89, 97), which produces and promotes the curli-regulating elements *csgA* and *csgB* (98) and also activates cellulose genes on the bacterial cellulose synthase (*bcs*) operon (89, 98). *S. flexneri csgA* and *csgB* have been shown to contribute to adherence and biofilm formation despite their classification as pseudogenes (99), but the *S. flexneri* cellulose synthase genes *bcsA* and *bcsC* possess frameshift mutations (100, 101). Lastly, *E. coli* biofilms contain colanic acid, which is regulated by the *wca* gene operon (102), but the glucosyl transferase genes *wcaC* and *wcaE* in *S. flexneri* are also annotated as pseudogenes (26). The *S. flexneri* outer membrane protein IcsA has been shown to promote biofilm formation, OspE1/OspE2 mediate host cell adherence in response to bile salts (15, 100, 103), and these proteins are unique to *Shigella* as they are encoded on the virulence plasmid (104).

c-di-GMP controls the expression of *csgD* in *E. coli*, a regulator of curli fibers essential for biofilm formation (90, 105); however, we did not observe an increase in *S. flexneri csgD* levels in response to VCA0956 expression (see Tables S1 and S2 in the supplemental material). The *S. flexneri* protein IcsA has been shown to contribute to biofilm formation in the presence of bile by increasing cell-to-cell interactions (100), but our data also

show that VCA0956 expression does not significantly alter IcsA expression at the transcript or protein level (Fig. S3). Other genes that promote *S. flexneri* colonic epithelial adherence and/or biofilm formation include *ospE1*, *ospE2*, *lpfAB*, the *fim* operon, and the *csg* operon (8, 15, 99, 103); however, none of these genes or operons were significantly regulated by VCA0956 induction in our study (Tables S1 and S2). It is certainly possible that c-di-GMP regulates biofilms in a posttranscriptional manner; for example, in *E. coli*, c-di-GMP directly binds to BcsA and BcsE to regulate cellulose biogenesis (89), and a recent study has demonstrated that extracellular polymeric substances are present in *S. flexneri* biofilms (15). While *bcsA* is annotated as a pseudogene in *S. flexneri*, *bcsE* remains intact and could potentially be responding directly to c-di-GMP to regulate biofilm formation through cellulose biosynthesis; this is supported by the observation that *S. flexneri* biofilms are sensitive to exogenous cellulase (99) and that an *S. flexneri* Δ*bcsE* mutant is defective in biofilm formation (Fig. 6A).

In *E. coli*, the MASE2 domain of DgcC interacts with PdeK and the cellulose synthetase subunit BcsB, which results in a localized c-di-GMP pool in close proximity to the cellulose synthase complex for BcsE and BcsA to bind (89, 93, 94). Here, we found that all our individual DGC knockouts in *S. flexneri* show reduced biofilm formation compared to the WT strain. This is consistent with *S. flexneri* DgcC retaining a similar role in regulating cellulose biosynthesis as its *E. coli* ortholog. DgcF also promotes biofilm formation in *E. coli* K-12 and *E. coli* CFT073 (45, 106), and DgcP promotes biofilm formation in uropathogenic *E. coli* CFT073 (45). Interestingly, an *E. coli* CFT073 Δ*dgcI* mutant increases adherence, which is contrary to the invasion phenotype we observed in our *S. flexneri* Δ*dgcI* mutant (Fig. 6B) (45).

There are many environmental barriers that *S. flexneri* must overcome to effectively cause disease, including pH stress in the stomach. We found that VCA0956 expression significantly reduces gene expression associated with acid resistance, and this corresponds with decreased acid shock survival (Fig. 4C and 5). Consistent with c-di-GMP repressing AR1 and AR2 genes, the *S. flexneri* Δ*dgcF* mutant strain exhibits increased acid tolerance relative to the WT strain (Fig. 7A). To our knowledge, this is the first study to connect c-di-GMP signaling and acid resistance. Because the addition of glutamate eliminates differences in *S. flexneri* acid shock survival in response to VCA0956 expression and in our DGC mutants, we postulate that the differential regulation of AR1 category genes is what drives c-di-GMP-mediated differences to acid shock survival.

S. flexneri uses a T3SS to invade and spread among host cells, and this T3SS is encoded on the virulence plasmid in the *mxi-spa* operon (4, 8, 22, 95, 107). We found that VCA0956 expression in *S. flexneri* leads to deficiencies in DOC-dependent invasion and plaque formation and also correlates with a reduction in RNA levels of *ipa* genes; we confirmed that IpaC protein levels were modestly reduced in response to VCA0956 expression (Fig. S3), although it is unclear if the reduction in *ipa* gene expression is sufficient to inhibit invasion and plaque formation or if other factors also contribute. Interestingly, we also found that in the absence of DOC, VCA0956 expression promotes *S. flexneri* host invasion, suggesting that exogenously increasing *S. flexneri* c-di-GMP levels could be dominant over the effects of DOC on host invasion.

One drawback to using VCA0956 to increase *S. flexneri* c-di-GMP levels is that the c-di-GMP concentration in the cell far exceeds normal, physiological levels and thus has the potential for creating abnormal effects; this could be the case for *S. flexneri* invasion and plaque formation, where both VCA0956 expression and our Δ*dgcC* and Δ*dgcF* strains exhibited defects in host cell invasion and, in the case of the Δ*dgcF* strain, plaque formation. Both *S. flexneri* host cell invasion and plaque formation are complex phenotypes involving many systems beyond the T3SS, and our data suggest that c-di-GMP regulates a wide range of targets in *S. flexneri*; we speculate that increasing c-di-GMP levels beyond normal ranges indirectly causes deficiencies in these phenotypes. For example, VCA0956 expression decreases the expression of several virulence genes required for invasion and plaque formation, such as *ipaC*, but also increases the expression of many

genes involved in nucleotide metabolism, including the GMP synthase gene *guaA*. We would expect that *S. flexneri* DGC mutants would exhibit reduced nucleotide metabolism and *guaA* expression, and a previous study has shown that an *S. flexneri* Δ*guaA* mutant is deficient in HeLa cell invasion and intracellular growth (108). It is also possible that DgcF and DgcC contribute to the regulation of *S. flexneri* invasion and plaque formation in a manner that is independent of the overall c-di-GMP level in the bacterium, commonly known as signaling specificity (34, 47). The mechanism driving reduced invasion and plaque formation of DGC mutants will be the subject of future studies.

MATERIALS AND METHODS

Media and growth conditions. *S. flexneri* strains were cultured at 37°C on tryptic soy broth (TSB) agar plates supplemented with Congo red (0.01% wt/vol), and red colonies were selected to maintain the *S. flexneri* virulence plasmid. Overnight cultures were grown in Luria-Bertani (LB) broth at 30°C; for experiments, these were subcultured 1:100 and grown at 37°C to an optical density at 600 nm (OD_{600}) of 0.5 to 1.0 (mid-log phase) prior to infection. Deoxycholate (DOC) was added as a supplement at 0.05% (wt/vol) where indicated to increase the efficiency of invasion of Henle-407 cultured cells (8). *S. flexneri* carrying pVCA0956 or pEVS143 was always grown with 50 μg/ml kanamycin. Supplementation of other antibiotics where indicated was as follows: 25 μg/ml ampicillin, 25 μg/ml chloramphenicol, and 20 μg/ml gentamicin. Unless otherwise indicated, IPTG was added at 100 μM.

Henle-407 cells (ATCC intestinal 407, CCL-6) were cultured at 37°C with 5% CO_2 in minimal essential medium (MEM; Gibco 61100-087) supplemented with 10% (wt/vol) Bacto tryptone phosphate broth (Difco), 10% (vol/vol) fetal bovine serum (Gibco 16140-071), 2 mM glutamine, and nonessential amino acids (Gibco 11140-050).

Bacterial strains and mutant generation. All bacterial strains, mutants, plasmids, and primer sequences are summarized in Table S5 in the supplemental material. The *S. flexneri* parent strain for this study was the *S. flexneri* 2457T (serotype 2a) (26). Mutants were generated using either the one-step inactivation method (109) or the P1 transduction method (110). Our Δ*dgcF* and Δ*dgcC* knockouts were generated using the one-step inactivation method by amplifying the chloramphenicol resistance cassette of pkD3 with primers containing ~50-bp homologous regions upstream and downstream of the target gene. Our Δ*dgcI* and Δ*dgcP* knockouts were generated using the bacteriophage P1 transduction method (110) with strains from the *E. coli* Keio collection. The DNA sequences of the mutants were confirmed using sequencing primers as listed in Table S5.

UPLC-MS/MS quantification of intracellular c-di-GMP. Quantification of intracellular levels of c-di-GMP was performed using ultraperformance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS). Subcultures were grown to mid-log phase at an OD_{600} between 0.6 and 0.9. Five milliliters of this culture was centrifuged for 30 s at maximum speed, and supernatant was removed immediately. The pellet was resuspended in 200 μl of cold extraction buffer containing 40% methanol, 40% acetonitrile, and 0.1 N formic acid. The tube was incubated for 30 min at −20°C. The tube was centrifuged again for 5 min at maximum speed to remove the insoluble fraction. The supernatant was collected and stored at −80°C. The extraction buffer was removed using a vacuum centrifuge before initiation of the mass spectrometry analysis. The residual pellet was resuspended in 100 μl water. Quantification of c-di-GMP was performed using a Quattro Premier XE mass spectrometer (Waters) coupled with an Acquity ultraperformance LC system (Waters) at the Mass Spectrometry & Metabolomics Core Facility at Michigan State University as previously described (111). To determine the intracellular concentrations of c-di-GMP, the concentration of c-di-GMP for each sample was divided by the estimated total volume of the extracted bacteria, based on mean phase-contrast microscopy measurements of *S. flexneri* cells. The product of bacterial cell number and the average volume of the bacterial cell was used to determine the total volume of the extracted bacteria.

Biofilm assay. Biofilm assays were set up in either borosilicate glass test tubes or 96-well plates and quantified by crystal violet staining as previously described (15). Overnight cultures were diluted 1:50 in TSB supplemented with 10 mM glucose and DOC (0.05%, wt/vol). Kanamycin was added to strains containing pEVS143-based plasmids, and IPTG was added where indicated. For 96-well plates, 120 μl of bacterial suspension was added to wells, and each strain was set up in octuplicate. Plates or tubes were incubated at 37°C for ~22 h, without shaking. After incubation, the OD_{600} was measured to ensure that there were no significant differences in growth. Medium was then removed, and wells or tubes were washed with phosphate-buffered saline (PBS). The plate or tubes were dried completely and then stained with crystal violet (0.5%, wt/vol) for 5 min at room temperature. Crystal violet was removed, and wells were washed with water 4 or 5 times. The plate was air dried for approximately 1 h, and then 200 μl ethanol (95%) was added to each well, or 2 ml ethanol (95%) was added to each tube. The plate or tubes were incubated on a shaker for 30 min at 4°C, and then the OD_{515} was measured using a BioTek H1 Synergy plate reader (plate), or the OD_{550} was measured using a spectrophotometer (tubes).

Cell culture assays. Invasion assays were performed as described previously, with modifications (6, 75). Henle-407 cells were grown in 6-well plates to ~20% confluence. Overnight bacterial cultures were diluted 1:100 and incubated at 37°C for 3 to 4 h with shaking to mid-log phase. The bacterial concentration was estimated by measuring the OD_{600}, cultures were centrifuged and resuspended in sterile saline to a concentration of 2×10^9 CFU per ml, and 100 μl of bacterial suspension was added to individual wells. Plates were centrifuged for 10 min at 200 × *g* and then incubated at 37°C, in 5% CO_2, for 30 min.

The wells with monolayers were then washed 4 times with PBS, and the medium was replaced with MEM with gentamicin. The plate was then incubated for 40 min at 37°C, in 5% CO_2. The wells were washed with PBS, and each well was stained with Wright-Giemsa stain. After drying, microscopy was used to score ~300 Henle-407 cells per well, using the criteria for a positively invaded cell as one containing 3 or more bacteria. The invasion percentage was calculated based on the number of invaded cells divided by the total cells counted.

The plaque assays were performed as described previously, with modifications (6, 67). Henle-407 cell monolayers were grown to 80% confluence in six-well plates. Overnight bacteria cultures were diluted 1:100 and incubated at 37°C for 3 to 4 h on a shaker to mid-log phase. The bacterial concentration was estimated by measuring the OD_{600}, the cultures were centrifuged and resuspended in sterile saline to a concentration of 5×10^4 CFU/ml, and 100 μl of bacterial suspension was added to individual wells. Plates were centrifuged for 10 min at 200 $\times$ *g* and then incubated at 37°C, in 5% CO_2, for 60 min. Each well was washed 4 times with PBS, and then the plate was incubated for 48 h at 37°C, in 5% CO_2, with medium containing 0.2% glucose and gentamicin. The wells were washed with PBS, and each well was stained with Wright-Giemsa stain and imaged using a Bio-Rad VersaDoc imaging system. The plaque size was digitally measured using ImageJ (112).

RNA extraction and sequencing. For RNA extractions, *S. flexneri* was subcultured from three independent overnight cultures at a dilution factor of 1:100 and grown with shaking at 37°C in 5 ml LB broth supplemented with 5 mM glucose, kanamycin, 0.05% DOC (wt/vol), and 100 μM IPTG where indicated. When the culture reached the mid-log phase of growth, 4 ml of the culture was pelleted by centrifugation, and then RNA was isolated using the Purelink RNA minikit (Thermo Fisher, catalog no. 12183020) using the manufacturer's protocol with TRIzol reagent. RNA samples were submitted to Genewiz for RNA sequencing.

Read mapping and gene expression analysis. Sequence reads were aligned to the *S. flexneri* 2457T genome (GenBank accession no. AE014073.1) the *S. flexneri* virulence plasmid pCP301 (GenBank accession no. AF386526.1), the R27 plasmid (GenBank accession no. AF250878), and the VCA0956 gene from *V. cholerae* N16961 (GenBank accession no. NC_002506) using Geneious software. Values of reads per kilobase per million (RPKM) were used for expression analysis to determine the $\log_2$ fold change, comparing *S. flexneri* carrying pVCA0956 grown with (induced) and without IPTG (uninduced). A binomial test with a false discovery rate correction was used to determine statistical significance ($P < 0.05$). A complete list of read mappings can be found in Table S1.

Bioinformatics analysis of *S. flexneri* DGC enzymes. To compare the domain structures for *S. flexneri* DGCs, MiSTDB software was used (87). Information about the input-output domains, number of transmembranes, and individual DGC sequences was collected. Details about the domain architecture with unique MIST identifiers is summarized in Table S4 in the supplemental material.

Acid resistance assay. Acid resistance assays were carried out using a previously published protocol with modification (81). *S. flexneri* was subcultured from overnight cultures at a dilution factor of 1:100 and grown with shaking at 37°C in 3 ml LB broth supplemented with kanamycin and 100 μM IPTG where indicated. When the cultures reached mid-log growth, the OD_{600} was read, and 5×10^8 cells were pelleted by centrifugation. Pellets were then resuspended in LB or LB adjusted to pH 2.5. Where indicated, LB was supplemented with 50 mM glutamate, 50 mM glutamine, or 50 mM Glu-Ala (Glutamax); addition of amino acids did not substantially alter the pH of the medium (pH of LB [2.5] plus Gln was 2.7; pH of LB [2.5] plus Glu was 2.8). Acid shock cultures were incubated statically for 1 h at 37°C, and then 10-μl volumes of serial dilutions in PBS (pH 7.3) were spot plated on LB agar. Percent survival was determined by dividing the number of CFU after 1 h in the acid shock cultures by the CFU of the LB cultures for each respective strain.

Immunoblotting. Immunoblot analyses were performed as previously described (76). Bacterial cultures were grown in LB broth at 37°C, supplemented with DOC. When the cells reached the mid-log phase of growth, the OD_{650} was read, and cells were collected by centrifugation and resuspended in SDS sample buffer (5% β-mercaptoethanol, 3% [wt/vol] SDS, 10% glycerol, 0.02% bromophenol blue, 63 mM Tris-Cl, pH 6.8) at a concentration of 2×10^9 CFU/ml. Supernatant proteins were precipitated with 10% (wt/vol) trichloroacetic acid as previously described (8). Samples were then boiled for 10 min and then electrophoresed in duplicate SDS-PAGE gels. After electrophoresis, one gel was stained with Coomassie blue, and the proteins from the other gels were transferred to a 0.45-μm-pore-size nitrocellulose membrane (Hybond-ECL; GE Healthcare) and incubated with the following antibodies: mouse monoclonal anti-IpaC, rabbit polyclonal anti-IcsA, monkey anti-*S. flexneri* convalescent-phase antiserum (E. V. Oaks, WRAIR), or mouse monoclonal horseradish peroxidase (HRP)-conjugated DYKDDDDK (Proteintech HRP-66008). Proteins were detected using HRP-conjugated goat anti-mouse, anti-human, or anti-rabbit antibody. Signals were detected by developing the blot with a Pierce ECL detection kit (Thermo-Fisher Scientific).

Statistical analysis. Figures and statistical analysis for this study were generated using GraphPad Prism, using a standard statistical significance cutoff of $P < 0.05$. RNA-seq statistical analysis was performed using Geneious with a binomial test ($P < 0.05$).

Data availability. Sequencing data were deposited in GEO under accession number GSE173759.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

SUPPLEMENTAL FILE 2, XLSX file, 1.7 MB.

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