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Functional Characterization of the Stringent Response Regulatory Gene *dksA* of *Vibrio cholerae* and Its Role in Modulation of Virulence Phenotypes

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In bacteria, nutrient deprivation evokes the stringent response, which is mediated by the small intracellular signaling molecule ppGpp. In Gram negatives, the RelA enzyme synthesizes and SpoT hydrolyzes ppGpp, although the latter protein also has weak synthetase activity. DksA, a recently identified RNA polymerase binding transcription factor, acts as a coregulator along with ppGpp for controlling the stringent response. Recently, we have shown that three genes, *relA*, *spoT*, and *relV*, govern cellular levels of ppGpp during various starvation stresses in the Gram-negative cholera pathogen *Vibrio cholerae*. Here we report functional characterization of the *dksA* gene of *V. cholerae* (*dksA*$_{Vc}$), coding for the protein DksA$_{Vc}$. Extensive genetic analyses of the Δ*dksA*$_{Vc}$ mutants suggest that DksA$_{Vc}$ is an important component involved in the stringent response in *V. cholerae*. Further analysis of mutants revealed that DksA$_{Vc}$ positively regulates various virulence-related processes, namely, motility, expression of the major secretory protease, called hemagglutinin protease (HAP), and production of cholera toxin (CT), under *in vitro* conditions. We found that DksA$_{Vc}$ upregulates expression of the sigma factor FliA (σ^{28}), a critical regulator of motility in *V. cholerae*. Altogether, it appears that apart from stringent-response regulation, DksA$_{Vc}$ also has important roles in fine regulation of virulence-related phenotypes of *V. cholerae*.

Expression of genes in microorganisms is a highly regulated process and often involves complex genetic circuits controlling several phenotypes for their growth and survival under various environmental conditions. This is further complicated if we consider that bacteria, including pathogens in nature, are found in complex communities. Thus, signaling mechanisms in bacteria must be robust in order for them to sustain various environmental onslaughts and to survive and grow through tremendous competition with the community microorganisms in a particular niche. As a result, bacteria have evolved with multiple gene regulatory circuits to sense and combat various environmental stresses. The most important one among such adaptive responses is the stringent response, where bacterial cells undergo rapid and complex metabolic adjustments through negative and positive regulation of gene expression during nutritional starvation. The global changes in gene expression associated with the stringent response are triggered mainly by the intracellular accumulation of two small molecules called guanosine 3′-diphosphate 5′-triphosphate (pppGpp) and guanosine 3′,5′-bis diphosphate (ppGpp), together called (p)ppGpp, and are characterized by negative regulation of rRNA transcription, positive regulation of amino acid biosynthesis, readjustment of metabolic pathways according to physiological requirements, and induction of stationary-phase genes needed for survival (10).

In Gram-negative organisms, including *Escherichia coli*, the products of the *relA* and *spoT* genes synthesize (p)ppGpp. However, SpoT is a bifunctional enzyme having strong hydrolyzing and weak (p)ppGpp synthetase activities (10, 32). Although the exact mechanism is not yet clearly known, it appears that (p)ppGpp binds to a site adjacent to, but not overlapping, the active site on the β and β′ subunits of the RNA polymerase (RNAP) core enzyme and affects gene transcription at the stage of initiation during open promoter complex formation (2, 31). However, recent studies indicate that (p)ppGpp alone is not involved in the process; rather, a small protein, DksA, the product of the *dksA* gene, acts as a coregulator to facilitate the function of (p)ppGpp during the stringent response (39–41). Like the *relA* and *spoT* genes, *dksA* is also conserved in Gram-negative bacteria (11, 41). From a structural point of view, DksA belongs to an unusual family of transcriptional regulators, whose members do not bind directly to the regulatory part of a gene but rather bind directly to the secondary channel of RNAP (36, 39, 41). The crystal structure of the DksA protein of *E. coli* indicates a globular domain and a coiled-coil structure with C_4 zinc finger motif (41). When DksA binds directly to RNAP, two highly conserved aspartic acid residues present at the tip of the coiled-coil domain of the protein help to stabilize the (p)ppGpp-Mg^{2+}-RNAP complex. Based on several reports, it appears that apart from participation in the stringent response, DksA is also involved in multiple cellular processes in different Gram-negative bacteria. Among these functions, the conspicuous ones are modulation of multiple gene expression (55), quorum sensing (QS) (8, 24), and virulence (24, 30, 33, 36, 46, 49, 55). Most recently, it has been shown that DksA along with (p)ppGpp is directly involved in regulation of transcription of *E. coli* flagellar genes and ribosomal protein coding genes (27, 28).

Although most of the DksA-related studies have so far been conducted in *E. coli*, at present our knowledge regarding how DksA modulates different gene functions in pathogens is limited.

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Previously we functionally characterized the *relA* and *spoT* genes of *Vibrio cholerae* (13, 14, 20, 38), a Gram-negative bacterium and the causative agent of the severe diarrheal disease cholera. We discovered that apart from the canonical *relA* and *spoT* genes, this pathogen also possesses a novel (p)ppGpp synthetase gene, *relV* (14), and thus (p)ppGpp metabolism is quite complex in this organism. However, very little information is currently available about the function of *V. cholerae* DksA ($DksA_{Vc}$). This study aims to explore further the role of $DksA_{Vc}$ in the stringent response. Since DksA in other enteric pathogens has been reported to be involved in regulation of pathogenicity, we wanted to check this possibility in the case of *V. cholerae*. Regulation of virulence genes in *V. cholerae* is quite complex, and several positive and negative regulators are involved in the process. Among these regulators, HapR, the master regulator of QS, plays a crucial role since virulence gene expression in this pathogen is QS dependent (56). At low cell density (LCD), when the cellular HapR level is low, expression of major virulence determinants, such as cholera toxin (CT)-, toxin-coregulated pilus-, and biofilm formation-related genes, is upregulated. Furthermore, at this condition, the cellular level of cyclic diguanylic acid (c-di-GMP), the newly identified second messenger, also remains high (19, 53). In contrast, at high cell density (HCD), the intracellular HapR concentration is increased, leading to repression of the above-described processes and upregulation of expression of the hemagglutinin protease gene *hapA*, which codes for the major protease HAP of *V. cholerae*. Several reports indicate that HAP is most likely involved in *V. cholerae*'s pathogenesis program (7, 16, 35, 47), including its role in detaching adhered *V. cholerae* cells on intestinal epithelial cell surfaces.

To study the function of $DksA_{Vc}$, the *dksA* gene locus (J. Craig Venter Institute annotation no. VC0596) was identified bioinformatically using the genome sequence information of the *V. cholerae* O1 El Tor strain N16961 (21). The gene was cloned and manipulated further to construct chromosomally deleted nonpolar $\Delta dksA_{Vc}$ strains. As with the *E. coli* $\Delta dksA$ mutant ($\Delta dksA_{Ec}$), $\Delta dksA_{Vc}$ mutant cells exhibited poor growth in M9 minimal (M9M) medium and sensitivity toward 3-amino-1,2,4-triazole (AT). However, unlike the case with *E. coli*, the $\Delta dksA_{Vc}$ mutant showed growth in serine-, methionine-, glycine-, and leucine (SMGL)-containing M9M medium. Furthermore, the $\Delta dksA_{Vc}$ mutant gave the following virulence-related phenotypes compared to its wild-type (Wt) strain: (i) decreased HAP production, (ii) decreased motility, and (iii) poor production of CT under *in vitro* conditions. The results clearly indicate that along with the stringent response, several other genes of *V. cholerae*, involved in pathogenicity, dissemination, and persistence in the environment, are also controlled by the circuit of the $DksA_{Vc}$ regulome.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Details of recombinant plasmid and strain constructions are provided in Supplement S1 in the supplemental material. Both *E. coli* and *V. cholerae* cells were routinely grown in Luria broth (LB) (Difco) at 37°C with shaking essentially as described previously (20). For plate culture, LB was used with 1.5% agar (Difco). Antibiotics (all from Sigma-Aldrich) were used at the following concentrations unless otherwise indicated: ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; kanamycin, 40 μg/ml; spectinomycin, 50 μg/ml; tetracycline, 10 μg/ml for *E. coli* and 1 μg/ml for *V. cholerae*. In some experiments, bacterial cells were grown in M9M medium (Sigma-Aldrich) containing 0.4% glucose as a carbon source (13). The Wt *E. coli* strain CF1648 (MG1655) (Table 1) has a frameshift mutation in the RNase PH-coding gene, leading to a weak requirement of uracil for its growth in M9M (23). It has been reported that the situation was further aggravated after deletion of the *dksA* gene of MG1655 (strain CF9240) (Table 1), which is then unable to grow in M9M without uracil (9). Therefore, we added 20 μg/ml of uracil (SRL Pvt. Ltd., India) as a supplement in M9M agar plates for growing the $\Delta dksA_{Ec}$ strain. Bacterial strains were maintained at −70°C in LB containing 20% sterile glycerol. To avoid development of any suppressor, all the mutant strains were minimally subcultured, and before any experiment they were directly inoculated from −70°C stock. The growth of bacterial culture was monitored spectrophotometrically by measuring the optical density at 600 nm (OD_{600}). The growth kinetic experiments were repeated at least three to five times, and their average values were plotted.

Molecular biological methods. Standard molecular biological methods (3) for chromosomal and plasmid DNA preparations, electroelution of DNA fragments, restriction enzyme digestion, DNA ligation, bacterial transformation, conjugation, agarose gel electrophoresis, etc., were followed unless stated otherwise. All restriction enzymes and nucleic acid-modifying enzymes were purchased from New England BioLabs, Inc., and were used essentially as directed by the manufacturer. Electrocompetent *V. cholerae* cells were prepared as described previously (13). Transformants were selected by plating transformed cells on LB agar plates containing appropriate antibiotics.

AT and SMGL tests. Sensitivity of bacterial strains toward the histidine analogue AT or in SMGL medium was examined essentially as described previously (13, 14). When needed, the amino acid L-histidine (Sigma-Aldrich) was added (4 μg/ml) to the AT medium.

Determination of intracellular (p)ppGpp by TLC. Intracellular accumulation of (p)ppGpp under amino acid or glucose starvation in various strains, including the $\Delta dksA_{Vc}$ mutant, was determined by the thin-layer chromatography (TLC) method essentially as described previously (13, 14, 20).

RT-PCR and qRT-PCR assays. For reverse transcriptase PCR (RT-PCR) and quantitative RT-PCR (qRT-PCR) assays, total cellular RNA was prepared from bacterial cells grown in LB to an OD_{600} of ~1 using TRIzol reagent (Invitrogen) as described by the vendor. The purity check and quantitation of the prepared RNA were done spectrophotometrically (3). A standard RT-PCR experiment was carried out using the Qiagen One Step RT-PCR kit as directed by the manufacturer (Qiagen, Germany). The PCR-amplified product was checked by agarose gel electrophoresis using appropriate DNA size markers. To confirm absence of any contaminating DNA in prepared RNA samples, PCR assay of each sample was also done with *Taq* DNA polymerase (Invitrogen). Lack of amplification in the absence of RT confirmed that the desired PCR product was generated only from cDNAs.

For qRT-PCR, cDNA was prepared from 1 μg of DNase I-treated RNA using SuperScriptIII RT (Invitrogen) essentially as described by the manufacturer. The qRT-PCR was done using either Power SYBR green PCR master mix (Applied Biosystems Inc.) or the One Step SYBR PrimeScript RT-PCR kit (TaKaRa, Japan) essentially as described by the manufacturer. The primer sets FliA-F/FliA-R and HapA-F/HapA-R (see Table S1 in the supplemental material) were used for qRT-PCR analysis. Relative expression values (R) were calculated using the equation $R = 2^{-(\Delta C_T \text{ target } - \text{ } C_T \text{ reference})}$, where C_T is the fractional threshold cycle. In each experiment, as an internal control, the *recA*-specific primers recA-F/

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype	Source or reference
V. cholerae		
N16961	Wild type, lacking *hapR* function, O1 serogroup, biotype El Tor, Sm^r	14
C6709	Wild type, *hapR*$^+$, O1 serogroup, biotype El Tor, Sm^r	20
N-DksA1	N16961 Δ*dksA*::*kan*; Km^r Sm^r	This study
C-DksA1	C6709 Δ*dksA*::*kan*; Km^r Sm^r	This study
NRVDK2	N-DksA1 Δ*relV*::*aadA1*; Km^r Sm^r Sp^r	This study
E. coli		
DH5α	F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* (ϕ80d*lacZ*ΔM15)	Promega
SM10λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir R6K*	20
S17-1λ*pir*	*thi proA hsdR recA*::RP4-2-Tc::Mu-1 *kan*::Tn*7* integrant λ*pir R6K*; Tp^r Sm^r	Lab stock
CF1648	Wild type MG1655	54
CF9240	CF1648 Δ*dksA*::*Tet*; Tt^r	9
Plasmids		
pDrive	pUC origin, high-copy-no. cloning vector; Ap^r Km^r	Qiagen
PBluescript II KS(+)	ColE1, high-copy-no. cloning vector; Ap^r	Stratagene
pKAS32	*rpsL* suicide vector with *ori*R6K *mob*RP4; Ap^r	Lab stock
pBR322	pMB1 origin, general-purpose cloning vector; Ap^r Tt^r	Lab stock
pUC4K	Source of kanamycin resistance gene cassette; Ap^r Km^r	Pharmacia
pBAD24	pBR322 origin, L-arabinose-inducible vector; Ap^r	Lab stock
pBS20	2.9-kb Δ*relV*::*aadA1* allele in pKAS32; Ap^r Sp^r	14
pJK537	2.3-kb *dksA* region of *E. coli* strain MG1655 in pBR322; Ap^r	9
pDDKW1	841-bp *dksA* region of *V. cholerae* strain N16961 containing *dksA* gene with its putative natural promoter cloned in pDrive; Ap^r Km^r	This study
pBSDA3.5	0.6-kb Δ*dksA* allele in pBluescript II KS(+); Ap^r Km^r	This study
pBSDA4.8	1.8-kb Δ*dksA*::*kan* allele in pBluescript II KS(+); Ap^r Km^r	This study
pKDK1	1.9-kb Δ*dksA*::*kan* allele in pKAS32; Ap^r Km^r	This study
$pDksA_{Vc}$	841- bp *dksA* region of *V. cholerae* strain N16961 from pDDKW1 subcloned pBR322; Ap^r	This study
$pDksA_{BAD}$	*V. cholerae dksA* ORF cloned in pBAD24; Ap^r	This study
$pFliA_{BAD}$	*V. cholerae fliA* ORF cloned in pBAD24; Ap^r	This study
$pRelV_{BAD}$	*V. cholerae relV* ORF cloned in pBAD24; Ap^r	This study

recA-R (see Table S1 in the supplemental material) were used. The experiments were repeated at least thrice using three different batches of prepared RNA.

Motility assay. Motility assay of *V. cholerae* strains was performed on LB soft-agar plates containing 0.3% agar (Difco) at 30°C as described previously (18), and a reading was taken after 8 to 10 h of incubation. Experiments were repeated at least thrice, and the average values were used.

HAP assays. HAP activity was studied by milk plate assay as described previously by Vance et al. (51). Each strain was examined thrice by milk plate assay, and an average was taken. HAP was also quantitated by azocasein assay as described previously (4). The amount of enzyme required for increasing 0.01 units in OD at 440 nm per hour was considered one azocasein unit.

GM_1-ELISA of CT. For detection of CT production by *V. cholerae* strains under *in vitro* conditions, the cells were grown in AKI medium (1.5% Bacto peptone [Difco], 0.4% yeast extract [Difco], 0.5% NaCl [Merck], and 0.3% sodium bicarbonate [Sigma-Aldrich]) essentially as described earlier by Iwanaga et al. (22). *V. cholerae* cells were initially grown statically in a test tube containing freshly prepared AKI medium at 37°C for 4 h, and then the culture was aseptically transferred to a sterile conical flask, followed by continuation of incubation overnight at 37°C with shaking (22). CT present in culture supernatant was assayed by GM_1-enzyme linked immunosorbent assay (ELISA) (29, 34) using pure CT (Sigma-Aldrich) and phosphate-buffered saline (10 mM, pH 7.2) as positive and negative controls, respectively. A standard curve of known CT concentrations was plotted and used to estimate the amount of CT present in each sample.

Assessment of CT production by rabbit ileal loop assay. *In vivo* CT production by *V. cholerae* strains, including mutants, was assayed by using the ligated rabbit ileal loop model essentially as described previously (15). Fluid accumulation (FA) in the ligated ileal loop was measured as the ratio of loop fluid volume to loop length and expressed as ml/cm, and an FA ratio of 1 or more than 1 was considered high production of CT under *in vivo* conditions. In all experiments, sterile 0.9% NaCl (normal saline) was used as a negative control, and as a positive control, Wt *V. cholerae* N16961 (Table 1) live culture was used. Each strain was tested at least thrice in three different animals. The experimental protocol used in this study was reviewed and approved by the institutional animal ethics committee of Indian Institute of Chemical Biology, Kolkata, India.

SEM. For microscopy, the *V. cholerae* sample was prepared as described previously (14, 45), and bacterial cells were examined using a scanning electron microscope (SEM) (model Vega II Lsu; Tescan, Czech Republic) at 10 kV. The images in the figures are representative of what was observed in 10 random fields in each of two independent experiments.

DNA sequencing. DNA sequencing reactions were carried out using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc.) essentially as recommended by the manufacturer. The samples were run on an ABI3130 genetic analyzer using the Pop-7 polymer (Applied Biosystems Inc.). Results were analyzed using the software DNA Sequencing Analysis V5.1 (Applied Biosystems Inc.).

Computational analyses. DNA sequence data were compiled and analyzed by using the DNASIS software program (Hitachi Corporation, Yokohama, Japan). The National Center for Biotechnology Information (NCBI) BLASTN program was used to search for homologous sequences

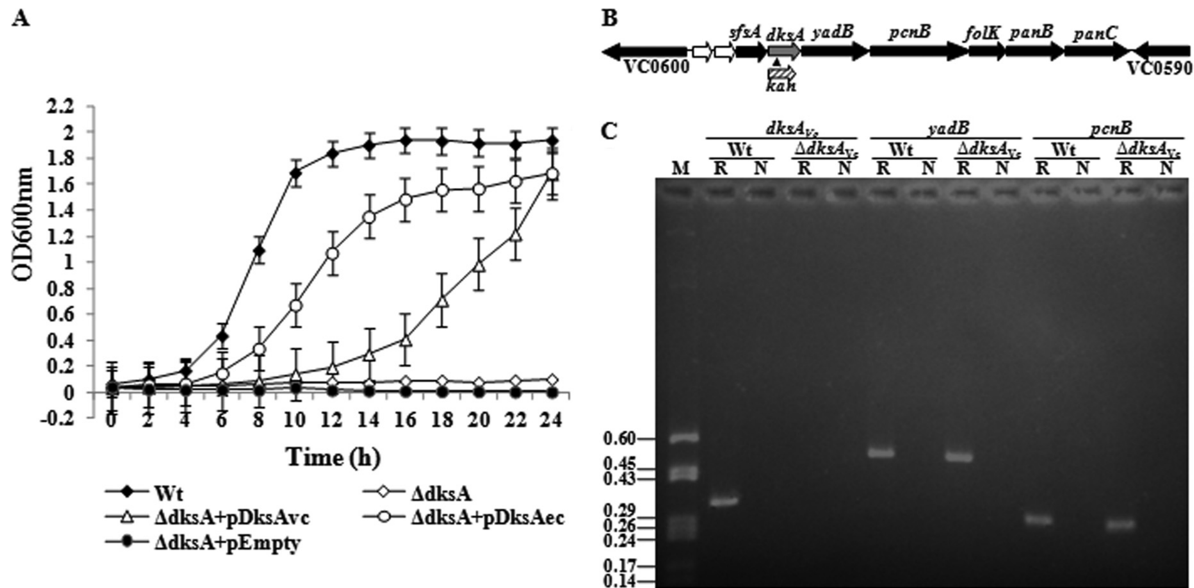

FIG 1 (A) Growth complementation of the $\Delta dksA_{Ec}$ strain with a functional $dksA_{Vc}$ gene in M9M medium. *E. coli* strains used are as follows: Wt, CF1648; ΔdksA, CF9240; ΔdksA+pDksAvc, CF9240(pDDKW1); ΔdksA+pDksAec, CF9240(pJK537); ΔdksA+pEmpty, CF9240(pDrive). Error bars indicate standard deviations. (B) Genomic arrangement of the *dksA* gene (gray arrow), including its flanking genetic determinants (VC0590 to VC0600), in *V. cholerae*. The direction of each arrow indicates the direction of transcription of a gene. VC0598 and VC0599 are two small hypothetical ORFs (white arrows). The insertion location of the kanamycin resistance gene (*kan*) cassette (small filled triangle) and its direction of transcription are also shown. (C) RT-PCR analysis to show that the deletion of $dksA_{Vc}$ did not hamper transcription of downstream genes. *V. cholerae* strains used are the Wt (C6709) and the $\Delta dksA_{Vc}$ mutant (C-DksA1). Lanes: M, pBluescript II KS(+) plasmid DNA digested with HaeIII, used as markers; sizes (in kb) of the DNA fragments are given in the left margin; R, RT with *Taq* DNA polymerase; N, only *Taq* DNA polymerase (used as a negative control).

in the database (www.ncbi.nlm.nih.gov). The open reading frames (ORFs) were subsequently subjected to a database search using the BLASTP program, version 2.2.15 (www.ncbi.nlm.nih.gov). For designing PCR and other primers, the Primer3 software program was used (http://frodo.wi.mit.edu/). Genomatix software (www.genomatix.de/cgi-bin/dialign/dialign.pl) was used for the alignment of protein sequences.

Statistical analysis. Where needed, pairwise comparison of data for each sample was analyzed for statistical significance using Student's *t* test.

RESULTS

Functional analysis of $dksA_{Vc}$. Bioinformatics analysis of the whole-genome-sequenced strain N16961 of *V. cholerae* (21) indicated that the large chromosome of the organism carries the $dksA_{Vc}$ gene with an open reading frame (ORF) of 447 bp (VC0596) having 66% identity with the sequence of the *E. coli dksA* gene ($dksA_{Ec}$). While $dksA_{Ec}$ codes for a 151-amino-acid-long DksA protein (here it will be designated $DksA_{Ec}$), $DksA_{Vc}$ is composed of 148 amino acids (41), with a calculated molecular mass of about 17.2 kDa (www.jcvi.org). BLASTP (blast.ncbi.nlm.nih.gov/blast.cgi) analysis of $DksA_{Vc}$ showed 77.8% identity and 84.7% similarity with $DksA_{Ec}$. For functional verification, the identified $dksA_{Vc}$ gene of the strain N16961 (Table 1) along with its natural promoter was cloned into the plasmid pBR322 or pDrive (Table 1), and the recombinant plasmid was designated $pDksA_{Vc}$ or pDDKW1 (Table 1), respectively. Brown et al. (9) previously reported that a $\Delta dksA_{Ec}$ strain is unable to grow in M9M medium, which is identical to the phenotype of an *E. coli* Δ*relA* Δ*spoT* mutant ($ppGpp^0$ strain). Introduction of the plasmid pDDKW1 into the $\Delta dksA_{Ec}$ strain CF9240 (Table 1) enabled growth of the strain in M9M salt solution, although at a lower rate than that of the Wt (Fig. 1A). On the other hand, CF9240 complemented with the $dksA_{Ec}$ gene through the plasmid pJK537 (Table 1) showed better growth in M9M medium than the $dksA_{Vc}$-complemented strain. However, after 24 h of incubation, both CF9240(pDDKW1) and CF9240(pJK537) reached to an OD_{600} value similar to that of the Wt strain, CF1648 (Fig. 1A). In sharp contrast, CF9240 carrying the empty vector failed to grow in M9M salt solution (Fig. 1A). We have also verified the growth of these strains in M9M agar plates after 18 to 24 h of incubation and found that only the Wt, $\Delta dksA_{Ec}$ strain carrying the plasmid pDDKW1, $pDksA_{Vc}$, or pJK537 could grow (data not shown). The complementation results are consistent with those reported earlier (9). Furthermore, the experimental results support that the promoter P_{dksA} of *V. cholerae* and the $DksA_{Vc}$ protein both are functional in *E. coli*.

To define the functions of $DksA_{Vc}$ in more detail, in-frame *dksA* deletion mutants of N16961 (lacking *hapR* function) and C6709 ($hapR^+$) were constructed by the positive selection method using the kanamycin resistance gene (*kan*) as a marker (see Supplement S1 in the supplemental material), and the $\Delta dksA_{Vc}$ mutants thus constructed were designated N-DksA1 and C-DksA1 (Table 1), respectively. Since the two mutants showed almost similar phenotypes, here they will be collectively called the $\Delta dksA_{Vc}$ mutants unless mentioned otherwise. Brown et al. suggested that $dksA_{Ec}$ could be present in an operon with the flanking genes *sfsA* and *yadB* (9). Preliminary examination of the $dksA_{Vc}$ locus (VC0596) appears to be organized in a similar fashion, where VC0597 and VC0595 are the *sfsA* and *yadB* genes, respectively (Fig. 1B). However, BioCyc analysis (http://biocyc.org/vcho/new-image?type=gene&object=vc0596) of the locus predicted that the gene $dksA_{Vc}$ could alone be a single transcriptional unit. Therefore, to confirm that deletion of the $dksA_{Vc}$ gene in strain C-DksA1 had no polar effect, we examined the transcript levels of two physically linked genes, *yadB* (VC0595) and *pcnB* (VC0594), present

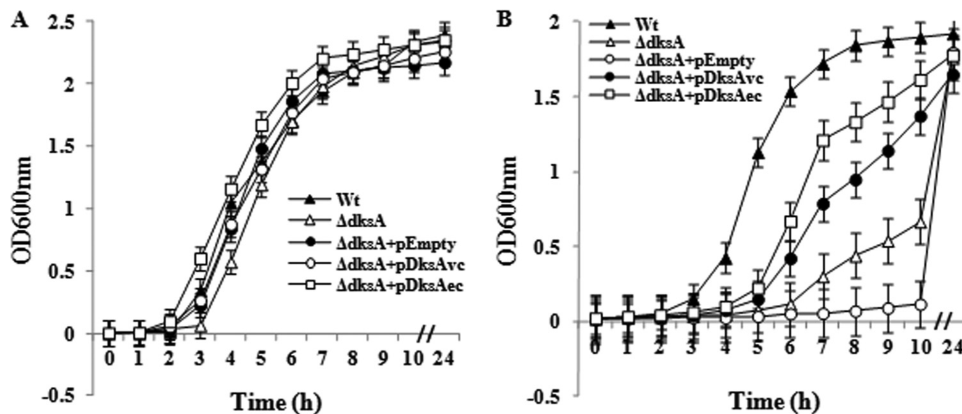

FIG 2 (A) *V. cholerae* strains, including the $dksA_{Vc}$ mutant, showed no growth defect in LB (nutrient-rich medium). Strains used are as follows: Wt, N16961; ΔdksA, N-DksA1; ΔdksA+pEmpty, N-DksA1(pDrive); ΔdksA+pDksAvc, N-DksA1(pDDKW1); ΔdksA+pDksAec, N-DksA1(pJK537). (B) Growth phenotypes of *V. cholerae* cells in M9M medium. Strains are as indicated for panel A. Error bars indicate standard deviations.

downstream of $dksA_{Vc}$ (VC0596) by employing the RT-PCR method using the specific primer sets VCO595-F/VC0595-R and PcnB-F/PcnB-R (see Table S1), respectively. While in both cases the desired cDNA of sizes 0.5 and 0.3 kb of *yadB* and *pcnB*, respectively, was generated, no cDNA was detectable for the $dksA_{Vc}$ gene using the specific primers Dksint-F/Dksrt-R (see Table S1), as shown in Fig. 1C. Similar results were obtained in the case of the N-DksA1 mutant strain (data not shown), indicating a similar arrangement of the locus in both *V. cholerae* strains used. The results confirmed the authenticity of deletion of the $dksA_{Vc}$ gene, and such deletion most likely had no polar effects on its downstream genes.

Initially, we checked cellular levels of (p)ppGpp in the $\Delta dksA_{Vc}$ mutant under amino acid- or glucose-starved conditions using the TLC method and found no significant change in the concentration of (p)ppGpp compared to that for the Wt (data not shown). This result is consistent with the report of Brown et al. (9), who found similar levels of (p)ppGpp in the $\Delta dksA_{Ec}$ and Wt strains. To analyze other phenotypes of the $\Delta dksA_{Vc}$ strain, we first compared its growth in nutritionally rich (LB) and poor (M9M) media. The $\Delta dksA_{Ec}$ mutant is unable to grow in M9M medium (Fig. 1A), but it grows as does the Wt in LB (9; this study). Although the $\Delta dksA_{Vc}$ mutant showed no growth defect in LB (Fig. 2A), there was significant growth retardation in M9M medium for ~5 h compared to growth of the Wt (Fig. 2B). The growth defect of the $\Delta dksA_{Vc}$ strain in M9M medium was partially corrected by expressing the $DksA_{Vc}$ or $DksA_{Ec}$ protein in *trans* through the plasmid pDDKW1 or pJK537 (Table 1), respectively, but not by the empty vector (Fig. 2B). To rule out the possibility that growth of the $\Delta dksA_{Vc}$ strain and the $\Delta dksA_{Vc}$ strain carrying the empty vector after overnight incubation was due to development of any suppressor, each overnight-grown culture was reinoculated separately into fresh M9M medium and their growth was monitored spectrophotometrically. Both of them showed growth patterns with ~5 h of an extended lag period, suggesting that they are indeed the $\Delta dksA_{Vc}$ mutant and not a suppressor (data not shown). The results support the view that the growth defect is due to a lack of the $DksA_{Vc}$ protein and not to the polar effect of deleting the gene. This further suggests that $DksA_{Ec}$ is functional in *V. cholerae*. We have already provided evidence that $DksA_{Vc}$ is functional in *E. coli*, and thus, functions of the DksA protein in this respect appear to be conserved between the two species.

It has been reported that $DksA_{Ec}$ is crucial for the function of (p)ppGpp and it acts as a cofactor by binding with the secondary channel of RNA polymerase, leading to positive regulation of amino acid biosynthesis operons (40). When $\Delta dksA_{Vc}$ cells were grown in M9M medium supplemented with all the amino acids, the mutant showed a growth pattern similar to that of the Wt (data not shown), which supports that a lack of $DksA_{Vc}$ probably leads to downregulation of amino acid biosynthesis operons even when the cells are $RelA^+$ $SpoT^+$, i.e., cells are $(p)ppGpp^+$. It is interesting to note that the $\Delta dksA_{Ec}$ strain CF9240 failed to grow in M9M medium even after overnight incubation at 37°C (Fig. 1A). In sharp contrast, the $\Delta dksA_{Vc}$ strain showed initiation of growth in M9M medium after ~5 h of incubation at a similar temperature and reached saturation ($OD_{600} > 1.8$) after overnight (16 h) incubation (Fig. 2B). Similar results were obtained when the growth phenotype of the $\Delta dksA_{Vc}$ mutant was compared with that of the $\Delta dksA_{Ec}$ strain along with appropriate control strains using an M9M agar plate assay (Fig. 3). Similarly, we also checked the growth sensitivity of $\Delta dksA_{Vc}$ cells toward AT and SMGL. The principal effect of AT (a histidine analog) is blockage of biosynthesis of the amino acid histidine (44). It has been demonstrated that the $\Delta dksA_{Ec}$ strain is unable to grow on AT medium (9). Although AT sensitivity could be overcome by an adequate amount of (p)ppGpp synthesis (54), as in the case of the Wt, it should be noted that the $\Delta dksA_{Ec}$ strain is a $(p)ppGpp^+$ strain and still failed to grow in AT medium. In SMGL agar plates the growth of a $(p)ppGpp^0$ strain is inhibited due to increased intracellular levels of methylenetetrahydrofolate (44, 50). This inhibitory effect of methylenetetrahydrofolate could also be overcome by optimal cellular levels of (p)ppGpp. According to Paul et al. (40), DksA and (p)ppGpp act synergistically to regulate the transcription of various amino acid biosynthetic pathway genes during the stringent response. Therefore, the $\Delta dksA_{Ec}$ strain should be sensitive to AT and SMGL. When these assays were performed with appropriate controls, interestingly, $\Delta dksA_{Vc}$ cells were able to grow in SMGL-containing (Fig. 3) but not in AT-containing (Fig. 4) medium. This difference in growth phenotypes between the $\Delta dksA_{Ec}$ and $\Delta dksA_{Vc}$ strains in M9M and SMGL media could be explained

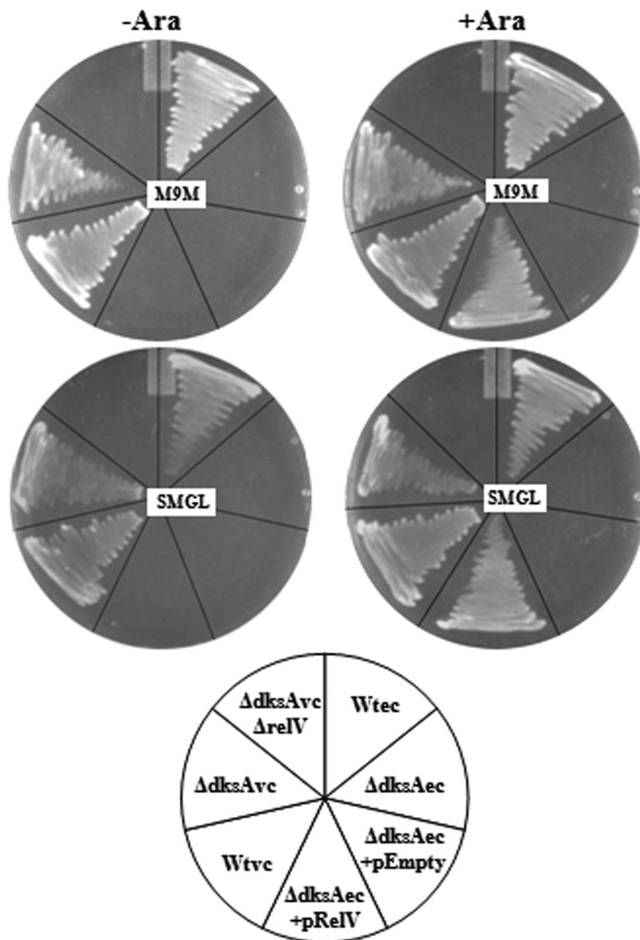

FIG 3 The functional *relV* gene confers growth on M9M agar and SMGL media in the Δ*dksA* background. Growth of different *V. cholerae* (vc) and *E. coli* (ec) strains on M9M and SMGL media without (−) or with (+) 0.01% L-arabinose (Ara) after 24 h is shown. Strains used are as follows: Wtvc, N16961; ΔdksAvc, N-DksA1; ΔdksAvcΔrelV, NRVDK2; Wtec, CF1648; ΔdksAec, CF9240; ΔdksAec+pEmpty, CF9240(pBAD24); ΔdksAec+pRelV, CF9240(pRelV$_{BAD}$).

as follows. It has recently been shown that the *V. cholerae* genome carries a novel (p)ppGpp synthetase gene, *relV*, apart from the canonical *relA* and *spoT* genes (14). We hypothesize that this could be due to the presence of the *relV* gene in *V. cholerae*, which probably helped Δ*dksA*$_{Vc}$ cells to overcome growth defects in M9M/SMGL medium through optimal production of (p)ppGpp. In favor of this hypothesis, one piece of indirect evidence is that *E. coli* lacks a *relV*-like gene and thus accumulation of excess (p)ppGpp is not possible although the cells are RelA$^+$ and SpoT$^+$, and probably for this reason, the Δ*dksA*$_{Ec}$ strain failed to grow in SMGL/M9M medium. If this is the case, then a *V. cholerae* Δ*dksA* Δ*relV* double mutant, like the Δ*dksA*$_{Ec}$ strain, will not be able to grow in SMGL/M9M medium. Therefore, we constructed a *V. cholerae* Δ*dksA* Δ*relV* double mutant strain, NRVDK2 (Table 1; see also Supplement S1 in the supplemental material), which, as hypothesized, failed to grow in M9M and SMGL (Fig. 3) media. It is to be noted that like the Δ*dksA*$_{Vc}$ mutant, NRVDK2 was unable to grow in AT medium (data not shown), and in this respect it behaved just like the Δ*dksA*$_{Ec}$ strain. It may be argued that the supplying of functional *relV* in Δ*dksA*$_{Ec}$ cells in *trans* may allow the strain to behave like a Δ*dksA*$_{Vc}$ strain. When the Δ*dksA*$_{Ec}$ strain CF9240 was transformed with the plasmid pRelV$_{BAD}$ (Table 1) carrying the *relV* ORF under the arabinose-inducible promoter (P$_{BAD}$), as rationalized, the strain CF9240(pRelV$_{BAD}$) showed growth on M9M and SMGL agar media, while CF9240 carrying the empty vector pBAD24 failed to grow (Fig. 3). On the other hand, AT sensitivity of the Δ*dksA*$_{Vc}$ mutant [a (p)ppGpp$^+$ strain] could be due to the lack of the DksA$_{Vc}$ protein, which seems to be essential for upregulation of the *his* operon of *V. cholerae*. Therefore, we thought that supplementation of histidine in AT medium should rescue the Δ*dksA*$_{Vc}$ mutant from histidine auxotrophy. In fact, the Δ*dksA*$_{Vc}$ mutant showed growth in AT agar medium containing the amino acid L-histidine, as shown in Fig. 4. This is also true for *E. coli*, since the Δ*dksA*$_{Ec}$ strain showed growth in an L-histidine-containing AT agar plate (Fig. 4).

DksA$_{Vc}$ is required for optimal production of HAP. After establishing the function of DksA$_{Vc}$ in stringent-response-related phenotypes, we wished to study further its role, if any, in virulence-related phenotypes. We found that the *dksA*$_{Vc}$-deleted strain C-DksA1 produced a 3-fold smaller amount of HAP after 24 h of growth than its parent HapR$^+$ HapA$^+$ DksA$^+$ Wt strain, C6709 (Fig. 5A). Furthermore, the qRT-PCR assay with the *hapA* gene-specific primers HapA-F/HapA-R (see Table S1 in the supplemental material) revealed a ~5-fold decrease in the *hapA* transcript level in the C-DksA1 mutant with respect to that for the Wt strain, C6709 (Fig. 5B). The result was also consistent with the direct measurement of HAP in culture supernatant by azocasein assay (Fig. 5A). It is well established that HAP is produced at stationary phase under HCD conditions (4). We found that HAP in the culture supernatant of the Wt strain is detectable after 6 h of growth

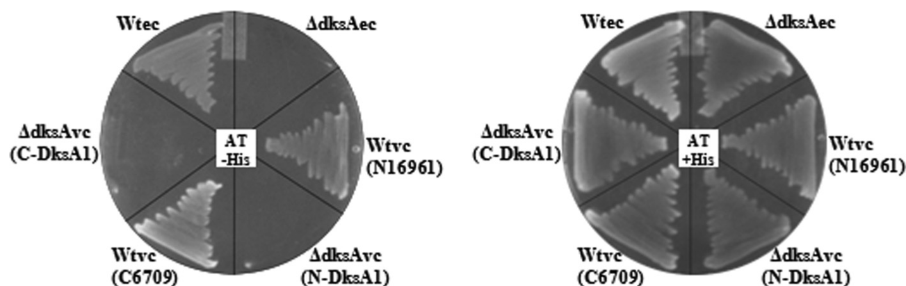

FIG 4 DksA is essential to overcome histidine auxotrophy caused by AT. Growth of different *V. cholerae* (vc) and *E. coli* (ec) strains in AT medium without (−) and with (+) 4 μg/ml L-histidine (His) after 24 h. Strains used are as follows: Wtec, CF1648; ΔdksAec, CF9240; Wtvc, N16961 or C6709; ΔdksAvc, N-DksA1 or C-DksA1.

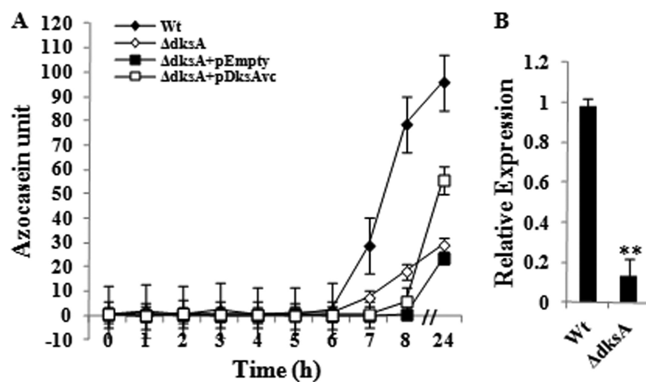

FIG 5 $DksA_{Vc}$ modulates HAP production in *V. cholerae*. (A) Kinetics of HAP production in various *V. cholerae* strains derived from the parental strain C6709. Error bars indicate standard deviations. Strains used are as follows: Wt, C6709; ΔdksA, C-DksA1; ΔdksA+pEmpty, C-DksA1(pBR322); Δ*dksA*+pDksAvc, C-DksA1($pDksA_{Vc}$). (B) Quantitative measurement of *hapA* transcript of *V. cholerae.* Relative expression of *hapA* in the Δ*dksA* strain (C-DksA1) with respect to that in the Wt (C6709) was measured by qRT-PCR. Error bars indicate standard deviations. Significant differences (**, $P < 0.01$) in expression are indicated from multiple comparison of each mutant versus the Wt.

and reached its maximum concentration in overnight stationary culture, as shown in Fig. 5A. However, when the C-DksA1 (Δ$dksA_{Vc}$) mutant strain was similarly tested, it showed a distinct shift in the timing of HAP production (from 8 h of growth), and the amount produced was also substantially less in a saturated culture than with the Wt strain, C6709 (Fig. 5A). Expression of $DksA_{Vc}$ in *trans* through the plasmid $pDksA_{Vc}$ in the C-DksA1 strain partially complemented the mutant phenotype and thus provided further evidence that the downregulation of HAP production in C-DksA1 was probably due to the lack of the $DksA_{Vc}$ protein (Fig. 5A). This is further supported by the fact that C-DksA1 carrying the empty vector pBR322 failed to complement (Fig. 5A).

$DksA_{Vc}$ positively regulates motility. Recently Åberg et al. (1) reported that *E. coli* cells deficient in $DksA_{Ec}$ are hyperflagellated, leading to hypermotility, and this observation was further supported by the work of Lemke et al. (27) in analyzing transcription of the flagellar biosynthesis genetic cascade. Österberg et al. (37) reported that *dksA* deletion leads to a decrease in motility in another Gram-negative bacterium, *Pseudomonas putida*. In this study, unlike the case with *E. coli*, the Δ$dksA_{Vc}$ strain showed about a 30% reduction in motility in a soft-agar plate assay, considering the motility of a Wt strain as 100% (Fig. 6A). Since the decreased motility of the Δ$dksA_{Vc}$ strain was complemented by expressing $DksA_{Vc}$ in *trans* through the plasmid $pDksA_{Vc}$ (Fig. 6A), it may be concluded that $DksA_{Vc}$ is involved in regulation of motility of *V. cholerae*.

Motility-related flagellar gene expression in *V. cholerae* is highly complex, and there are four distinct levels (class I to IV) of the gene regulation cascade (43). As with the QS pathway, several motility genes are transcribed with the help of sigma factor RpoN, or σ^{54} (25, 43). The master regulatory gene (class I category) is *flrA*, the product of which in the presence of σ^{54} RNA polymerase holoenzyme activates several class II genes, including *flrC* and *fliA* (which codes for sigma factor 28, or σ^{28}). FlrC, with the help of the σ^{54} holoenzyme, promotes expression of the class III genes, including the flagellin gene *flaA*. Finally, the σ^{28} RNA polymerase holoenzyme promotes expression of the class IV genes, including the flagellar motor component genes *motABY* (43). It is noteworthy that unlike the *V. cholerae* Δ*rpoN* and Δ*fliA* mutants (both of which are nonmotile), the Δ$dksA_{Vc}$ mutant strain showed motility (decreased from that of the Wt) as revealed by a soft agar assay (Fig. 6A). Furthermore, SEM analysis of the Wt and the Δ$dksA_{Vc}$ mutant showed the presence of a single polar flagellum in more than 90% of cells (Fig. 6B), suggesting normal flagellation of both the strains. Interestingly, SEM analysis revealed distinct elongated morphology of Δ$dksA_{Vc}$ cells compared to that of the Wt (Fig. 6B). Since Δ$dksA_{Vc}$ cells were hypomotile with their intact flagella, this suggested that flagellar motor gene functions are most probably affected, instead of functions of genes related to flagellar synthesis. As mentioned above, expression of the flagellar motor component genes *motABY* in *V. cholerae* is controlled by the sigma factor *fliA*, or σ^{28} (43). In other bacteria, DksA has been shown to be involved in regulation of expression of σ^{28} (12, 27). Therefore, we hypothesize that $DksA_{Vc}$ may carry out a similar function. To examine this, we performed qRT-PCR experiments by using total cellular RNA of the Δ$dksA_{Vc}$ mutant along with the Wt as a control, and the result indicated about a 2-fold downregulation of expression of the σ^{28} gene in the Δ$dksA_{Vc}$ genetic background (Fig. 6C). Thus, it seems that $DksA_{Vc}$ is most likely needed for optimal expression of σ^{28} of *V. cholerae*. To further confirm, the *fliA* ORF (21) of *V. cholerae* was cloned under an arabinose-inducible promoter, generating the plasmid $pFliA_{BAD}$ (Table 1) (see Supplement S1 in the supplemental material) and introduced into the Δ$dksA_{Vc}$ strain. Controlled expression of FliA (σ^{28}) through the plasmid $pFliA_{BAD}$ partially complemented the motility defect of the Δ$dksA_{Vc}$ strain, while the empty vector pBAD24 or $pFliA_{BAD}$ without arabinose induction failed to complement (Fig. 6D). Thus, it appears that $DksA_{Vc}$ positively regulates σ^{28} for its optimal expression.

$DksA_{Vc}$ modulates CT production under *in vitro* conditions. Since $DksA_{Vc}$ appears to be involved in modulation of expression of HAP as well as regulation of motility, both of which are pathogenicity-related phenomena, we checked the status of production of CT, the principal virulence factor of *V. cholerae*, in Δ$dksA_{Vc}$ cells. To do this, both Δ$dksA_{Vc}$ and its isogenic Wt strain were grown in CT-inducing AKI medium (for details, see Materials and Methods), and the amount of CT produced was measured by GM1-ELISA. Interestingly, the Δ$dksA_{Vc}$ strain produced significantly less CT than the Wt (Fig. 7). Since this observation is true for both the $dksA_{Vc}$ mutants derived from C6709 (Fig. 7) and N16961 (data not shown), it appears that $DksA_{Vc}$ most likely has a role in CT production under *in vitro* conditions, and this is most probably not strain specific. When the $DksA_{Vc}$ protein was expressed in the Δ$dksA_{Vc}$ strain C-DksA1 through the plasmid $pDksA_{BAD}$ (Table 1) using 0.1% L-arabinose as an inducer, it complemented CT production in the mutant (Fig. 7). A similar result was obtained when N-DksA1($pDksA_{BAD}$) was examined (data not shown). We also wished to know whether the *in vitro* defect in CT production by Δ$dksA_{Vc}$ mutant cells is also true in an *in vivo* situation. Therefore, the strains were tested for CT production in rabbit ileal loops. However, the Δ$dksA_{Vc}$ mutant and the Wt showed similar FA ratios (for details, see Materials and Methods) of about 1.2 ml/cm, indicating *in vivo* induction of an unknown factor(s), which could easily overcome the lack of $DksA_{Vc}$. These results support the view that the signaling cascade in CT production by *V. cholerae* differs under *in vitro* and *in vivo* conditions (26).

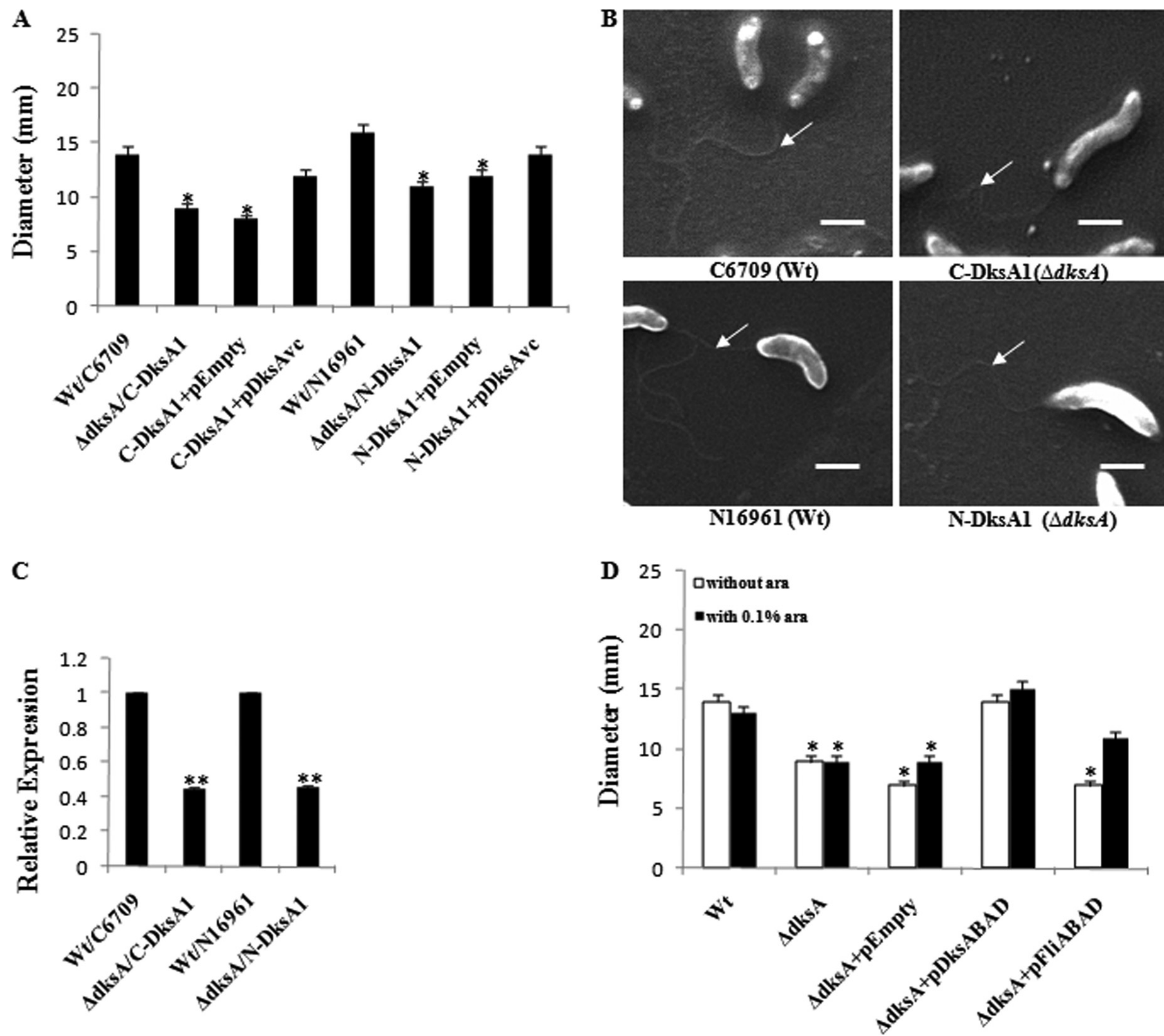

FIG 6 $DksA_{Vc}$ is involved in regulation of motility of *V. cholerae*. (A) Motility assay of *V. cholerae* strains was carried out on LB soft agar plates. Strains used are as indicated. Error bars indicate standard deviations. Significant differences (*, $P < 0.05$) in motility are indicated from multiple comparison of each mutant with Wt strains. (B) SEM analysis of *V. cholerae* cells. Strains used are as indicated. White arrows indicate flagella. Bars correspond to 1 μm. (C) Relative expression of the *fliA* gene of *V. cholerae*, determined by qRT-PCR assay. Significant differences (**, $P < 0.01$) in *fliA* transcript levels between mutant and Wt strains are indicated. Error bars indicate standard deviations. (D) Complementation of motility defect of Δ*dksA* mutant strain C-DksA1. Strains are as indicated. Significant differences (*, $P < 0.05$) in motility are indicated from multiple comparisons of mutant, gene-complementing plasmid, or empty vector (pBAD24) strains with the Wt strain, C6709.

DISCUSSION

In the present study, we have for the first time functionally characterized the stringent-response-related *dksA* gene of the cholera pathogen *V. cholerae*. Our experimental results suggest that $DksA_{Vc}$ is indeed involved in the stringent response in conjunction with (p)ppGpp. We showed that the $DksA_{Ec}$ and $DksA_{Vc}$ proteins are functionally similar and both of them are active in homologous and heterologous genetic backgrounds (Fig. 1 and 2). It has previously been reported from this laboratory that unlike the case with *E. coli*, the intracellular level of (p)ppGpp in *V. cholerae* is governed by three enzymes, RelA, SpoT, and RelV (14), and we have provided evidence that RelV through its (p)ppGpp synthetase activity indeed helps the $\Delta dksA_{Vc}$ mutant to grow in M9M salt solution or agar plate (Fig. 2 and 3) after a certain period of lag time (~5 h). This is also true in the case of SMGL agar medium (Fig. 3), which is not possible for the $\Delta dksA_{Ec}$ mutant since it naturally lacks the *relV* gene. This is further supported by expressing the *relV* gene in the $\Delta dksA_{Ec}$ strain, which rescued the growth defect of the mutant in M9M and SMGL media, and the strain behaved like the $\Delta dksA_{Vc}$ mutant (Fig. 3). It should further be noted that although DksA has been proposed as an essential cofactor for the action of (p)ppGpp, our experimental results suggest that most probably the cellular concentration of (p)ppGpp is crucial for bacterial growth, which might work in a DksA-independent manner. Alternatively, the RelV protein itself may be involved in the process, which needs further investigation. We found that the $\Delta dksA_{Vc}$ mutant failed to grow in AT medium (Fig. 4). AT is a histidine analog and blocks protein synthesis by inhibiting histidine biosynthesis. It appears that $DksA_{Vc}$ is essential to overcome this inhibitory effect of AT in *V. cholerae*. In fact, this may be the case, since Paul

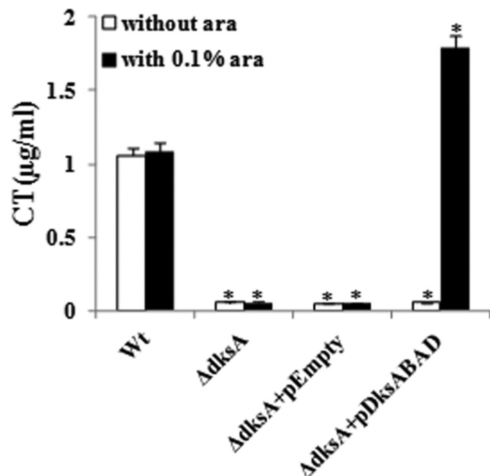

FIG 7 Deletion of $dksA_{Vc}$ affects CT production. Strains used are as follows: Wt, C6709; ΔdksA, C-DksA1; ΔdksA+pEmpty, C-DksA1(pBAD24); ΔdksA+pDksABAD, C-DksA1(pDksA_{BAD}). Error bars indicate standard deviations. Significant differences (*, $P < 0.05$) in CT production are indicated from multiple comparisons of each mutant with the Wt strain, C6709.

et al. (40) have shown that $DksA_{Ec}$ is absolutely needed for upregulation of histidine biosynthesis in *E. coli*.

Apart from regulation of the stringent response, our experimental results also suggest for the first time that $DksA_{Vc}$ is likely to be involved in fine regulation of important virulence-related phenotypes in clinical *V. cholerae* strains. We found that $DksA_{Vc}$ positively regulates HAP production (Fig. 5). In support of our study, it may be mentioned here that mutation in the *dksA* gene of *P. aeruginosa*, a Gram-negative organism, also led to significant downregulation of expression of the secreted elastase enzyme LasB (24), a highly homologous protein of HAP. Our results also suggest that $DksA_{Vc}$ positively controls expression of the critical motility regulator σ^{28} of *V. cholerae*, and this could be one of the reasons for the decreased-motility phenotype showed by the $DksA_{Vc}$-negative strain (Fig. 6). Dalebroux et al. (12) have recently reported that for flagellar morphogenesis in the Gram-negative human pathogen *Legionella pneumophila*, DksA is required for basal σ^{28} promoter activity. Furthermore, it has also been shown that deletion of the *dksA* gene of *P. putida* leads to a significant motility defect (37). Although our SEM analysis indicated normal flagellation (Fig. 6), we believe that downregulation of σ^{28} expression in the $\Delta dksA_{Vc}$ strain possibly affected the flagellar motor functions, which are under the control of σ^{28} (43). In sharp contrast, recently $DksA_{Ec}$ has been shown to be a negative regulator of σ^{28} expression in *E. coli* (1, 27). Although the basis of these opposite activities of DksA in *V. cholerae* and *E. coli* is currently unknown, it could be due to a difference in their lifestyle.

Our study indicates that CT production under *in vitro* conditions is positively regulated by $DksA_{Vc}$ (Fig. 7). CT production by *V. cholerae* cells is a highly regulated process and is QS dependent (56). At LCD, when cellular levels of the QS master regulator HapR are low, AphA, a positive transcriptional regulator of virulence gene expression in *V. cholerae*, is derepressed, leading to a series of reactions which ultimately allow *V. cholerae* cells to express the virulence master regulator ToxT followed by production of the principal virulence factor CT. It should also be noted that the cellular level of the newly established second messenger c-di-GMP is critical for biofilm formation, virulence factor production, and motility in *V. cholerae* (5, 42, 48, 53). c-di-GMP is synthesized in bacteria by the action of the GGDEF domain, containing the diguanylate cyclase enzyme, on two molecules of GTP and degraded by the EAL/HD-GYP domain, containing phosphodiesterase enzymes (17). It should be noted that the *V. cholerae* genome codes for a large numbers of GGDEF/EAL domain-containing proteins (6, 17), and thus, maintenance of the cellular level of c-di-GMP appears to be controlled by a complex regulatory network about which our current knowledge is limited. A high cellular level of c-di-GMP negatively regulates CT production and motility (5, 42, 48), the phenotypes of the $\Delta dksA_{Vc}$ mutant observed in this study. In a recent study, the authors have shown that c-di-GMP regulates the production of HAP in a negative manner, because an artificial increase in the cellular c-di-GMP pool through overexpression of a diguanylate cyclase caused poor expression of HAP (52). Thus, it cannot be ruled out that $DksA_{Vc}$ possibly acts as a direct/indirect negative regulator in critically maintaining the intracellular c-di-GMP pool in *V. cholerae*, which needs to be addressed. Our preliminary experimental results (R.R. Pal, S. Bag, S. Dasgupta, and R.K. Bhadra, unpublished observation) suggest that overexpression of a c-di-GMP-degrading phosphodiesterase (VCA0681) in the $\Delta dksA_{Vc}$ mutant could rescue the motility defect.

One enigma of our finding is that CT production by the $\Delta dksA_{Vc}$ strain is significantly reduced only under *in vitro* conditions and not in the *in vivo* situation, where $DksA_{Vc}$ appears to not be required (see Results), suggesting possible host-specific activation of some unknown factor(s) in overcoming the deficiency of $DksA_{Vc}$. The result further indicates that our knowledge regarding signaling pathways under *in vitro* and *in vivo* conditions is extremely limited. It is plausible, however, that CT production may be needed by the pathogen when it is residing in a particular environmental niche for its protection/survival, and under that condition, $DksA_{Vc}$ may facilitate the process.

Last, it is noteworthy that the $\Delta dksA_{Vc}$ strains described in this study were not growth defective when cultivated in nutritionally rich media (Fig. 2), and our experiments conducted to assess the virulence-related phenotypes of the $\Delta dksA_{Vc}$ strains were always done in nutritionally rich medium. Therefore, we believe that phenotypes shown by the $\Delta dksA_{Vc}$ strains are most likely due to the lack of the $DksA_{Vc}$ protein and not to any growth defect of cells. Altogether, this study suggests that different global regulators, namely, $DksA_{Vc}$, FliA (σ^{28}), etc., exert positive and negative effects on various crucial genetic circuits involved in expression of virulence determinants, including motility of the cholera pathogen, and further investigations are needed for a clear understanding of how this is achieved.

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