DksA-HapR-RpoS axis regulates haemagglutinin protease production in *Vibrio cholerae*

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**Abstract**

DksA acts as a co-factor for the intracellular small signalling molecule ppGpp during the stringent response. We recently reported that the expression of the haemagglutinin protease (HAP), which is needed for shedding of the cholera pathogen *Vibrio cholerae* during the late phase of infection, is significantly downregulated in *V. cholerae* ΔdksA mutant (ΔdksA 

So far, it has been shown that HAP production by *V. cholerae* cells is critically regulated by HapR and also by RpoS. Here, we provide evidence that *V. cholerae* DksA (DksA 

We show that in ΔdksA 

Moreover, the expression of exponential phase regulatory protein Fis, a known negative regulator of HapR, was found to continue even during the stationary phase in ΔdksA 

Extensive reporter construct-based and quantitative reverse-transcriptase PCR (qRT-PCR) analyses supported that RpoS is distinctly downregulated at the post-transcriptional/translational levels in stationary phase-grown ΔdksA 

Since HAP expression through HapR and RpoS is stationary phase-specific in *V. cholerae*, it appears that DksA 

Moreover, experimental evidence provided in this study clearly supports that DksA 

**INTRODUCTION**

In order to survive and grow in any environment, microorganisms must regulate the expression of a wide variety of genes involving multiple genetic circuits. For pathogens, the situation is further complicated due to the presence of multiple virulence genes and their coordinated expression in a host environment. Since the evolution of bacterial pathogens is believed to be due to the acquisition of virulence genes/cassettes by innocuous progenitor cells, it is, therefore, highly expected that survival, growth and virulence should also be under critical regulations interlinking many ancestral gene regulatory networks. Inside the host, pathogens face a plethora of physicochemical challenges, including nutritional scarcity. Nutritional insufficiency is an important signal in any living organism and about which our knowledge is still limited. Nutritional deficiency in prokaryotes evokes the stringent response, a well-studied regulatory network involving several genes, and the situation is efficiently managed through the generation of two intracellular small molecules, guanosine 3’-diphosphate 5’-triphosphate (pppGpp) and guanosine 3’,5’-bis(diphosphate) (ppGpp), together called (p)ppGpp [1–4]. However, several lines of evidence suggest that (p)ppGpp alone is unable to control gene expression; instead, a 17.5 kDa small protein molecule, called DksA, is needed as a co-factor [5, 6]. Interestingly, like (p)ppGpp, DksA also binds with the secondary channel of RNA polymerase and thus it is an unusual transcription factor [5–8].

Apart from regulation of the stringent response, DksA has been shown to be needed for expression of several virulence genes in different bacterial pathogens [9–15]. DksA has been shown to be involved in the regulation of quorum sensing (QS) in the opportunistic human pathogen *Pseudomonas aeruginosa* [11, 16]. In *Vibrio cholerae*, QS is a highly regulated process [17]. At low cell density, the intracellular concentration of QS master regulator HapR is low, which allows cells to express major virulence determinants like...
cholera toxin (CT), toxin co-regulated pilus (TCP) and biofilm formation. At high cell density (HCD), when intracellular HapR concentration is high, CT, TCP and biofilm formation are downregulated and production of a major protease, called haemagglutinin protease (HAP), is upregulated [17–21]. HAP helps in detaching adhered V. cholerae cells from the intestinal epithelial cell surface and thus it is an important virulence factor of the pathogen [22–24]. Apart from HapR, the stationary phase sigma factor RpoS is also involved in the regulation of HAP production [25, 26]. Recent studies have shown that deletion of the dksA gene of V. cholerae (hereafter called dksA\textsubscript{Vc}) leads to fivelfold decrease of hapA transcripts compared to that of wild-type (WT) V. cholerae cells [15]. Similarly, the two-component system VarS/VarA and the carbon storage regulatory network involving CsrA/BCD constitute another pathway which is involved in the regulation of HapR in V. cholerae. The VarS/VarA-CsrA/BCD system controls QS in V. cholerae through a cascade of reactions where CsrA, with the help of activated LuxO protein, regulates the expression of four redundant sRNAs called quorum regulatory RNAs 1–4 (Qrr1–4). Qrr1-4 sRNAs bind with the hapR transcripts and block its translation [27]. Furthermore, Fis, a highly conserved exponential phase small nucleoid protein, positively regulates the expression of Qrr1-4 sRNAs in a LuxO-dependent manner and thus affect the stability of hapR transcripts in V. cholerae [28].

In this study, we tried to understand how DksA\textsubscript{Vc} modulates HAP production in V. cholerae. Extensive genetic analysis using V. cholerae ΔdksA\textsubscript{Vc} mutant cells indicates that DksA\textsubscript{Vc} positively regulates the expression of the QS master regulator HapR by modulation of the levels of the CsrB/C/D sRNAs and the global exponential phase regulator Fis. Furthermore, DksA\textsubscript{Vc} also appears to be a positive regulator of the stationary phase sigma factor RpoS, and thus DksA\textsubscript{Vc} is epistatic to HapR and RpoS and comprises a complex regulatory circuit in regulating the expression of HAP.

**METHODS**

**Bacterial strains, plasmids and growth conditions**

Details of the bacterial strains and plasmids used in this study are given in Table 1. Details of the plasmid and strain constructions are provided in the online Supplementary Material. Bacterial strains were routinely grown in LB medium to early log (OD\textsubscript{600} = 0.2), mid log (OD\textsubscript{600} = 0.4–0.6), early stationary (OD\textsubscript{600} = 1.0–1.5) and late stationary (OD\textsubscript{600} = 2.0) growth phases using TRI Reagent (Sigma-Aldrich, USA) essentially as described by the supplier. The purity check and quantification of the prepared RNA were done spectrophotometrically. qRT-PCR was done using the Power SYBR green RNA to C\textsubscript{T} One step kit (Life Technology, USA) essentially as described by the manufacturer and reported earlier [15, 26]. The primer sets hapRrtm-F/R, rpoStrtm-F/R, fisrtm-F/R (Table S1) were used for qRT-PCR analysis. Relative expression values (R) were calculated using the equation \( R = 2^{-\Delta\Delta C_{T}} \), where C\textsubscript{T} is the fractional threshold cycle. The recA gene was used as a reference to normalize the expression values of target genes [15, 26]. Expression of the recA gene was studied using the primers recA-F/recA-R (Table S1).

**Enzyme assays**

HAP production was quantified by azocasein assay as described previously [15]. One azocasein unit was considered as the amount of enzyme required for increasing 0.01 OD units at 440 nm per h. The β-galactosidase activity was measured essentially as described earlier [31]. Briefly, cells were grown to stationary phase and the OD\textsubscript{600} of bacterial culture was measured. As a substrate, O-nitrophenyl-β-D-galactopyranoside (ONPG) was used and, after the enzyme–substrate reaction, absorbance of the coloured solution was measured at OD\textsubscript{420} nm. The β-galactosidase activity was determined using the formula 1000(OD\textsubscript{420} t x x/OD\textsubscript{600} value), where t is the total time of the reaction after addition of ONPG until colour development and v is the total volume of the enzyme extract per reaction.

**DNA sequencing and computational analyses**

DNA sequencing was done using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, USA) essentially as instructed by the company. The samples were run on an ABI3130 Genetic Analyzer (Applied
DNA sequence data were compiled and analysed by using Chromas 1.45 (www.techneilsum.com.au/ chromas.html). DNA sequences were obtained from JCVI (www.jcvi.org) and protein domain information was obtained from KEGG (www.genome.jp/kegg/). BLASTN and BLASTP programs were used to search for homologous nucleotide and protein sequences, respectively, in the database (www.ncbi.nlm.nih.gov). For designing primers, Primer3 software (http://frodo.wi.mit.edu/) was used. For designing qRT-PCR primers, Primer Express 3.0 software (Applied Biosystems, USA) was used.

### Statistical analysis

Where needed, pair wise comparison of data for each sample was analysed for statistical significance using Student’s t-test.
RESULTS AND DISCUSSION

DksA\textsubscript{VC} epistatically modulates HapR for optimal HAP production

During the course of this study, a rugose colony of \(\Delta dksA\textsubscript{VC}\) strain C-DksA1 (Table 1) was isolated fortuitously during laboratory subculturing and was named CDK1 (Table 1). Since rugose variant develops in \(V.\) cholerae due to null mutation in the \(hapR\) gene [32], therefore, it is expected that the strain may carry a mutation in this gene. This strain showed negligible HAP production (data not shown). To confirm this, the entire 1.3 kb \(hapR\) gene along with its natural promoter region was PCR-amplified with the primers HapR-F1/HapR-R1 (Table S1) and sequenced. BLAST analysis of the sequence indicated that there is indeed a single nucleotide insertion at the nucleotide position 102, which caused a frameshift (fs) and thus led to the production of a 61 aa residue long truncated HapR protein rather than the functional 203 aa containing WT protein (data not shown). We also checked the \(\Delta dksA\textsubscript{VC}\) locus of the mutant and found intactness of its \(\Delta dksA\textsubscript{VC}:\text{kan}\) allele by PCR assay and sequencing (data not shown). Thus, the isolated strain apparently carried two mutant alleles in its genome and its genotype is \(\Delta dksA\textsubscript{VC}:hapR\) (Table 1). To further confirm that the strain only had these two mutations and did not carry any other mutation which could arise due to subculturing, we generated an \(hapR\) deletion mutant in the \(\Delta dksA\textsubscript{VC}\) strain C-DksA1 (available from the online Supplementary Material). This strain was named C-DksA1\textsubscript{hapR} (Table 1) and it also showed negligible HAP production. Since HapR is known to autoregulate its own expression, not only by downregulating its promoter but also by upregulating the expression of the \(qrr\) sRNA genes [33], \(hapR\) was introduced back into the chromosome in a single copy to avoid any non-physiological effects due to overexpression of HapR from a multicopy plasmid. Revertants were generated in which either \(hapR\) or \(dksA\textsubscript{VC}\) or both were reverted back into the chromosome in a single copy. The different revertants constructed in this study are shown in Fig. 1(a). The revertants derived from C-DksA1\textsubscript{hapR}, i.e. CDK1-H1 (\(DksA\textsubscript{VC},\text{HapR}\)), CDK1-R2 (\(DksA\textsuperscript{+}\text{VC},\text{HapR}\)) and CDK1-RH1 (\(DksA\textsuperscript{+}\text{VC},\text{HapR}\)) (Table 1), were examined by performing azocasein assay in order to determine the interplay between \(DksA\textsubscript{VC}\) and HapR in HAP production. WT (C6709), \(\Delta dksA\textsubscript{VC}\) (C-DksA1) and \(\Delta dksA\textsubscript{VC},\text{HapR}\) (C-DksA1\textsubscript{HapR}) (Table 1) were used as controls. It was found that the presence of functional DksA\textsubscript{VC} alone in the absence of HapR in the strain CDK1-R2 (\(dksA\textsuperscript{+}\text{VC},\text{HapR}\)) is unable to complement the HAP production defect of the \(\Delta dksA\textsubscript{VC},\text{HapR}\) strain C-DksA1\textsubscript{hapR}. On the other hand, when \(hapR\) was reverted back in the \(\Delta dksA\textsubscript{VC},\text{HapR}\) (C-DksA1\textsubscript{HapR}) and \(\Delta dksA\textsubscript{VC}\) (C-DksA1\textsuperscript{R2-HapR}) strains, they behaved like the \(\Delta dksA\textsubscript{VC}\) (C-DksA1) mutant and WT (C6709), respectively (Fig. 1b). Since mutation of the \(hapR\) gene leads to strong downregulation of HAP production but the \(dksA\textsubscript{VC}\) deleted strain is still able to express HAP, although in decreased amount (Fig. 1b), the results suggest that DksA\textsubscript{VC} finely regulates HAP production by epistatically modulating the HapR expression in \(V.\) cholerae. The above results indicate that DksA\textsubscript{VC} is epistatic to HapR; our next attempt was to determine how DksA\textsubscript{VC} regulates HapR.

DksA\textsubscript{VC} positively regulates the expression of \(hapR\) at both the transcriptional and post-transcriptional levels

HapR is a master regulator of the QS genetic regulatory circuit of \(V.\) cholerae and the virulence factor expression by the pathogen. Since we have provided evidence that DksA\textsubscript{VC} is epistatic to HapR, our next attempt was to determine the mechanism by which it regulates HapR expression. Our qRT-PCR analysis revealed an ~fourfold reduction in the \(hapR\) transcript level in the \(\Delta dksA\textsubscript{VC}\) mutant compared to its isogenic WT cells, as shown in Fig. 2(a). For further support we also checked the promoter activity of \(hapR\) in the \(\Delta dksA\textsubscript{VC}\) genetic background. To do this, the promoter region of \(hapR\) was fused with the promoterless lacZ gene present in a reporter plasmid and the construct was designated pBhapRPLz (Table 1).
Galactosidase assay was carried out by introducing the transcriptional reporter in both the ΔdksA<sub>Vc</sub> and WT backgrounds. β-Galactosidase assay using ΔdksA<sub>Vc</sub> mutants as background revealed an ~2.8-fold downregulation of the hapR promoter activity compared to that of WT cells (Fig. 2b). Since qRT-PCR is a measure of mRNA levels, based on the above results, it can be said that DksA<sub>Vc</sub> apart from providing a transcriptional control may also regulate HapR at the post-transcriptional level. To prove this, an hapR translational reporter plasmid, named pHapRfusLz (Table 1), was constructed and it was introduced in both the ΔdksA<sub>Vc</sub> and WT cells followed by β-galactosidase assay, which revealed an ~3.3-fold decrease in the expression level of HapR in the ΔdksA<sub>Vc</sub> strain compared to that of the isogenic WT parent strain (Fig. 2c). Pal et al. [15] have previously reported that the expression of the hapA gene is ~fivefold downregulated in ΔdksA<sub>Vc</sub> cells. It is to be noted that the lasB gene of <i>P. aeruginosa</i>, which codes for the elastase enzyme, is highly homologous to the hapA gene of <i>V. cholerae</i>. Furthermore, expression of HAP and LasB is QS-dependent and regulated by the production of small molecules called autoinducers. The lasB gene expression requires the transcriptional regulator LasR [34].

<i>P. aeruginosa</i> ΔdksA<sub>Vc</sub> mutant has been reported to produce poor levels of elastase, and different experimental results allowed the authors to conclude that LasB is regulated by DksA at the post-transcriptional level [11]. However, contrary to this result found in <i>P. aeruginosa</i>, the expression of HAP by <i>V. cholerae</i> cells appears to be regulated at the transcriptional level. Moreover, Jude et al. [11] have also reported that in <i>dksA</i> mutant the expression of the LasR regulator of <i>P. aeruginosa</i> was not affected. All these observations prompted us to check the expression status of HapR, the main transcriptional regulator of HAP, under a ΔdksA<sub>Vc</sub> genetic background. It was shown earlier that HapR strongly binds to the promoter region of the <i>hapA</i> gene [35]. In this study, we have shown that DksA<sub>Vc</sub> positively regulates the expression of HapR and it is epistatic to HapR. Although the exact reason for this difference in regulation of protease production by <i>V. cholerae</i> and <i>P. aeruginosa</i> is currently unknown, it could be possible that the sites of infection by these pathogens are diverse and thus this could be an example of niche-specific adaptive regulation by DksA. The intracellular HapR level in <i>V. cholerae</i> has been shown to be regulated through other genetic circuits such as: (i) a LuxO-dependent QS pathway using the sRNAs Qrr1–4; (ii) by a two-component signalling system VarS/VarA, which is linked with the global carbon storage regulatory system CsrA/BCD; and (iii) by small nucleotide binding protein Fis [27, 28]. Therefore, attempts were made to understand whether DksA<sub>Vc</sub> is somehow linked with these alternative regulatory systems, as discussed in the following sections.

**DksA<sub>Vc</sub> positively regulates the expression of sRNAs CsrB/C/D**

In <i>V. cholerae</i>, the VarS/VarA two-component regulatory system controls the expression of three redundant sRNAs CsrB, C and D. These sRNAs in turn regulate the activity of the global regulatory protein CsrA, which acts as a positive regulator of the LuxO-Qrr sRNA genetic circuit to control the stability of the hapR transcripts and thereby post-

Fig. 2. DksA<sub>Vc</sub> positively regulates the expression of hapR. (a) Relative expression of hapR in the ΔdksA<sub>Vc</sub> strain (C-DksA1) with respect to WT (C6709) cells is shown. Values represent the average of three independent experiments (n=3). Error bars indicate standard deviation (**P<0.01). (b) β-Galactosidase assay to determine the promoter activity of hapR. Strains used are: WT ΔlacZ, C6709ΔlacZ and ΔdksA<sub>Vc</sub>ΔlacZ (C-DksA1ΔlacZ) carrying the transcriptional reporter plasmid pRhlPRLz containing the hapR promoter region fused with the <i>V. cholerae</i> lacZ ORF, respectively. Values represent the average of three independent experiments (n=3). Error bars indicate standard deviation (P<0.05). (c) β-Galactosidase assay to determine the HapR expression levels in the strains WT ΔlacZ, C6709ΔlacZ and ΔdksA<sub>Vc</sub>ΔlacZ (C-DksA1ΔlacZ) carrying the transcriptional reporter plasmid pHapRfusLz. Values represent the average of three independent experiments (n=3). Error bars indicate standard deviation. *P<0.05.
transcriptionally regulates hapR expression [27]. Since we have shown that DksA_{Vc} somehow regulates the expression of HapR at the post-transcriptional/translational level, it was therefore necessary to know whether DksA_{Vc} is needed to influence the VarS/VarA-CsrA/BCD genetic circuit. We initially hypothesized that the expression of the CsrB/C/D sRNA coding genes csrB, csrC and csrD could be downregulated in ΔdksA_{Vc} strain C-DksA1. To verify this, the promoter region of the csrB, csrC or csrD gene [27] was fused with the promoterless lacZ gene of V. cholerae and thus generated the promoter-reporter recombinant plasmids pBcsrBPLz, pBcsrCPLz or pBcsrDPLz (Table 1), respectively, followed by introduction of each of these constructs into WT or ΔdksA_{Vc} cells. When β-galactosidase activity of these plasmid-bearing strains was measured and compared, there was an −three-, −two- or −fourfold reduction of promoter activity of the csrB, csrC or csrD genes, respectively, in ΔdksA_{Vc} genetic background compared to that of isogenic WT strain (Fig. 3a−c). Furthermore, the defect in HAP production by ΔdksA_{Vc} cells could be overcome by overexpression of CsrB/C/D in trans (Fig. 3d). Lenz et al. [27] have shown that, in the absence of the CsrB/C/D sRNAs, the unbound global regulatory protein CsrA downregulates the expression of hapR in a QS-dependent manner. However, it was then reported that CsrA can also regulate the expression of hapR in a QS-independent manner [36]. Additionally, E. coli DksA protein has been shown to regulate the expression of the csrB/C genes which are also known to sequester the global regulatory protein CsrA [37]. In summary, in the absence of DksA_{Vc}, the expression of the csrB/C/D genes encoding the CsrB/C/D sRNAs is downregulated leading to an increase in the level of free intracellular CsrA followed by downregulation of HapR.

**DksA_{Vc} regulates Fis, a negative regulator of HapR**

It has been shown that Fis negatively regulates HapR in *V. cholerae* [28]. Fis is a small nucleoid-associated protein found during the exponential growth phase in bacteria [38]. In *E. coli* ΔdksA mutant, however, transcription of *fis* extends into the late exponential and stationary growth phases, suggesting the importance of DksA in growth phase dependent regulation of *fis* [39]. We wished to examine the status of *fis* expression levels in *V. cholerae* ΔdksA_{Vc} mutant strain C-DksA1 in comparison to the WT strain C6709. We found that the *fis* transcripts of the *V. cholerae* ΔdksA_{Vc} mutant were −4.5-fold higher in the early log phase (OD=0.2) and −twofold higher in the mid-log (OD=0.4−0.6) phase of growth. On the other hand, the *fis* transcript levels of both ΔdksA_{Vc} and WT cells of stationary phase were found to be much lower compared to their respective concentrations in exponentially growing cells. However, the *fis* transcript levels were −twofold higher in the early stationary phase (OD=1.0−1.5) and −fivefold higher in the late stationary (OD=2.0) phase of growth in the ΔdksA_{Vc} cells compared to that of the isogenic WT parent strain (Fig. 4a). Interestingly, compared to the *fis* transcript level of the WT strain which almost vanishes during the late stationary phase, in ΔdksA_{Vc} cells it was significantly higher. Thus, in the absence of DksA_{Vc}, it appears that the expression of *fis* is not stringently controlled and it continued even in the stationary phase of growth. As mentioned previously, Fis negatively affects HapR [28], and it seems therefore that modulation of HAP production during the stationary growth phase could be indirectly controlled by DksA_{Vc} by downregulating the expression of *fis* when cells are entering into the stationary phase. It is to be noted that apart from defect in HAP production, the ΔdksA_{Vc} mutant also showed shifting of HAP production timing compared to WT. While WT cells showed initiation of HAP production after about 6 h of growth (stationary phase), there was a further −2 h delay in HAP production by the ΔdksA_{Vc} mutant ([115]; Fig. 1b). Since expression of *fis* remains high in all growth phases in ΔdksA_{Vc} cells, we hypothesized that in the absence of DksA_{Vc}, intracellular Fis (a negative regulator of HapR) concentration goes up leading to downregulation of HapR and delay in HAP production. This hypothesis was supported by the fact that when *fis* was overexpressed in WT cells of *V. cholerae* through the recombinant plasmid construct pFisBAD (Table 1), it showed a distinct shift in timing of HAP production, a phenotype exhibited by the ΔdksA_{Vc} cells (Fig. 4b). A shift in HAP production was also found in uninoculated bacterial culture which could be due to leaky expression from the P_{BAD} promoter of the vector pBAD24 (Table 1). In this experiment, ΔdksA_{Vc} cells were taken as a control. However, except for a delay in HAP production, overexpression of Fis did not affect overall HAP production as measured in overnight incubated culture supernatant of WT; in contrast, HAP production was distinctly affected and delayed in the case of ΔdksA_{Vc} cells (Fig. 4b). It is to be noted here that *V. cholerae* requires both the growth phase regulated CsrB/C/D sRNA system as well as Fis to control density-dependent gene expression [28]. In a ΔdksA_{Vc} mutant, apart from Fis, the expression of the csrB/C/D genes is also affected (Fig. 3), which is not the case in Fis overexpressing WT cells. Our results also support a previous finding where the ΔcsrB/C/D strain showed poor production of HapR and therefore a decrease in expression of the luxCDABE operon of *V. harveyi* [27]. On the other hand, a Δ*fsi* mutant of *V. cholerae* shows a density-dependent bioluminescence profile which is much shallower than the profile of WT cells. In the absence of Fis, the expression of the *V. harveyi luxCDABE* operon, which is regulated by HapR, was found to be upregulated early in the log phase resulting in an increase in bioluminescence at the early time points of growth; however, at later time points it showed a bioluminescence profile comparable to that of the WT strain. In *V. cholerae*, Fis together with phosphorylated LuxO upregulates the expression of the Qrr1–4 sRNAs, which in turn decreases the stability of the *hapR* mRNA [28]. Moreover, Fis cannot function alone in the absence of LuxO/phosphorylated LuxO. Since, Fis expression is regulated in a density-dependent manner, its downstream target genes are also regulated in a same manner [28]. In summary, it appears that in ΔdksA_{Vc} cells, high levels of Fis are found even in the HCD-specific stationary growth phase and, along with this, low levels of CsrB/C/D sRNAs may be responsible for high expression of Qrr1–4 sRNAs, which ultimately leads to defective and delayed HAP production. However, further experiments are needed to understand the mechanism of negative regulation of Fis by DksA_{Vc}.
DksA\textsubscript{Vc} positively regulates the expression of the stationary phase sigma factor RpoS

Apart from HapR, the stationary phase sigma factor RpoS also positively regulates HAP production in \textit{V. cholerae} [25, 26] and a \textit{V. cholerae} ΔrpoS mutant of C6709 constructed by us also produced low levels of HAP (data not shown). Therefore, to find out whether DksA\textsubscript{Vc} is involved in regulation of RpoS, we wished to determine the expression of the \textit{V. cholerae} rpoS gene in the ΔdksA\textsubscript{Vc} genetic background using WT rpoS expression as a control. To test this, total cellular RNA was isolated from WT and ΔdksA\textsubscript{Vc} cells grown till stationary phase and qRT-PCR assay was carried out with the rpoS gene-specific primers. Interestingly, there was ~5.7-fold diminished expression of the rpoS gene in

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**Fig. 3.** DksA\textsubscript{Vc} regulates the expression of the genes encoding csrB, csrC and csrD sRNAs. \(\beta\)-Galactosidase assay to determine the promoter activities of (a) csrB, (b) csrC and (c) csrD in the strains WT ΔlacZ (C6709 ΔlacZ) and ΔdksA\textsubscript{Vc} ΔlacZ (C-DksA1 ΔlacZ) carrying the plasmids pBcsrBPLz, pBcsrCPLz and pBcsrDPLz. Values represent the average of three independent experiments (\(n=3\)). Error bars indicate standard deviation (*\(P<0.05\)). (d) HAP production by various \textit{V. cholerae} strains overexpressing dksA\textsubscript{Vc}, csrB, csrC or csrD gene. Strains used are: WT (C6709), ΔdksA\textsubscript{Vc} (C-DksA1) carrying the empty vector (pBAD24) [ΔdksA\textsubscript{Vc} (pBAD24)] or DksA\textsubscript{Vc} expressing plasmid (pDksA\textsubscript{BAD}) [ΔdksA\textsubscript{Vc} (pDksA\textsubscript{BAD})] or CsrB expressing plasmid (pCsrB\textsubscript{BAD}) [ΔdksA\textsubscript{Vc} (pCsrB\textsubscript{BAD})] or CsrC expressing plasmid (pCsrC\textsubscript{BAD}) [ΔdksA\textsubscript{Vc} (pCsrC\textsubscript{BAD})] or CsrD expressing plasmid (pCsrD\textsubscript{BAD}) [ΔdksA\textsubscript{Vc} (pCsrD\textsubscript{BAD})]. Values represent the average of two independent experiments (\(n=2\)). Error bars indicate standard deviation (*\(P<0.05\)).
ΔdksA\textsubscript{Vc} cells compared to its parent WT strain (Fig. 5a). To understand whether DksA\textsubscript{Vc} regulates the promoter activity of RpoS, the promoter region of rpoS was fused with the promoterless lacZ gene present in a reporter plasmid and the construct was designated pBrpoS\textsubscript{PLz} (Table 1). The recombinant reporter plasmid pBrpoS\textsubscript{PLz} was introduced in WT and ΔdksA\textsubscript{Vc} strains followed by β-galactosidase assay, which revealed only a modest −1.8-fold downregulation in the rpoS promoter activity in the ΔdksA\textsubscript{Vc} mutant strain compared to isogenic WT cells (Fig. 5b). RpoS regulation is very complex and in E. coli it is regulated at transcriptional, post-transcriptional/translational levels and also at the level of protein stability [26, 40–44]. Our qRT-PCR and reporter assay-based experimental results indicate that DksA\textsubscript{Vc} most likely regulates RpoS at the post-transcriptional level. To understand whether DksA\textsubscript{Vc} regulates RpoS at translational level, an rpoS translational reporter construct containing the rpoS promoter region and its ORF fused in-frame with the lacZ ORF was generated and named pRpoS\textsubscript{fusLz} (Table 1). This construct was introduced in ΔdksA\textsubscript{Vc} and WT cells and β-galactosidase assay was performed, which revealed −fourfold decrease in the expression of rpoS in ΔdksA\textsubscript{Vc} cells compared to the isogenic WT strain (Fig. 5c). The above results suggest that DksA\textsubscript{Vc} most likely regulates rpoS expression at post-transcriptional/translational levels. However, further experiments are needed to confirm this. It is to be noted that Brown \textit{et al.} [45] have shown that DksA regulates expression of RpoS at the translational level in \textit{E. coli}. Furthermore, DksA has been shown to be involved in the regulation of the rpoS gene of \textit{Salmonella enterica} serovar Typhimurium. In fact, the region between the eighth and 73rd codons of the rpoS ORF was found to be required for carrying out the translational regulation by DksA [9]. Jelssen \textit{et al.} [46] have reported that in \textit{V. cholerae}, HapR positively controls the expression of rpoS, although the exact mechanism is not known. Future studies are needed to understand whether DksA\textsubscript{Vc} directly binds the rpoS transcript and thereby regulates its translation or it is an indirect effect. Whatever the mechanism, the experimental results of this study clearly indicate that the regulation of RpoS is indeed also highly complex in \textit{V. cholerae}.

**Conclusion**

The present study aimed to determine the mechanism by which DksA\textsubscript{Vc} regulates HAP production in \textit{V. cholerae}. In the host environment, HAP helps \textit{V. cholerae} cells to detach from intestinal epithelial cell surfaces and ultimately escape into the environmental water bodies. Apart from pathogenesis, HAP plays an important role during starvation survival by enabling \textit{V. cholerae} to digest the proteinaceous organic matter dissolved in water leading to the liberation of nutritional amino acid substrates [47]. In this study, we provide experimental evidence that DksA\textsubscript{Vc} positively regulates HapR by controlling some of the regulators of the QS circuitry such as the CsrB/C/D system and Fis. We have also shown that DksA\textsubscript{Vc} most likely post-transcriptionally regulates RpoS. Since HapR, RpoS and, ultimately, HAP are all associated with the stationary phase of growth in \textit{V. cholerae}, it appears that DksA\textsubscript{Vc} is indeed a critical regulator of these stationary phase genes. Expression of DksA\textsubscript{Vc} appears to be constitutive and its expression is detectable in equal intensities in all growth phases (unpublished observation). DksA is a co-factor for the small intracellular signalling molecule (p)ppGpp, and (p)ppGpp has previously been shown to be important for the regulation of stationary phase genes [48–50]. Moreover, Holley \textit{et al.} [51] have shown the importance of both DksA and (p)ppGpp in regulation of stationary phase genes in the pathogen \textit{Haemophilus ducreyi}. Under laboratory conditions, entry into the
stationary phase generally represents a stressed condition for bacterial cells due to nutrient limitation resulting in depletion of energy. Since DksA is expressed constitutively, it is the concentration of (p)ppGpp which fluctuates during different phases of growth, remaining at a basal level during the exponential phase and with its concentration increasing during the stationary phase [1, 48]. Since DksA acts as an essential co-factor of (p)ppGpp, in the absence of this regulatory protein, bacterial cells behave like a (p)ppGpp null mutant or (p)ppGpp$^0$ mutant. Through our initial experiments, we have found that a (p)ppGpp$^0$ mutant shows similar phenotypes to a $\Delta$dksA$^Vc$ mutant, i.e. decreased expressions of HapR, RpoS and HAP production (unpublished observation). Thus, DksA$^Vc$ links nutrient limitation to upregulation of stationary phase genes such as hapR, rpoS and hapA which are associated with the virulence of V. cholerae. To our knowledge, this is the first report that links DksA$^Vc$ to the regulation of HapR and RpoS, the principal regulators of HAP production in V. cholerae. A working model is proposed in Fig. 6.

Finally, it is to be noted that, other than HAP, HapR negatively affects biofilm and CT production in V. cholerae [18, 19]. Since $\Delta$dksA$^{Vc}$ showed downregulation of HapR, therefore, it was expected that there would be an increase in biofilm formation by the mutant cells; when such an experiment was performed, $\Delta$dksA$^{Vc}$ exhibited increased biofilm formation (unpublished observation). On the other hand, we reported decreased CT production by $\Delta$dksA$^{Vc}$ cells [15], but in this study we found downregulation of HapR in $\Delta$dksA$^{Vc}$, which should lead to excess CT production. Although currently this apparent anomaly in regulation of CT production by $\Delta$dksA$^{Vc}$ cells with respect to HapR is not clear, our preliminary qRT-PCR experiments indicated increased CT transcript levels in $\Delta$dksA$^{Vc}$ compared to WT cells, suggesting probable post-transcriptional/translational regulation of CT by DksA$^{Vc}$ (unpublished observation).

Fig. 5. DksA$^{Vc}$ positively regulates the expression of rpoS. (a) Relative expression of rpoS in $\Delta$dksA$^{Vc}$ strain (C-DksA1) with respect to WT (C6709) cells is shown. Values represent the average of three independent experiments ($n=3$). Error bars indicate standard deviation (**$P<0.01$). (b) $\beta$-Galactosidase assay to determine the promoter activity of rpoS. Strains used are: WT ΔlacZ (C6709ΔlacZ) and $\Delta$dksA$^{Vc}$ΔlacZ (C-DksA1ΔlacZ) carrying the reporter plasmid pBrpoSPLz (insert contains rpoS promoter region fused with the V. cholerae lacZ ORF). Values represent the average of three independent experiments ($n=3$). Error bars indicate standard deviation (*$P<0.05$). (c) $\beta$-Galactosidase assay to determine the RpoS expression levels in the strains WT ΔlacZ (C6709ΔlacZ) and $\Delta$dksA$^{Vc}$ΔlacZ (C-DksA1ΔlacZ) carrying the translational reporter plasmid pRpoSfusLz. Values represent the average of three independent experiments ($n=3$). Error bars indicate standard deviation (*$P<0.05$).

Fig. 6. Proposed working model showing the complex regulatory link among DksA$^{Vc}$ and HapR, RpoS, CsrB/C/D sRNAs and Fis for optimal expression of HAP during the stationary phase of growth by V. cholerae cells (see details in text). Arrows and ‘?’ indicate activation and inhibition of expression, respectively.
Further investigations are underway in our laboratory to resolve the complex regulation of CT production by DksA_{VC}.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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