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Stringent Response Regulation of Biofilm Formation in *Vibrio cholerae*

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Biofilm formation is a key factor in *Vibrio cholerae* environmental survival and host colonization. Production of biofilm enables *V. cholerae* to survive and persist in aquatic environments and aids in the passage through the gastric acid barrier to allow access to the small intestine. The genes involved in biofilm formation are regulated by the transcriptional activators *vpsR* and *vpsT*, which are in turn transcriptionally regulated by a number of environmental signals. In this study, the role of the stringent response in biofilm formation was examined. *V. cholerae* mutants deficient in stringent response had a reduced ability to form biofilms, although they were not completely deficient in biofilm formation. There are three (p)ppGpp synthases in *V. cholerae*: RelA, SpoT, and RelV. All three synthases were necessary for *vpsR* transcription, with RelV showing the strongest effect. RelA was the only synthase that was necessary for *vpsT* expression. Stringent response regulation of *vpsR* and *vpsT* was shown to partially occur through *rpoS*. Biofilm formation in *V. cholerae* is controlled by a complex regulatory apparatus, with negative regulators of biofilm gene expression, such as quorum sensing, and positive regulators of biofilm genes, including stringent response, interacting to ensure that biofilm formation is coordinated with the environment.

Vibrio cholerae is a Gram-negative gammaproteobacterium that is the causative agent of the epidemic disease cholera. A facultative human pathogen, *V. cholerae* is found in aquatic environments, but when ingested by a human host, *V. cholerae* is able to colonize the small intestine and produce a massive watery diarrhea. Host colonization is accompanied by rapid growth of bacterial cells that are returned to the environment to begin a new infectious cycle. In the environment, *V. cholerae* attaches to the surfaces of aquatic organisms and abiotic material and forms biofilms (12, 33). The formation of a biofilm community aids the organism in the initial stages of host interaction in that biofilms protect the organisms from harsh environmental conditions and help them survive passage through the gastric acid barrier (4, 17, 92). Once *V. cholerae* reaches the small intestine, planktonic cells use flagellar motility to penetrate the mucosal layer and colonize the intestinal microvilli (21, 41, 49, 61, 69). In the small intestine, the bacteria express the major virulence factors toxin-coregulated pilus (TCP), which aids in bacterial aggregation in the small intestine, and cholera toxin (CT), which produces the watery diarrhea and facilitates dissemination from the host (16, 37, 67). Recently shed *V. cholerae* bacteria are hyperinfectious, aiding in the rapid spread of the disease, and this hyperinfectious state includes an increased propensity to form biofilms in aqueous environments (3, 10, 17, 51).

Biofilm formation in *V. cholerae* is under the control of a complex array of regulatory factors that respond to a variety of environmental signals, including salinity, bile, calcium, and phosphate (8, 32, 64, 73). The genes involved in production of biofilms are found primarily in the two *vps* (*Vibrio* exopolysaccharide) operons, one consisting of *vpsU* and *vpsA* to *-K* and the other consisting of *vpsL* to *-Q*, along with nearby associated genes (18, 20, 89). There are two known transcriptional activators of the *vps* operons, VpsR and VpsT (11, 87). VpsR is a response regulator that belongs to the NtrC family (87). VpsT is a member of the UhpA (FixJ) class of transcriptional regulators (11). VpsR is a stronger transcriptional activator than VpsT. *vpsT* mutants are able to induce *vps* gene expression and form a biofilm, albeit to a lesser degree than wild-type cells, whereas *vpsR* mutants are completely defective in biofilm formation (5).

For the *V. cholerae* O1 serogroup, several global regulatory systems, including quorum sensing, the second messengers 3′,5′-cyclic diguanylic acid (c-di-GMP) and cyclic AMP (cAMP), and the sigma factors RpoS and RpoN, regulate transcription of *vpsR* and *vpsT*, with some factors directly regulating the *vps* genes (46, 47, 54, 65, 79, 88, 93). Many of these regulatory systems intersect, suggesting that there is precise control of biofilm gene expression. Quorum sensing regulates expression of *vpsR* and *vpsT* through the expression of *hapR*. At low cell density, *hapR* is not expressed, relieving repression of *vpsR* and *vpsT* and allowing expression of genes involved in biofilm formation. At high cell density, *hapR* is expressed, and *vpsR* and *vpsT* are repressed (27, 88). HapR has been shown to regulate *vpsT* through direct promoter binding (83), but there have been conflicting reports as to whether HapR directly controls *vpsR* gene expression. Hammer and Bassler showed no effect on *vpsR* expression in a *hapR* deletion strain, while Yildiz et al. reported that HapR does regulate *vpsR*. However, these discrepancies could have been the result of strain differences, as two different *V. cholerae* O1 El Tor strains were used (27, 83, 88).

The stringent response is a low-nutrient stress response that occurs when bacteria encounter nutrient-poor environments and initiates changes in gene regulation to maximize use of the available resources. Stringent response is induced when there is an increased concentration of the second messengers pppGpp (guanosine 3′-diphosphate 5′triphosphate) and ppGpp [guanosine 3′5′-bis(diphosphate)], together termed (p)ppGpp. An increased concentration of (p)ppGpp causes significant changes in

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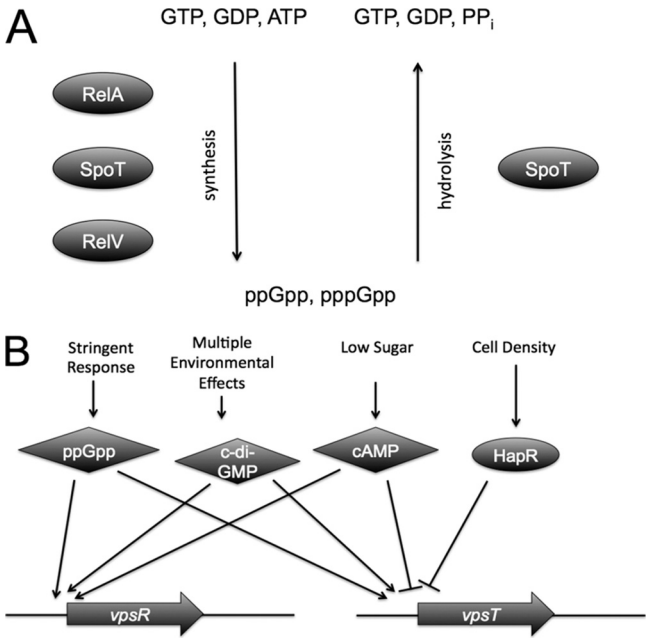

FIG 1 Stringent response and *V. cholerae* biofilm expression. (A) Three factors regulate (p)ppGpp synthesis and hydrolysis in *V. cholerae*: RelA, SpoT, and RelV. All three factors are able to synthesize ppGpp and pppGpp from GDP and GTP, but only SpoT is able to hydrolyze (p)ppGpp. (B) The stringent response mediator (p)ppGpp is one of several global regulators that affect biofilm formation in *V. cholerae*. (p)ppGpp and c-di-GMP both induce expression of VpsR and VpsT, the transcriptional activators of biofilm formation. cAMP induces expression of VpsR but inhibits expression of VpsT. High cell density induces the quorum-sensing regulator HapR, which represses expression of VpsT and possibly VpsR (see the text for references).

gene expression that result in cessation of growth and induction of specific stress responses (63). Many bacteria have only one regulator of (p)ppGpp concentration, Rel. Rel can phosphorylate GDP or GTP to produce (p)ppGpp and can hydrolyze (p)ppGpp back to GDP or GTP to allow growth after nutrient restrictions are alleviated. The beta- and gammaproteobacteria have two (p)ppGpp synthases, RelA and SpoT, but only SpoT can hydrolyze (p)ppGpp (Fig. 1A) (63). Although these proteins have extensive homology, RelA contains a defective hydrolase domain. *V. cholerae* has a third (p)ppGpp synthase, RelV, which is smaller than RelA and SpoT and has homology to other small (p)ppGpp synthases in distantly related organisms such as *Streptococcus mutans* and *Bacillus subtilis* (14, 15, 43, 60). RelV has synthase activity, but not hydrolase activity, and while *relV* homologs are present in other *Vibrio* species and closely related organisms, *relV* does not appear to be very well conserved in the gammaproteobacteria. An increase in intracellular (p)ppGpp affects the affinity of RNA polymerase for certain promoters and sigma factors. When uncharged tRNAs enter the A site of the ribosome, RelA synthase is activated and generates (p)ppGpp from GDP/GTP (29). In *Escherichia coli*, low carbon, nitrogen, phosphate, or iron induces SpoT synthase activity (59, 76, 80, 81). In addition, defects in fatty acid metabolism induce the SpoT synthase (71). The small, RelV-like (p)ppGpp synthases have been discovered only recently; thus, not much is known about their regulation or activity. It has been reported that, in *V. cholerae*, RelV is induced by low carbon and membrane stress (15). Two different groups have examined the role of *relA* in *V. cholerae* pathogenesis (28, 74). One study found that *relA* mutants were defective in production of CT and TCP and in colonization of suckling mice (28). The second study found that *relA* mutants had no effect on colonization of suckling mice or on production of CT and TCP (74). Neither group examined effects of *spoT* or *relV* on these processes, and examination of the full stringent response regulatory pathway may be necessary to resolve the differences seen in those studies.

Stringent response has been shown to regulate biofilm formation in *E. coli* and *S. mutans* (1, 9, 42). In addition, it has been found to be involved in the regulation of many bacterial virulence factors (13). From this, we hypothesized that the stringent response may have a role in regulating *V. cholerae* biofilm formation, particularly when *V. cholerae* is forming biofilms in aquatic environments, where there may be low nutrient availability (34). In order to test our hypothesis, we constructed *relA*, *relV*, *relA spoT*, *relA relV*, and *relA spoT relV* mutants to characterize the effects of (p)ppGpp on biofilm formation. We found that there were defects in biofilm formation in strains lacking (p)ppGpp synthases. We also found that while RelA was the sole (p)ppGpp synthase necessary for activation of *vpsT*, all three synthases activated *vpsR*, with RelV having the largest role, suggesting that different environmental stresses have different effects on biofilm formation. Furthermore, we found that (p)ppGpp regulation of *vpsR* and *vpsT* occurred through *rpoS*, the stationary-phase sigma factor. We also investigated the interaction between stringent response and quorum sensing and found that (p)ppGpp and HapR act on biofilm gene expression in opposing manners.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are listed in Table 1. All experiments were performed in LB medium, and antibiotics were used in the following concentrations: for ampicillin, 50 μg/ml; for streptomycin, 100 μg/ml; for kanamycin, 50 μg/ml; and for chloramphenicol, 1 μg/ml. DH5αλpir and SM10λpir were used for subcloning and strain construction (55). *V. cholerae* was propagated at 37°C in LB medium unless otherwise noted. Construction of *relA* and *spoT* in-frame deletions was described previously (66). Strains containing in-frame *relV* deletions were constructed by generating *relV* upstream and downstream regions by the use of PCR. All primers are listed in Table 2. The upstream fragment was digested with BamHI and SpeI and the downstream fragment was digested with SpeI and NotI, and they were ligated into *sacB*-containing suicide vector pWM91 (52), yielding pHH4. The pHH4 vector was mated to the appropriate *V. cholerae* strain, and *sacB* counterselection was used to create in-frame deletions. Strains containing in-frame *hapR* deletions were constructed by generating *hapR* upstream and downstream regions by the use of PCR and the primers listed in Table 2. The upstream fragment was digested with XhoI and SpeI and the downstream fragment was digested with SpeI and NotI, and they were ligated into *sacB*-containing suicide vector pWM91, yielding pDR330. The pDR330 vector was mated to the appropriate *V. cholerae* strains, and *sacB* counterselection was used to create *hapR* in-frame deletions. Plasmids pDR321, pDR322, and pHH17 were derivatives of pBAD33, pBAD18-kan, and pBAD24, respectively (25). A promoterless *lacZ* gene and a 5′ multicloning sequence were introduced into the plasmids by digesting plasmid pHT304-18Z (2) with KpnI and HindIII and ligated into these sites in pBAD33, yielding pHH7, or with EcoRI and PstI and inserted into pBAD18-kan, yielding pHH6, or with EcoRI and HindIII and ligated into pBAD24, yielding pDR323. The *vpsT* and *vpsR* promoters were amplified by PCR from genomic DNA template from *V. cholerae* El Tor strain N16961Sm. For *vpsT*, PCR was used to incorporate HindIII and PstI sites on the 5′ and 3′ ends, respectively. The PCR product was digested by HindIII and PstI and ligated into the corresponding sites

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or feature(s)[a]	Reference or source
Strains		
N16961Sm	WT O1 El Tor (St^r)	22
DR200	N16961Sm Δ*relA*	66
N16961*relAspoT*	N16961Sm Δ*relA* Δ*spoT*	66
HH4	N16961Sm Δ*relV*	This work
HH5	N16961Sm Δ*relA* Δ*relV*	This work
HH6	N16961Sm Δ*relA* Δ*spoT* Δ*relV*	This work
DR234	N16961Sm Δ*relA* Δ*spoT* Δ*relV* Δ*lacZ*	This work
DR235	N16961Sm Δ*relA* Δ*spoT* Δ*lacZ*	This work
DR236	N16961Sm Δ*relV* Δ*lacZ*	This work
DR237	N16961Sm Δ*relA* Δ*relV* Δ*lacZ*	This work
DR238	N16961Sm Δ*relA* Δ*lacZ*	This work
DR247	N16961Sm Δ*rpoS* Δ*lacZ*	This work
DR248	N16961Sm Δ*relA* Δ*spoT* Δ*relV* Δ*rpoS* Δ*lacZ*	This work
DR258	N16961Sm Δ*lacZ*	This work
C6706	WT O1 El Tor (St^r)	78
HH9	C6706 Δ*relA* Δ*spoT* Δ*relV*	This work
HH33	C6706 Δ*lacZ*	This work
DR253	C6706 Δ*hapR*	This work
DR254	C6706 Δ*relA* Δ*spoT* Δ*relV* Δ*hapR*	This work
DR233	C6706 Δ*relA* Δ*spoT* Δ*relV* Δ*lacZ*	This work
DR256	C6706 Δ*hapR* Δ*lacZ*	This work
DR257	C6706 Δ*relA* Δ*spoT* Δ*relV* Δ*hapR* Δ*lacZ*	This work
Plasmids		
pDR321	P_{vpsT}::*lacZ*	This work
pDR322	P_{vpsR}::*lacZ*	This work
pDR330	*hapR* knockout vector	This work
pDR345	P_{BAD}::*relA*	This work
pHH4	*relV* knockout vector	This work
pHH17	P_{rpoS}::*lacZ*	This work

[a] St^r, streptomycin resistant; WT, wild type.

TABLE 2 Oligonucleotide sequences

Primer name	Sequence[a]
relV upstream 5′	TGCGGCCGCCACTGTCATGCTGATTCGCCA
relV upstream 3′	CACTAGTCACTCTCCTTAGCTTGCGCTG
relV downstream 5′	TACTAGTTGAGCAGATCCAAACCATTGA
relV downstream 3′	AGGATCCATCGTGATAATCTGGAACTGA
hapR upstream 5′	ACTCGAGCAACATCTCGACCAAAACGTT
hapR upstream 3′	AGAACTAGTTTCTTGGGCAGCACAAAG
hapR downstream 5′	ACACTAGTAGGGGTATATCCTTGCCAATT
hapR downstream 3′	GTGCGGCCGCACCCAATTCACTTCAACGTCC
vpsR promoter 5′	TACCTGCAGTGAACGATGCTGAAGACCAAG
vpsR promoter 3′	TATCTAGAGGTACTGAATCCATACGGAAT
vpsT promoter 5′	TGTAAGCTTTTTCTGATTCATTGCGCTATC
vpsT promoter 3′	CGACTGCAGCTCCTAACACATCAAGGCTAA
rpoS promoter 5′	GCAAGCTTTCTGATCAGTTACAACGATCT
rpoS promoter 3′	TATCTAGAAGCGGCCTCCCCCTGGCAACT
relA 5′	GAGAATTCATGGTTGCGGTACGAAGCGCA
relA 3′	TCGGTACCTTAACCTAAGCGTTTGACCAA
gyrA forward	TCAAAGTCTCTGAGCGTAACGGCA
gyrA reverse	TACCAGTGTACCGGCATTGGTGAT
vpsR forward	TGGCGAAAGTGGTACTGGGAAAGA
vpsR reverse	CCAAGACCAAACAGCTCGCTTTCA
vpsT forward	ACCTCTTTCGCATCAGGACAACTG
vpsT reverse	CCTTTGGCGCTGGAAATTACACCA
vpsA forward	TCACGCAGTACCACTTTGCACCTA
vpsA reverse	GCCAACAACGCATCAATCACCGTA
vpsL forward	AAAGGCGATCCACGAGTTACACGA
vpsL reverse	ATGGTGCGATATTGCTCGTTGTGC

[a] Underlined sequences indicate restriction sites.

in pHH7 to produce pDR321 [P_{vpsT}::*lacZ*]. The *vpsR* promoter was amplified by PCR to incorporate PstI and XbaI sites on the 5′ and 3′ ends, respectively. The PCR product was digested by PstI and XbaI and ligated into the corresponding sites in pHH6 to produce pDR322 [P_{vpsR}::*lacZ*]. The *rpoS* promoter was amplified by PCR, and HindIII and XbaI sites were incorporated on the 5′ and 3′ ends, respectively. The PCR product was digested by HindIII and XbaI and ligated into the corresponding sites in pDR323, producing pHH17 [P_{rpoS}::*lacZ*]. Plasmid pDR332 is a derivative of pDSW208 (84). The *relA* gene was amplified by PCR using a genomic DNA template from *V. cholerae* El Tor strain N16961Sm to incorporate EcoRI and KpnI sites on the 5′ and 3′ ends, respectively. The PCR product was digested with EcoRI and KpnI and ligated into the corresponding sites in pBAD24 to produce pDR345 [P_{BAD}::*relA*].

In order to generate growth curves, an overnight culture was subcultured at a 1/100 dilution in LB medium and grown at 37°C with shaking. A_{600} was measured every 30 min. A representative example is presented.

Determination of (p)ppGpp synthesis. We measured (p)ppGpp as previously described with slight modification (66). To observe (p)ppGpp concentrations in actively growing cultures, we diluted an overnight culture into fresh LB medium supplemented with [^{32}P]orthophosphate (Perkin-Elmer) (100 μCi/ml), incubated the cultures at 37°C with shaking, and prepared cell extracts once the cultures reached mid-logarithmic-phase growth ($A_{600} \sim 0.4$). The cultures were extracted with formic acid on ice. After three freeze/thaw cycles, we removed cell debris by centrifugation. Supernatants were spotted on polyethyleneimine cellulose F thin-layer chromatography (TLC) plates. The plates were developed in 1.5 M KH_2PO_4 (pH 3.5) buffer and visualized by autoradiography. For biofilm cultures, we prepared a 1:10 dilution of overnight cultures with fresh LB medium containing [^{32}P]orthophosphate with static incubation at 37°C. Extracts were prepared and visualized using the same protocol as that used with the actively growing cultures.

Biofilm assays. Biofilm assays were performed using the method of O'Toole and Kolter (62). Overnight cultures were inoculated at a 1:100 dilution into 1 ml of LB medium using borosilicate glass tubes (10 by 75 mm) or into 100 μl of LB medium in two duplicate 96-well plates. Biofilm formation was encouraged by incubating stationary cultures at 37°C for 30 h. The cultures were removed, and the tubes were rinsed in 1× phosphate-buffered saline and filled with crystal violet stain. After 10 min, the stain was removed and the tubes were rinsed. The biofilm was resuspended with dimethyl sulfoxide (DMSO), and the A_{570} of the DMSO-crystal violet suspension was measured. Experiments were performed in triplicate in at least three separate trials, and the results of a typical assay are shown. For biofilm assays using strains N16961/pBAD24 and N16961Sm/pDR345, 0.025% arabinose was added at the time of inoculation. In order to eliminate bias from growth defect data (see Fig. 3), we did the biofilm assays in duplicate 96-well plates. After the 30-h incubation, one 96-well plate was used to determine cell density, and the biofilms were resuspended and the A_{600} was measured. The other plate was used for crystal violet staining. The ratio of A_{570}/A_{600} was used to normalize measurement of biofilm formation to bacterial growth. Statistical analyses were performed using GraphPad Prism 4 software.

β-Galactosidase assays. β-Galactosidase assays were performed using a modified version of the method of Miller (24, 53). To examine gene expression in stationary phase, overnight cultures of strains were subcultured in LB medium, and A_{600} was measured. Assays were performed in 96-well culture plates with 75 μl of culture. Cultures and Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4,10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol) were mixed at a ratio of 1:1. Cells were permeabilized with 0.1% sodium dodecyl sulfate (SDS) and 15 μl of chloroform. A 20-μl volume of ONPG (2-nitrophenyl-β-D-galactopyranoside) (4 mg/ml) was added to each well and mixed to initiate the reaction. Reactions were stopped with the addition of 75 μl of 1 M Na_2CO_3. Absorbance was

measured at A_{420} and A_{600}. β-Galactosidase activity was determined as an average of data determined with three cultures and was calculated by the following formula: activity = 1,000 × [A_{420}/(A_{600} × time × volume)]. Experiments were performed at least three times, and figures shown are from a representative experiment.

RNA isolation and qRT-PCR. Total RNA was isolated from *V. cholerae* strains grown to mid-logarithmic phase in LB broth for analysis of *vpsR* and *vpsT* expression. Total RNA was isolated from *V. cholerae* strains in LB broth in the stationary phase for analysis of *vpsA* and *vpsL* expression. Where described, cultures were treated with 10 mM serine hydroxamate for 10 min before RNA was isolated. The RNeasy kit (Qiagen) was used to isolate RNA, and the samples were treated with DNA-free DNase (Ambion) to remove DNA. The RNA samples were analyzed using quantitative real-time reverse transcription-PCR (qRT-PCR) and a SensiMix SYBR One-Step kit (Bioline) with SYBR green. Primers used in the reaction were the *gyrA*, *vpsR*, *vpsT*, *vpsA*, and *vpsL* forward and reverse oligonucleotides (listed in Table 2). Relative expression values were calculated as $2^{-\Delta(CT\ \text{target}\ -\ CT\ \text{reference})}$, where *CT* is the fractional threshold cycle. The *gyrA* gene was used as the reference to normalize expression values. Two technical replicates from each of three experimental replicate experiments were used for each strain and each set of conditions. Reaction mixtures lacking RNA were used as negative controls for each set of primers.

RESULTS

Growth conditions affect (p)ppGpp levels. We constructed *relA*, *relV*, *relA relV*, *relA spoT*, and *relA spoT relV* mutants to determine their roles in regulating (p)ppGpp concentration. We were unable to test a *spoT* single mutant or a *spoT relV* double mutant, because SpoT (p)ppGpp hydrolysis is required while *relA* is present (86). We visualized nucleotides present in each mutant strain in logarithmically growing cultures and biofilm cultures (Fig. 2). We observed that the wild-type strain and the *relA spoT* mutant produced the most (p)ppGpp (Fig. 2A). Presumably, the high (p)ppGpp levels were due to the wild-type strain having all three (p)ppGpp synthases whereas the *relA spoT* strain lacked the SpoT (p)ppGpp hydrolase but still contained the RelV synthase, making it unable to hydrolyze any (p)ppGpp synthesized by RelV. There was less (p)ppGpp in the *relA* mutant, there were very low detectable levels in the *relA relV* mutant, and there was almost no detectable (p)ppGpp in the *relA spoT relV* mutant. The biofilm cultures showed (p)ppGpp levels (Fig. 2B) similar to those of the logarithmically grown cultures (Fig. 2A), with the exception of the *relA* mutant, where the biofilm showed less (p)ppGpp than was seen in the logarithmically grown culture. Interestingly, the other noticeable difference between the biofilm cultures and actively growing cultures was the ratio of ppGpp to pppGpp (Fig. 2A and B). Biofilm cultures had a high ppGpp/pppGpp ratio, and actively growing cultures had a lower ppGpp/pppGpp ratio. It has been reported that ppGpp is more capable of stringent response induction than pppGpp (63), suggesting that in our biofilm cultures, the stress response-inducing ppGpp form is present, while in the actively growing cultures, the less active pppGpp form is present. This also suggests that there may be additional regulation of stringent response: in addition to the activity of the RelA and RelV synthases, there may be regulation of pppGpp conversion to ppGpp. We performed growth curve analyses on the mutant strains to determine the effects of the (p)ppGpp synthase mutations on growth (Fig. 2C). All the mutants grew at approximately the same rate as the wild type, except for the *relA spoT* mutant. The *relA spoT* mutant had a (p)ppGpp synthase (RelV) but no hydrolase. As shown in Fig. 2B, this strain produced a considerable amount of (p)ppGpp, likely causing the growth defect.

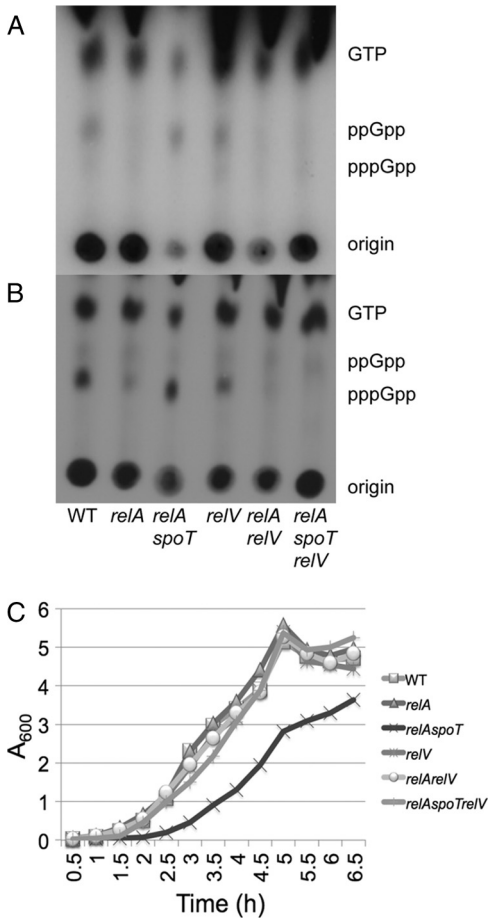

FIG 2 Production of (p)ppGpp in wild-type and synthase-mutant *V. cholerae* strains in (A) actively growing cultures and (B) stationary biofilm cultures. N16961 and in-frame deletions of the indicated genes were used. Each culture was grown in LB medium incubated with [^{32}P]orthophosphate. TLC was performed on culture extracts to identify nucleotides. (C) Growth curves of the N16961 wild type (WT) and the (p)ppGpp synthase mutants.

Stringent response mutants are deficient in biofilm formation. We tested whether stringent response played a role in biofilm formation by examining the effects of our single, double, and triple mutations of the (p)ppGpp synthases on biofilm formation in the naturally occurring *hapR*-frameshift N16961 strain. In order to control for growth defects in the *relA spoT* strain (Fig. 2C), we used the ratio of biofilm formation (A_{570}) to culture density (A_{600}) in the biofilm assays (Fig. 3A and B) (38, 74, 91). The *relA spoT* growth defect in biofilm cultures was less severe than in logarithmic-growth-phase cultures, with the *relA spoT* mutant showing an approximately 30% lower A_{600} for both the N16961 and C6706 strain backgrounds (Fig. 2C and data not shown [but included in calculations performed to produce the data shown in Fig. 3A and B]). We found that the *relA spoT relV* triple mutant showed a significant decrease in the production of biofilm compared to the wild-type strain (Fig. 3A). While the *relA spoT* double mutant showed very little biofilm formation, as observed by crystal violet staining (Fig. 3A, fourth tube), this strain produced high levels of (p)ppGpp, inhibiting cell growth, due to lack of SpoT hydrolase

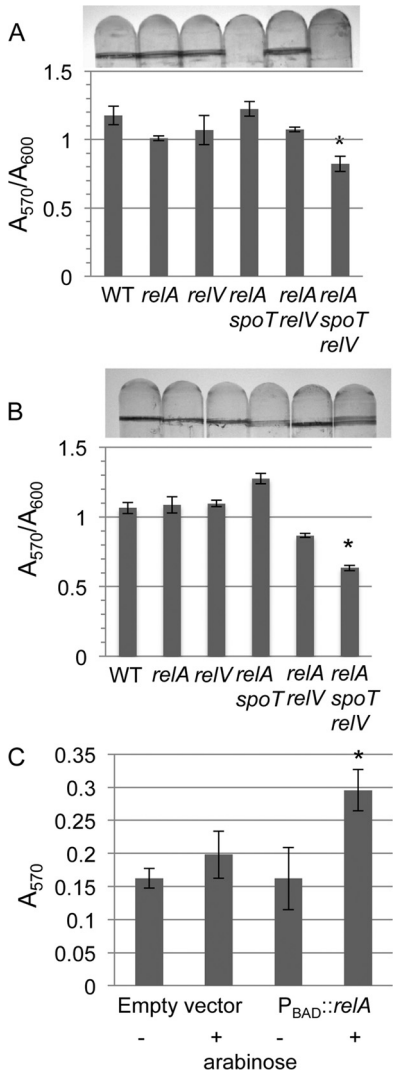

FIG 3 Stringent response regulates biofilm formation. Biofilm assays were performed in both N16961 and C6706 backgrounds to test involvement of the *relA*, *spoT*, *and relV* gene products in biofilm formation. (A) N16961-derived strains. (B) C6706-derived strains. (C) Overexpression of RelA induces biofilm formation. An N16961 strain containing the *relA* fusion gene under the control of the P_{BAD} promoter was incubated with 0.025% arabinose to induce expression of *relA*. (A and B) Biofilm formation was normalized to cell growth by calculating the ratio of A_{570}/A_{600} (crystal violet staining to culture density). Data were analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test. For panel A, the asterisk indicates a significant ($P < 0.01$) difference compared to the wild type. In addition, there were significant differences for *relA* versus *relA spoT* and *relV* versus *relA relV* ($P < 0.05$), *relA relV* versus *relA spoT relV* ($P < 0.01$), and *relA* versus *relA relV*, *relA* versus *relA spoT relV*, *relV* versus *relA spoT relV*, and *relA relV* versus *relA spoT relV* ($P < 0.001$). For panel B, the asterisk indicates a significant ($P < 0.01$) difference compared to every value indicated without an asterisk. For panel C, the asterisk indicates a significant ($P < 0.01$) difference compared to uninduced cultures.

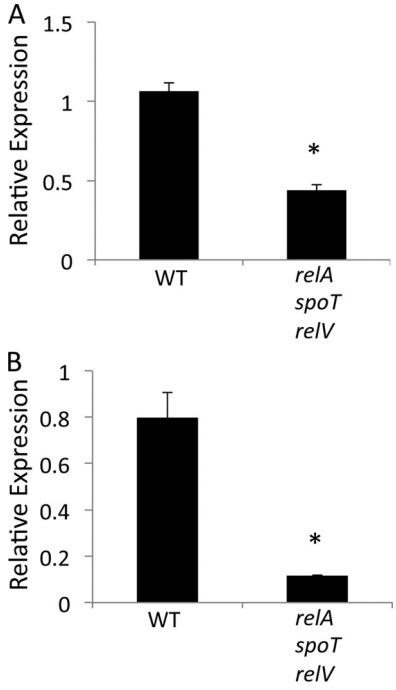

FIG 4 Expression of *vpsA* and *vpsL* in stringent response mutants. *vpsA* expression (A) and *vpsL* expression (B) in the indicated strains were measured using qRT-PCR. Data were analyzed using a one-tailed Student's *t* test. Asterisks indicate a significant ($P < 0.05$) difference.

activity (Fig. 2B). When biofilm formation was integrated with total cell growth, there was no observable defect in biofilm formation in the *relA spoT* strain (Fig. 3A). Interestingly, while there appeared to be a complete loss of biofilm in the *relA spoT relV* mutant (Fig. 3A, sixth tube) in the crystal violet-stained culture tubes, there was still measurable biofilm according to the optical density data. The dominant crystal violet staining of the glass tubes was from the pellicle, suggesting the possibility that the stringent response may have specific effects on production of the pellicle of the biofilm.

As quorum sensing is also involved in regulating biofilm formation and N16961 does not produce HapR, we tested whether the stringent response affects biofilm formation in *hapR*-positive (*hapR*$^+$) strain C6706 (93). In this background, we found that the *relA spoT relV* triple mutant showed a significant defect in biofilm formation (Fig. 3B). Just as in the N16961 strain (Fig. 3A), we observed a defect in biofilm formation in the *relA spoT* double mutant that was attributable to defects in cell growth, with no significant loss when taking growth into account (Fig. 3B). There was a decrease in crystal violet staining (Fig. 3B, fourth tube), indicating less biofilm, but also a decrease in cell growth due to a lack of SpoT hydrolase, so that when biofilm formation was normalized to cell growth, there was no significant defect in biofilm formation (Fig. 3B). While the *relA spoT relV* mutants in both the N16961 and C6706 backgrounds still produced biofilm, the mutants produced 30% less in the N16961 background (Fig. 3A) and 40% less in the C6706 background (Fig. 3B), indicating that the stringent response played a role in this process. To determine whether the decrease in biofilm formation was due to decreased expression of the *vps* genes, we compared *vpsA* and *vpsL* expression in wild-type and *relA spoT relV* mutants. Both *vpsA* expression and *vpsL* expression were significantly lower in the stringent response mutants (Fig. 4A and B), further supporting a role in stringent response regulation of biofilm.

The decrease in biofilm formation in (p)ppGpp-deficient strains suggested that increasing the (p)ppGpp concentration would result in a concomitant increase in biofilm production. Overexpression of RelA increases intracellular concentrations of

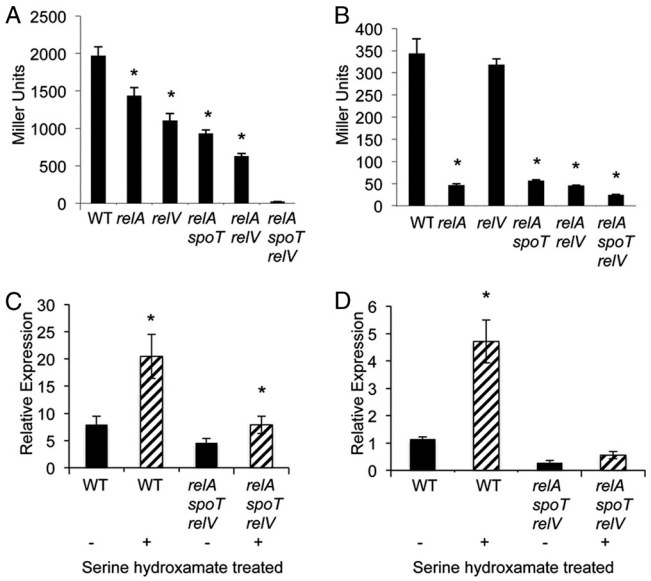

FIG 5 (p)ppGpp synthases have specific effects on *vpsR* and *vpsT* expression. (A and B) We constructed *vpsR* and *vpsT* promoter fusions with the *lacZ* gene and measured β-galactosidase activity in each N16961 strain background as indicated. (A) *vpsR* expression. (B) *vpsT* expression. (C and D) *vpsR* expression (C) and *vpsT* expression (D) were measured using qRT-PCR in the indicated strains. Serine hydroxamate was added to the culture to induce the stringent response. Solid bars indicate untreated cultures, and striped bars indicate cultures treated with serine hydroxamate. *vpsR* and *vpsT* transcript levels were normalized to *gyrA* levels and compared in a wild-type and *relA spoT relV* strain. (A and B) Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. For panel A, asterisks indicate a significant difference compared to the wild type (at least $P < 0.01$). For panel B, asterisks indicate a significant difference compared to the wild type ($P < 0.001$). For panels C and D, data were analyzed using a one-tailed Student's *t* test. Asterisks indicate a significant difference between the mock-treated and serine hydroxamate-treated cultures ($P < 0.05$).

(p)ppGpp but allows some bacterial growth (70). We used the *V. cholerae* N16961 strain containing a plasmid expressing *relA* under the control of the arabinose-inducible P_{BAD} promoter and compared induced and uninduced cultures. We found that overexpression of *relA* increased biofilm formation (Fig. 3C). Taken together, the data show that deletion of the (p)ppGpp synthases led to a decrease in biofilm and that overexpression of *relA* led to an increase in biofilm, indicating a role for the stringent response in the regulation of biofilm production.

Stringent response regulates *vpsR* and *vpsT* expression. As (p)ppGpp synthase mutants generated biofilms that were less robust than wild-type strains, we tested whether the stringent response affected expression of the *vpsR* and *vpsT* genes, which encode the transcriptional activators of the *V. cholerae vps* operons. We constructed *vpsR-lacZ* and *vpsT-lacZ* promoter fusions to test for transcription of these genes by measuring β-galactosidase activity. We tested the effects of our mutations on *vpsR* and *vpsT* transcription in stationary-phase cultures. Wild-type *V. cholerae* produced robust expression of the *vpsR* promoter (Fig. 5A). Both *relA* and *relV* mutants showed decreased expression of *vpsR*. While we were unable to test *spoT* activity individually, the *relA spoT* double mutant showed significantly lower *vpsR* expression than the *relA* mutant and the *relA spoT relV* mutant showed significantly lower *vpsR* expression than the *relA relV* mutant, indicating that *spoT* has a role in regulating *vpsR*. The *relA relV* double mutant showed a larger decrease in *vpsR* expression than either of the single mutants, while the triple mutant (*relA spoT relV*) strain had very little *vpsR* expression. These results indicate that all three (p)ppGpp synthases are important for *vpsR* expression, with RelV having the largest impact. Interestingly, *vpsT* regulation was different from *vpsR* regulation (Fig. 5A and B). Only *relA* appeared to have any significant effect on *vpsT* expression. No differences in *vpsT* expression were shown by a *relA* single mutant or *relA spoT* or *relA relV* double mutants. This indicates that *relA* was the most significant regulator of *vpsT*. Interestingly, the *relA spoT* mutant had decreased expression of *vpsR* and *vpsT* (Fig. 5A and B) but no biofilm formation defect (Fig. 3A and B). If expression of the regulators had decreased, a concomitant decrease in biofilm would be expected. It is possible that the normal biofilm phenotype displayed by the *relA spoT* mutant is due to a combination of lowered biofilm gene expression with slower growth. The *relA spoT* mutant has slightly reduced *vpsR* expression compared to the wild type and much less *vpsT* expression (Fig. 5A and B). VpsR is a stronger inducer of biofilm formation than VpsT, so there is still some expression of biofilm in *vpsT* mutants (5). In combination with the lowered expression, there is slower growth, so it is possible that there is as much biofilm material produced per cell by the mutant as by the wild type.

Deletion of (p)ppGpp synthases led to loss of the ability to form biofilm and a decrease in expression of *vpsR* and *vpsT*, so we tested whether induction of the stringent response increased *vpsR* and *vpsT* transcription. We treated cultures with serine hydroxamate, an amino acid analog that inhibits protein translation, to induce stringent response, and then compared *vpsR* and *vpsT* expression in the serine hydroxamate-treated cultures to expression in mock-treated cultures by the use of quantitative real-time PCR. We found that *vpsR* expression increased 2.5-fold and *vpsT* expression increased 4-fold in the stringent response-induced cultures (Fig. 5C and D). We performed the same experiment on the *relA spoT relV* strain and found that serine hydroxamate treatment had a much milder effect, with a small increase in *vpsR* expression and no statistically significant increase in *vpsT* expression (Fig. 5C and D). This suggests that the increase in *vpsR* and *vpsT* expression was specifically due to increased levels of (p)ppGpp. While there was a statistically significant increase in *vpsR* expression even in the absence of (p)ppGpp (Fig. 5C), this may have been due to broad effects of the presence of serine hydroxamate. Serine hydroxamate-induced disruption of translation may impact processes other than the stringent response, leading to induction of other regulatory pathways that induce *vpsR*. That *vpsR* induction was lower in the (p)ppGpp-null cells than in the wild-type strain suggests a specific role for (p)ppGpp in *vpsR* regulation.

These results were obtained from stationary-phase cultures but not from actual biofilms. We attempted to analyze gene expression in biofilms by the use of both β-galactosidase assays and qRT-PCR. When we repeated the β-galactosidase assays using biofilm cultures rather than stationary-phase cultures, there was no production of β-galactosidase when using any construct. When we attempted to measure gene expression in biofilm cultures by the use of qRT-PCR, we were unable to acquire quality RNA from biofilm cultures. So these results must be interpreted in the context of cultures that were not biofilms but were instead cultures that had reached the stationary phase and were transitioning to form biofilms. However, the data we present using sta-

tionary-phase bacteria are important for understanding how biofilm formation occurs, because the initial gene regulation that brings about biofilm formation occurs in bacteria that are planktonic.

Stringent response regulation of biofilm is partially dependent on *rpoS*. Increased (p)ppGpp synthesis allows bacteria to adapt to the stationary phase quickly and induce additional stress responses. *rpoS* encodes σ^s, the stationary-phase σ factor, which is induced by (p)ppGpp in *E. coli* (23). We tested the effects of stringent response on *rpoS* expression in *V. cholerae*. We constructed an *rpoS-lacZ* promoter fusion to test *rpoS* activation in the different (p)ppGpp synthase mutant backgrounds. All mutants showed some decrease in *rpoS* expression, indicating that (p)ppGpp is important for *rpoS* expression in *V. cholerae* (Fig. 6A). RelA had the largest effect on *rpoS* expression, with the *relA* single mutant and *relA relV* double mutant producing the lowest levels of *rpoS* expression (Fig. 6A). These results suggest that RelA had a significant role in *rpoS* expression. The *relA* strain produced higher *rpoS* expression than the *relA spoT* strain, indicating that it is possible that the SpoT activity was operating in opposition to that of RelA and RelV, possibly due to its (p)ppGpp hydrolase activity (Fig. 6A). In the RelA-expressing strains, there was strong *rpoS* expression (Fig. 6A). In a *relA spoT* double mutant, there was moderate *rpoS* expression (Fig. 6A), presumably due to the presence of RelV-produced (p)ppGpp (Fig. 2). While the *relA spoT relV* triple mutant showed higher *rpoS* expression than the *relA* or *relA relV* mutants, the difference was not statistically significant.

We then tested whether loss of σ^s would affect expression of *vpsR* and *vpsT*. We tested *vpsR* and *vpsT* expression in *rpoS* mutants and in stringent response-defective (*relA spoT relV*) *rpoS* mutants. The *rpoS* mutant showed a decrease in *vpsR* expression compared to the wild type but not as much of a decrease as the *relA spoT relV* mutant (Fig. 6B). This indicated that, although the stringent response affected *vpsR* expression through σ^s, it also acted on *vpsR* via a non-σ^s mechanism. In the *relA spoT relV rpoS* mutant, there was a further decrease in *vpsR* expression beyond what was seen in the *rpoS* mutant, although the decrease was not as low as that seen in the *relA spoT relV* mutant (Fig. 6B). This suggested that the stringent response was necessary for *vpsR* expression and that this was partially mediated through stringent response induction of *rpoS*. The *rpoS* mutation produced a decrease similar to that seen with expression of *vpsT* (Fig. 6C). Expression of *vpsT* in the *relA spoT relV rpoS* mutant was not much different from expression in the *relA spoT relV* strain, further differentiating *vpsT* regulation from *vpsR* regulation (Fig. 6B and C). These results suggested that (p)ppGpp regulated both *vpsR* and *vpsT* through induction of *rpoS*. However, *rpoS* is not the only factor necessary; for stringent response regulation of *vpsR*, additional factors must be involved.

Stringent response is necessary for positive regulation of biofilm formation. Quorum sensing in *V. cholerae* is mediated through the transcriptional regulator HapR. To test the various contributions of (p)ppGpp and *hapR* to biofilm formation, we used *hapR*$^+$ strain C6706 (Fig. 7). We compared the abilities of the C6706 wild type, a *hapR* mutant, the stringent response-defective mutant (*relA spoT relV*), and a combined *relA spoT relV hapR* mutant to form biofilms. We found that *hapR* mutants produced more biofilm than wild-type cultures, supporting studies that have shown that HapR is a repressor of biofilm formation (Fig. 7A) (27, 83, 88, 93). The *relA spoT relV* mutant had less biofilm

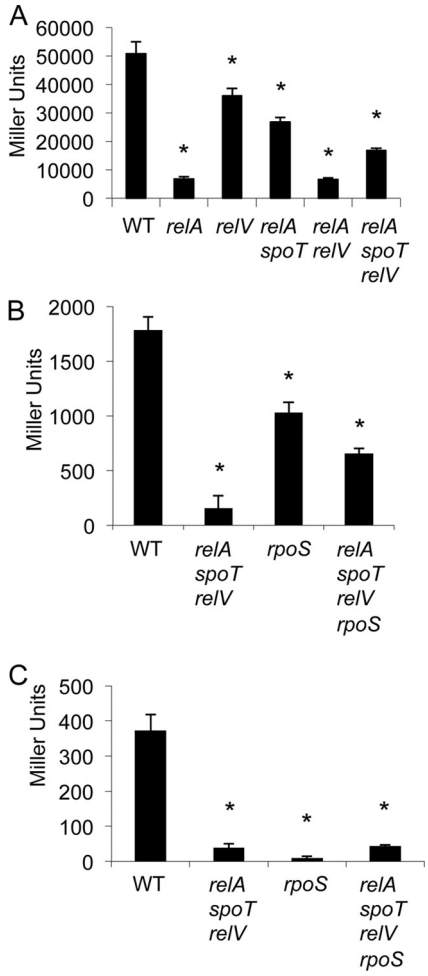

FIG 6 Stringent response regulation of *rpoS* affects *vpsR* and *vpsT* expression. Promoter fusions of *rpoS*, *vpsR*, and *vpsT* and β-galactosidase assays were used to measure gene expression in wild-type N16961-derived cells and the indicated mutant strains. (A) *rpoS* expression. (B) *vpsR* expression. (C) *vpsT* expression. Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. For panel A, asterisks indicate a significant difference compared to the wild type ($P < 0.01$). In addition, there were significant differences for *relA* versus *relV*, *relA* versus *relA spoT*, *relV* versus *relA relV*, and *relV* versus *relA spoT relV* ($P < 0.001$). For panel B, asterisks indicate a significant difference compared to all others ($P < 0.01$ for all [except $P < 0.05$ for *rpoS* versus *relA spoT relV rpoS*]). In addition, there were significant differences for *rpoS* versus *relA spoT relV rpoS* ($P < 0.05$), *relA spoT relV* versus *relA spoT relV rpoS* ($P < 0.01$), and *relA spoT relV* versus *rpoS* ($P < 0.001$). (C) Asterisks indicate a significant difference compared to the wild type ($P < 0.001$).

than the wild type (Fig. 7A), as it did in the experiment whose results are shown in Fig. 3B. The *relA spoT relV hapR* mutant produced more biofilm than the stringent response-defective *hapR*$^+$ strain (Fig. 7A), indicating that the stringent response and HapR worked in opposite manners. HapR repressed biofilm formation, and the *hapR* mutants increased biofilm formation, even in a stringent response-defective strain. This suggested that while HapR repressed biofilm, the stringent response and other factors were still necessary to induce expression of the biofilm genes. Even in a *relA spoT relV* mutant, there are factors that can induce the production of biofilm (c-di-GMP, cAMP, and other factors), but the effectiveness of these factors may depend on the *V. cholerae* population density, which in turn regulates HapR activity.

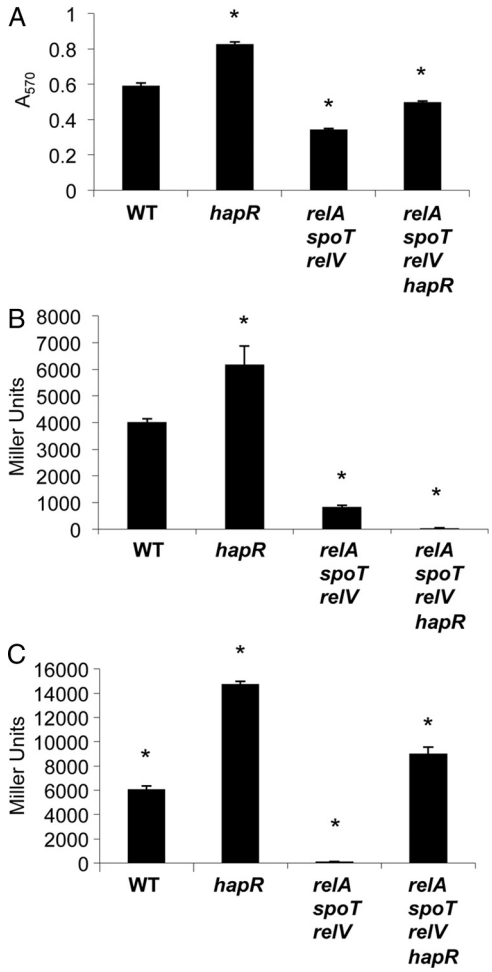

FIG 7 Effects of stringent response and HapR on biofilm formation and *vpsR* and *vpsT* expression. (A) Biofilm assays were performed in the indicated C6706-derived strains. (B and C) *vpsR* expression (B) and *vpsT* expression (C) were measured using β-galactosidase assays in wild-type cultures and in *hapR* and (p)ppGpp-null cultures. Data were analyzed using one-way ANOVA and Bonferroni's multiple comparison test. Asterisks indicate a significant difference from all other strains ($P < 0.01$ for panel A and $P < 0.001$ for panels B and C).

Due to the differential effects of HapR and stringent response on production of biofilm, we investigated the effect of these regulators on *vpsR* and *vpsT* expression. Both *vpsR* and *vpsT* were expressed at higher levels in the *hapR* mutant, confirming that HapR is a repressor of *vpsR* and *vpsT* (Fig. 7B and C). We also found that, while HapR repressed some biofilm formation, as seen by comparing the wild-type and *hapR* strains (Fig. 7A), without the stringent response there was very little expression of either *vpsR* or *vpsT* even in *hapR* deletion strains. This confirmed that removing HapR repression was not enough for maximal biofilm production. We also tested the effects of combining the *relA spoT relV* mutations with *hapR*. A *relA spoT relV hapR* mutant showed very little expression of *vpsR*, which was expected due to loss of both positive and negative regulators of *vpsR* (Fig. 7B). Interestingly, *vpsT* showed an increase in expression in *relA spoT relV hapR* cells (Fig. 7C). It is known that c-di-GMP induces *vpsT* expression, which may explain the increase in the absence of the stringent response, and perhaps other factors are involved as well (83).

DISCUSSION

Interpreting and responding to environmental signals is crucial for survival of pathogenic bacteria both within and outside the host. *V. cholerae* forms biofilms in aquatic reservoirs in order to allow the organism to conserve resources and promote environmental survival and to assist the bacteria during their passage through the high-acid environment of the stomach (56, 68, 89, 92). A number of regulatory systems, environmental factors, and intracellular molecules affect biofilm formation, including quorum sensing, c-di-GMP, cAMP-CRP, phosphate, salinity, calcium, indole, nucleosides, and environmental sugar (7, 8, 26, 30, 31, 45–48, 50, 57, 65, 73, 77, 79, 93). In the work presented here, stringent response was shown to play a role in biofilm formation in *V. cholerae*. While biofilm formation was able to occur absent stringent response, stringent response was necessary for full production of biofilm and for full production of the transcriptional activators VpsR and VpsT (Fig. 3 and 5).

V. cholerae has three (p)ppGpp synthases, RelA, SpoT, and RelV, and these synthases have differing effects on biofilm formation. RelV is the (p)ppGpp synthase that most affects *vpsR* expression, while RelA is the most important for *vpsT* expression, suggesting that the different (p)ppGpp synthases have distinct roles in biofilm formation. However, removing any single (p)ppGpp synthase did not significantly impair biofilm production; it was only in *relA spoT relV* mutants that biofilm formation was affected. RelA is the strongest *V. cholerae* (p)ppGpp synthase (Fig. 2) and can induce expression of both VpsR and VpsT. RelV is a weaker synthase than RelA but is able to induce expression of VpsR. In a *vpsT* mutant, biofilm genes are still expressed, suggesting that *relA* mutants should be able to form biofilm even though *vpsT* is not induced (5). This study showed that the stringent response regulated transcription of *vpsR* and *vpsT* in distinct manners, sometimes affecting the transcription of one but not the other. Earlier studies had shown that VpsR can activate *vpsT* transcription and vice versa, suggesting that factors that affect expression of one regulator should affect both (5, 11). Other studies have shown that quorum sensing and c-di-GMP can affect transcription of either *vpsR* or *vpsT* without affecting transcription of the other, perhaps due to the timing of regulation, and the stringent response may act like those other regulators (6, 44, 72, 83).

A previous study showed that *relA* mutants have normal biofilm formation, although those researchers did not test the effects of *spoT* or *relV* (74). This is consistent with our results, in that loss of RelA activity did not have a significant effect on biofilm formation even though there was some loss of expression of *vpsR* and *vpsT*. How the different synthases produce different effects on *vpsR* and *vpsT* expression is an intriguing issue. The differing effects may be due to differences in the (p)ppGpp concentration. There may be a threshold of (p)ppGpp concentration necessary for *vpsT* expression such that, if that threshold is reached, *vpsT* is expressed. As RelA is the strongest synthase, it may be the only synthase that achieves the threshold (p)ppGpp concentration required for *vpsT* expression. Full *vpsR* expression may require higher levels of (p)ppGpp, making the activity of all three synthases necessary. However, this would not adequately explain RelV-dependent induction of *vpsR*. Another possibility is that there is localized production of (p)ppGpp. RelA is thought to transiently associate with ribosomes and produce (p)ppGpp when uncharged tRNAs enter the A site of the ribosome (29, 85). Where

RelV is located in the cell is not known. Perhaps the location of a (p)ppGpp synthase leads to differences in local (p)ppGpp concentrations, affecting promoter activity in a small region of the cell. If (p)ppGpp generated by RelV is produced closer to the *vpsR* promoter than that generated by RelA, it could explain why RelV has a stronger effect on *vpsR* activity despite RelA being a stronger (p)ppGpp synthase. The differing effects of RelA and RelV may allow *V. cholerae* to coordinate biofilm gene expression with specific environmental effects, such as the presence of a low-carbon source that specifically induces RelV (15).

In this study, we demonstrated that the stringent response induced expression of the stationary-phase sigma factor σ^s (*rpoS*) in *V. cholerae*. Each (p)ppGpp synthase has an effect on *rpoS* expression (Fig. 6A). Both *vpsR* expression and *vpsT* expression are dependent on *rpoS* (Fig. 6B and C). Expression of *vpsT* is completely dependent on *rpoS*, while there is still some expression of *vpsR* in the *rpoS* mutant. The results of this study suggest that σ^s is necessary for biofilm formation, which conflicts with two previous studies that provided evidence that σ^s is a negative regulator of biofilm (58, 88). It is possible that σ^s has a complex role in the regulation of biofilm. As shown in Fig. 6B, the *rpoS* mutant had less *vpsR* expression than the wild-type strain but more than the *relA spoT relV* mutant. However, the *relA spoT relV rpoS* mutant strain had an increase in *vpsR* expression relative to the *relA spoT relV* strain. This suggests that σ^s may have different effects on biofilm formation depending on whether stringent response is induced or not. In the absence of (p)ppGpp, σ^s may repress *vpsR*, but in the presence of (p)ppGpp it may induce *vpsR*. These conflicting studies used a different *V. cholerae* strain, A1552, and it is possible that A1552 either is defective in (p)ppGpp synthesis or has other regulatory differences from N16961 and C6706 that affect biofilm formation.

Several global regulatory systems coordinate *V. cholerae* virulence gene expression with environmental conditions (Fig. 1B). Quorum sensing regulates biofilm, CT, and TCP, with high cell density inducing biofilm and repressing CT and TCP expression and low cell density repressing biofilm and inducing CT and TCP expression (26, 27, 39, 83, 93; reviewed in reference 90). (p)ppGpp and two other second messengers, c-di-GMP and cAMP (through its interaction with the cAMP receptor protein [CRP]), also regulate expression of these virulence factors (46, 47, 54, 65, 79, 88, 93). These three second messengers, as well as quorum sensing, are likely to be activated at similar times in the *V. cholerae* life cycle. Low glucose levels induce adenylyl cyclase expression, and a number of environmental factors, including phosphate, light, oxygen, bile, and nutrient starvation, induce c-di-GMP synthesis (35). Low carbon, nitrogen, and iron levels and phosphate stress induce stringent response. The composition of nutrients present in aquatic environments is quite variable. For instance, in North American waters, assimilable organic carbon levels range from 1 to 2,000 $\mu g\ liter^{-1}$, and *V. cholerae* needs at least 100 $\mu g\ liter^{-1}$ for growth (40, 82). It is likely that aquatic environments often produce low-nutrient stress responses mediated by (p)ppGpp, c-di-GMP, and cAMP. In the gut, there is a rich nutrient environment, leading to low expression of the second messengers. Rich nutrient environments are associated with rapid growth, linking quorum sensing to rich environments.

Why do so many regulatory systems affect biofilm formation in *V. cholerae*? Biofilm may be so important that redundancy is built into the system, so that the loss of any one regulatory factor does not lead to loss of biofilm. One piece of evidence in favor of the idea of redundancy is that pandemic *V. cholerae* strains often have defects in the quorum sensing system, suggesting that decoupling cell density from regulation of virulence factors has no harmful effect on long-term survival of *V. cholerae* (36). There is also evidence that the multiple regulatory systems provide fine-tuning of biofilm genes. The data presented here suggest that the stringent response is only one factor in regulation of biofilm formation and that there is still production of biofilm in the absence of stringent response regulators (Fig. 3A and B). There are reports that diguanylate cyclases and phosphodiesterases, the factors that synthesize and degrade c-di-GMP, regulate HapR (5, 48) and that HapR regulates diguanylate cyclases and phosphodiesterases (26, 83). In addition, cAMP has been reported to regulate diguanylate cyclases and phosphodiesterases and HapR (19, 45, 46, 75). The phosphate-responsive regulator PhoB also regulates diguanylate cyclases and phosphodiesterases (64). It is possible that the stringent response regulates some of these factors, and these factors may even regulate expression of *relA*, *spoT*, and *relV*. Beyond the global regulatory systems, other factors, such as salinity, calcium, indole, nucleosides, and environmental sugar, regulate biofilm formation (8, 30, 31, 57, 73). Understanding how these regulatory systems interact is necessary to determine how *V. cholerae* is able to coordinate biofilm formation with environmental conditions.

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