Mutation in the relA Gene of *Vibrio cholerae* Affects In Vitro and In Vivo Expression of Virulence Factors

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The *relA* gene product determines the level of (p)ppGpp, the effector nucleotides of the bacterial stringent response that are also involved in the regulation of other functions, like antibiotic production and quorum sensing. In order to explore the possible involvement of *relA* in the regulation of virulence of *Vibrio cholerae*, a *relA* homolog from the organism (*relA*$_{VCH}$) was cloned and sequenced. The *relA*$_{VCH}$ gene encodes a 738-amino-acid protein having functions similar to those of other gram-negative bacteria, including *Escherichia coli*. A ∆*relA::kan* allele was generated by replacing ~31% of the open reading frame of wild-type *relA* of *V. cholerae* El Tor strain C6709 with a kanamycin resistance gene. The *V. cholerae relA* mutant strain thus generated, SHK17, failed to accumulate (p)ppGpp upon amino acid deprivation. Interestingly, compared to the wild type, C6709, the mutant strain SHK17 exhibited significantly reduced in vitro production of two principal virulence factors, cholera toxin (CT) and toxin-coregulated pilus (TCP), under virulence gene-inducing conditions. In vivo experiments carried out in rabbit ileal loop and suckling mouse models also confirmed our in vitro results. The data suggest that (p)ppGpp is essential for maximal expression of CT and TCP during in vitro growth, as well as during intestinal infection by virulent *V. cholerae*. Northern blot and reverse transcriptase PCR analyses indicated significant reduction in the transcript levels of both virulence factors in the *relA* mutant strain SHK17. Such marked alteration of virulence phenotypes in SHK17 appears most likely to be due to down regulation of transcript levels of toxR and toxT, the two most important virulence regulatory genes of *V. cholerae*. In SHK17, the altered expression of the two outer membrane porin proteins, OmpU and OmpT, indicated that the *relA* mutation most likely affects the ToxR-dependent virulence regulatory pathway, because it had been shown earlier that ToxR directly regulates their expression independently of ToxT.

*Vibrio cholerae* is a facultative anaerobic gram-negative bacterium and the causative agent of the severe diarrheal disease cholera. In addition to residing temporarily in the intestinal lumen of humans during the diseased state, *V. cholerae* has its natural niche in the aquatic environment, residing in the free-living aquatic flora found in estuarine areas and in association with crustaceans and mollusks (25). The strains of *V. cholerae* that cause epidemic cholera belong to serogroups O1 and O139 (3, 4, 28, 41, 50). The O1 serogroup is again divided into two biotypes, classical and El Tor (28). Strains other than O1 and O139 are known as non-O1/non-O139 vibrios.

A pathogen in its natural environment and host-associated state is subjected to a plethora of stresses, such as fluctuations in pH, salinity, osmolarity, oxygen tension, temperature, and nutritional availability. These offer selective pressure to a bacterium, eliciting various adaptive responses for its survival. The adaptive response to nutritional stress encompassing rapid and complex cellular adjustments is called the stringent response. The *relA* gene has been identified as the genetic determinant responsible for the stringent response to amino acid starvation in prokaryotes. The hallmark of stringent response is the RelA-catalyzed cellular increase of hyperphosphorylated guanosine nucleotides, ppGpp and pppGpp, collectively called (p)ppGpp (6), which leads to the rapid inhibition of syntheses of stable RNAs, ribosomes, and proteins, and ultimately to the arrest of cell growth (6). However, the regulation of the level of (p)ppGpp is crucial for cell viability and depends on its rates of synthesis and degradation. Degradation of (p)ppGpp is carried out in *Escherichia coli* and other gram-negative organisms by the product of the spoT gene, which has (p)ppGpp 3'-pyrophosphohydrolase activity (6). Furthermore, SpoT also possesses a weak (p)ppGpp synthetase activity, as revealed by the presence of a basal level of (p)ppGpp in *relA* null mutants that disappears in *relA spoT* double mutants (52).

The various cellular responses to increased (p)ppGpp concentrations have been studied in a number of microorganisms, where it was found that (p)ppGpp has a role in the synthesis and accumulation of stationary-phase sigma factor, σS (18), in antibiotic and pigment production (7, 8) and quorum sensing (49) and in developmental processes (21). Recently, it has been shown that (p)ppGpp has a role in the coordination of *Legionella pneumophila* virulence with entry into the stationary phase (20) and that *Listeria monocytogenes relA* mutants are impaired in surface-attached growth and virulence (45). The *relA* gene has also been cloned and characterized from a number of pathogens other than the two mentioned above, but its exact role in virulence is not known (43, 51).

At present, no information is available about the function of the *relA* gene in *V. cholerae*, and more importantly, whether it has any role in the coordination of the bacterial virulence response is not known. The virulence cascade in *V. cholerae* entails the activation of two transmembrane proteins, TcpP and ToxR, which act synergistically at the *toxT* promoter (29).
TABLE 1. Bacterial strains, plasmids, and oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant genotype and/or phenotype</th>
<th>Source or reference</th>
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<tbody>
<tr>
<td><strong>V. cholerae</strong></td>
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<tr>
<td>569B</td>
<td>Wild type (O1 classical)</td>
<td></td>
</tr>
<tr>
<td>C6709</td>
<td>Wild type (O1 El Tor); Sm'</td>
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<tr>
<td>C6709-R</td>
<td>C6709 Sm' Rif'</td>
<td>J. J. Mekalanos</td>
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<tr>
<td>SHK17</td>
<td>C6709 ΔrelA::kan Sm' Km'</td>
<td>This study</td>
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<td>DH5α</td>
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<td>Promega</td>
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<tr>
<td>CF1648</td>
<td>Wild-type MG1655</td>
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<tr>
<td>SM100pir</td>
<td>thi thr leu tonA lacY supE recA::RP4-2-Tc::Mu λpir R6K</td>
<td>36</td>
</tr>
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<td><strong>Plasmids</strong></td>
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<tr>
<td>pBluescript KS(+)</td>
<td>ColE1: high-copy-number cloning vector; Ap'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pUC4K</td>
<td>Source of the kanamycin resistance gene cassette; Ap' Km'</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pGPT704</td>
<td>Suicide vector; pBR322 derivative with oriR6K mobRP4; Ap'</td>
<td>36</td>
</tr>
<tr>
<td>pREL邨VCH</td>
<td>3.21-kb PCR-amplified relA gene region of V. cholerae strain 569B in pBluescript KS(+)</td>
<td>This study</td>
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<td>pSHC4</td>
<td>1.7-kb HindIII fragment of pREL邨VCH cloned into the HindIII site of pBluescript KS(+)</td>
<td>This study</td>
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<tr>
<td>pSH99.5</td>
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<td>pSH99.6</td>
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<td>pSHEG17</td>
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<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>VCR-R</td>
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<td>This study</td>
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<td>This study</td>
</tr>
<tr>
<td>ctxB-R</td>
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<td>This study</td>
</tr>
<tr>
<td>tcpA-F</td>
<td>5'-CAGATAAGAAGAACCGGTACAGAG-3'</td>
<td>This study</td>
</tr>
<tr>
<td>tcpA-R</td>
<td>5'-GAAAAGGACCTTCTTACAGCGTTG-3'</td>
<td>This study</td>
</tr>
<tr>
<td>toxT-F</td>
<td>5'-ACTGTCGACGCCGAAGCATATTCAGAGA-3'</td>
<td>This study</td>
</tr>
<tr>
<td>toxT-R</td>
<td>5'-CGCGGATCCATAATCGAAATTAGGAAGT-3'</td>
<td>This study</td>
</tr>
<tr>
<td>toxR-F</td>
<td>5'-CGGGATCTTGTTTGGGATTTAGGACAC-3'</td>
<td>This study</td>
</tr>
<tr>
<td>toxR-R</td>
<td>5'-CGGGATCTTACACACACCTTGTATGAGTGC-3'</td>
<td>This study</td>
</tr>
<tr>
<td>PMBEX1</td>
<td>5'-ACGAAATTTCAATGGTGTCCGGTGTCG-3'</td>
<td>This study</td>
</tr>
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</table>

ToxT then activates the expression of the two major virulence determinants of the cascade, ctxAB and tcpA (29). Due to the pioneering work done by Miller and his colleagues on ToxR (37) and because of its important role in activating the virulence genes, the regulatory cascade controlling the virulence gene expression is called the ToxR regulon. ToxR also directly regulates the expression of the outer membrane porin proteins OmpU and OmpT in a separate branch of the ToxR cascade (9). While ToxR positively regulates the expression of the OmpU protein (11), OmpT is transcriptionally repressed (31). In the present study, we report the cloning and sequencing of the relA gene of V. cholerae and the construction of a deletion-insertion relA mutant strain. Surprisingly, the relA gene mutation in V. cholerae revealed that (p)ppGpp, synthesized by RelA, plays a significant role both in vitro and in vitro in the regulation of the expression of two principal virulence factors, cholera toxin (CT) and toxin-coregulated pilus (TCP), most likely by modulating the expression of the transcriptional activator ToxR.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, plasmids, and oligonucleotides used in this study are listed in Table 1. The C6709-R strain was a spontaneous rifampin-resistant (Rif') mutant derived from the wild-type El Tor O1 strain C6709 (Table 1). Competition experiments showed that the C6709-R strain was as fit for growth in vitro and colonization in the suckling mouse model (see below) as the parental strain, C6709 (data not shown). Both E. coli and V. cholerae cells were routinely grown at 37°C in Luria-Bertani broth (LB) with shaking. For plate culture, LB was used with 1.5% agar. For optimal CT production by the El Tor strains of V. cholerae, AKI conditions were used (26, 27). Under AKI conditions, V. cholerae cells are grown in AKI medium without sodium bicarbonate (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl) statically at 37°C for 4 h, and the culture is then shifted to overnight shaking at 37°C (26, 27). For functional assay of relA, AT medium containing the histidine biosynthesis inhibitor 3-amino-1,2,4-triazole (AT) was prepared essentially as described by Rudd et al. (42), except that it was based on M63 salt solution. For in vivo 3P, labeling, MOPS (morpholinopropanesulfonic acid)-glucose minimal medium containing 0.4 mM phosphate, 0.4% glucose, 40 μg of each amino acid (Sigma)/ml, and 20 μg of each of the nucleosides (Sigma)/ml was used (33). Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; chloramphenicol, 30 μg/ml; rifampin, 5 μg/ml; kanamycin, 50 μg/ml for E. coli and 40 μg/ml for V. cholerae. The growth kinetics of the bacterial culture was studied spectrophotometrically by measuring the optical density of the culture at 600 nm.

Determination of intracellular (p)ppGpp concentrations. The strains were screened for patterns of (p)ppGpp accumulation essentially as described by Cashel (5), with the following changes. An overnight bacterial culture grown in MOPS-glucose minimal medium containing 2 mM phosphate and other supplements (as stated above) was washed and resuspended in low-phosphate (0.4 mM) MOPS-glucose minimal supplemented with nucleosides and amino acids except serine and grown to an optical density of 0.2 at 600 nm. For detection
of [(p)pGpp in rich medium, the cells were resuspended in MOPS-glucose minimal medium supplemented with nucleosides and all the amino acids (33). At this stage, the cells were uniformly labeled with [32P]Pi-PiP0 (100 μCi/ml) (BRIT, Mumbai, India), and amino acid starvation was simulated by the addition of 500 μg of 1 M α-ketoglutarate (Spectro Chemical, Mumbai, India). The infection of (p)pGpp from V. cholerae cells, the method described by Ojha et al. (39) was followed. Briefly, samples were withdrawn at various time intervals, centrifuged and washed with 10 mM Tris (pH 8.0) buffer, resuspended in the same buffer containing 20 μg of lysozyme (Sigma/ml), and kept on ice for 20 min. Lysis of the cells was done with 1% sodium dodecyl sulfate (SDS), and [(p)pGpp was recovered and subjected to a final volume of 2 μM formic acid and analyzed by one-dimensional polyethyleneimine-coated thin-layer chromatography (TLC). The TLC plate (Merck) was developed with 1.5 M KH2PO4 buffer (pH 3.4), air dried, and exposed to X-ray film (Kodak) for 24 h at ~70°C for autoradiography. The authenticity of [(p)pGpp was determined by its comigration with [(p)pGpp from the 3.21-kb DNA fragment was cloned into an

DNA manipulations and cloning of the relA gene of V. cholerae. Standard molecular biological methods were followed for chromosomal and plasmid DNA preparation, as described in DNA manipulation of DNA were followed (9).

Nucleotide sequencing and analysis. DNA sequencing was done by using the Applied Biosystem (ABI) BigDye Terminator Cycle Sequencing kit (Perkin-Elmer) and the ABI PRISM automatic DNA sequencer (model 377). DNA sequence data were compiled and analyzed by using the DNASIS computer program. Amino acid sequence homology searching was done using the BLASTX program (1).

Construction of the relA deletion mutant of V. cholerae. To inactivate the relA gene of V. cholerae O1 El Tor strain C6709 (Table 1), the plasmid pRELVC1 (Table 1) was digested with the enzymes EcoRV and HindII, and the resulting 1.24-kb fragment, carrying the N-terminal region of the relA gene (Fig. 2), which was replaced with a 1.24-kb PstI-cut kanamycin resistance gene block of the vector pUC4K (Table 1). The resulting recombinant plasmid, pSHK17, was then mobilized conjunctly from E. coli SM10pir (Table 1). For creating a deletion-insertion allele, pSHEG17 was digested with the enzyme NcoI to remove a 660-bp internal fragment of the relA gene of V. cholerae (Fig. 2), which was replaced with a 1.24-kb PstI-cut kanamycin cassette by colony hybridization using linearized pGPT04 DNA and the 1.24-kb kanamycin gene block, respectively, as a probe. The presence of the new ΔrelA::kan allele in the V. cholerae C6709 chromosome (Table 1) was confirmed by Southern blot hybridization analysis (see Fig. 2) and also by sequencing with the primer PMBEX1 (Table 1). The V. cholerae relA mutant was grown on rich medium, the resultant colonies were discarded, and the total protein contents were adjusted to the same value according to protein determinations. Samples (5 μl) were plated directly on polyvinylidene difluoride membranes (Immobilon). The color intensity was measured at 492 nm in an ELISA reader (Bio-Rad).

RT-PCR assay. Reverse transcriptase (RT) PCR experiments were carried out as follows. Equal amounts of total cellular RNA from all experimental samples were first treated with RNase-free DNase I (Life Technologies) and incubated at room temperature for 15 min to eliminate any contaminating DNA from the RNA sample. The reaction was terminated by adding 2.5 mM EDTA, and the DNase I enzyme (Life Technologies) was then heat inactivated at 65°C for 10 min.

Immunological detection of OmpU, OmpT, and TcpA. Isolation of outer membrane proteins. V. cholerae C6709 and its isogenic mutant SHK17 grown under AKI conditions were harvested and sonicated, and the resulting cell lysate was separated into subcellular fractions as described previously (13). The outer membrane fraction was solubilized, and outer membrane proteins, together with whole-cell lysate proteins, were analyzed by SDS–12.5% polyacrylamide gel electrophoresis (PAGE), followed by staining with Coomassie blue or by Western blot analysis. To ensure equal loading of the protein samples, the total protein concentrations in the samples were determined using a commercial protein determination kit (Bio-Rad). The sizes of proteins were estimated using commercially available (Genei, Bangalore, India, or In-vitrogen) protein molecular weight standards. Contamination in the outer membrane preparation due to inner membrane was always <5% as determined by cytochrome assay, as described previously (13).

1. Immunological detection of OmpU, OmpT, and TcpA. Western blot analysis was performed with rabbit polyclonal antiserum against V. cholerae OmpU and OmpT (kindly provided by K. E. Kloese, University of Texas Health Science Center, San Antonio, Tex.) followed by goat anti-rabbit immunoglobulin G–alcaline phosphatase conjugate (Sigma). The blots were developed with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate) substrates by standard techniques (2). For TcpA protein detection, bacterial-cell pellets obtained from 10 ml of culture grown under AKI conditions were resuspended in 100 μl of phosphate-buffered saline (10 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl), and one part was stained with ethidium bromide and visualized with UV light to confirm equal loading of all samples. The other part of the gel was used for Northern blot experiments. The probes used were PCR amplified utilizing the ctxA-F and ctxB-R primers for the ctxAB gene, and toxT-F and toxT-R primers for the toxT gene (Table 1). The PCR conditions were as follows: one cycle of initial denaturation at 94°C for 5 min, followed by 30 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of elongation/kb at 72°C. The amplification reaction was completed with an additional cycle of 7 min at 72°C. Quantification of the mRNA bands was done using Quantity 1 software (Bio-Rad).
FIG. 1. Genetic organization of the relA region of the *V. cholerae* chromosome and comparison with those of different gram-negative organisms. The large arrows represent ORFs. The thick and thin lines represent intergenic regions and chromosomal DNA, respectively. The small arrows indicate the positions of the two primers, VCR-F and VCR-R, used to amplify the *relA* gene region (3.21 kb) of *V. cholerae*. The *tmaA* gene codes for an RNA methyltransferase (23); *mazG* codes for a protein of unknown function in *V. cholerae* (23) and *E. coli* (38), but in *S. enterica* serovar Typhimurium it codes for a pyrophosphatase (38); STM2955, *chpA*, and *chpR* code for a PemK-like growth inhibitor, for a suppressor of ChpA, and for a putative transcriptional regulator, respectively.

**RESULTS**

Cloning of the *relA* homolog of *V. cholerae* and its DNA sequence analyses. The 3.21-kb *relA* locus of *V. cholerae* (Fig. 1) was PCR amplified from the genomic DNA of the classical O1 strain 569B using the oligonucleotides VCR-F and VCR-R (Table 1) as shown in Fig. 1, and the amplified fragment was cloned as described in Materials and Methods. The recombinant plasmid thus generated was designated pREL*VC* (Table 1). The entire 3.21-kb insert DNA was sequenced after several subclones were constructed (Table 1). The sequence analysis of the 3.21-kb DNA revealed only one complete open reading frame (ORF) of 2.22 kb from nucleotide positions 399 to 2615. A similarity search using the BLAST program with the nonredundant peptide sequence database revealed 100% identity at the amino acid level with the deduced RelA protein of El Tor strain N16961 (23), as well as a high degree of similarity to all known RelA homologs of other bacteria (6, 16, 38). The result indicates that the cloned fragment containing the 2.22-kb ORF is most likely a *relA*-like gene, and it has been designated *relA* _VC*. However, the predicted RelA protein of *V. cholerae* consists of 738 amino acid residues compared to the 744 residues of *Vibrio* sp. strain 14 (16), *Salmonella enterica* serovar Typhimurium (38), and *E. coli* (38). Thus, the putative *relA* ORF of 2.22 kb should code for a protein with a molecular mass of ~81 kDa. Analysis of the upstream and downstream nucleotide sequences of *relA* _VC* revealed the presence of *tmaA* and *mazG*-like genes (Fig. 1), respectively, indicating that the *relA* locus of *V. cholerae* has a genetic organization similar to those of other gram-negative organisms (6, 16, 38). Complementation and functional analyses (5, 33, 42) of pREL*VC* using *E. coli* wild-type CF1648 and its *relA* mutant strains CF1652 and CF1693 (Table 1) confirmed that the cloned insert in pREL*VC* indeed carries a *V. cholerae* *relA* homolog whose product is responsible for the synthesis of (p)pppGpp (details of this work will be published elsewhere).

**Disruption of the *relA* gene and its effect on (p)pppGpp accumulation.** The El Tor strain C6709 was chosen for this study...
FIG. 2. (A) Schematic diagram (not to scale) showing the strategy for the construction of a relA deletion-insertion mutant strain, SHK17, of *V. cholerae* O1 El Tor. Restriction maps of the relA regions of the parental and mutant strains are shown. The thick lines represent gene regions, and the dashed lines represent vector DNA. The arrows indicate the direction of transcription of a gene. The thin lines indicate intergenic regions or chromosomal DNA. The hatched bar represents the *Eco*RV-*Pst*I fragment of the relA*VCH* gene used as a probe in Southern hybridization studies, as shown in panel B. The plasmid pSHK17, containing the kanamycin resistance gene cassette (kan) within the relA*VCH* gene, was introduced into strain C6709 by conjugation, and the recombinant (shown by an open arrow), in which the ΔrelA:kan allele had replaced the wild-type relA, was isolated as described in Materials and Methods. Restriction enzyme sites: EV, *Eco*RV; Hc, *Hinc*II; N, *Nde*I; P, *Pst*I.

(B) Confirmation of the relA mutation in SHK17 as a double-crossover event by Southern analysis. The *Eco*RV-*Pst*I fragment (shown in panel A) of the relA gene of *V. cholerae* was used for hybridization studies. Chromosomal DNA was digested with different restriction enzymes. For a
because this biotype of *V. cholerae* is the current pandemic strain (28) and there are data available from various virulence-related studies with the strain (15, 30, 35). Database searches and alignment of the RelA protein of *V. cholerae* with those of other organisms (38) revealed that the catalytic domain, or the (p)pGpp-synthesizing region of RelA, is highly conserved and putatively lies in a stretch of 200 amino acids between residues 170 and 370 near the N-terminal region (data not shown), which comprises ~600 nucleotides. We constructed a deletion-insertion allele, Δ*relA::kan*, in which a 660-bp internal region of *relA* overlapping the putative region coding for the catalytic domain was replaced by a kanamycin resistance gene cassette. This allele was recombined into the chromosome of the wild-type *V. cholerae* O1 El Tor strain C6709 as described in Materials and Methods. The *V. cholerae relA* mutant strain thus constructed was designated SHK17. Southern hybridization analyses of the mutant SHK17 and of the wild-type parent strain, C6709, using *HincII, Nsil*, and *EcoRV* enzymes confirmed that the *relA* mutant was generated by a double-crossover event. Hybridization of a *HincII* digest of the wild-type chromosomal DNA using the *EcoRV-ParI* fragment of the *relA*/*cII* gene (Fig. 2A) as a probe showed two bands of 2.05 and 1.3 kb (Fig. 2B), and the result is consistent with the *V. cholerae* El Tor whole-genome sequence data (23). In the case of the mutant, the 2.05-kb *HincII* band of the wild-type strain was replaced with a 2.63-kb hybridizing fragment (Fig. 2B). The increase in size of this *HincII* band in SHK17 is 0.58 kb, which is due to the insertion of the 1.24-kb kanamycin resistance gene cassette in place of the 0.66-kb internal *NsiI* fragment (see below) of the *relA*/*cII* gene (Fig. 2A), as described in Materials and Methods. The other *HincII* fragment (1.3 kb) showed no change in size in SHK17, since the two *HincII* sites (23) generating this fragment are present downstream of the site of allelic exchange (Fig. 2A). Similar analysis using the other restriction enzymes, *NsiI*, provided further evidence of the authenticity of the constructed *relA* mutant strain SHK17. The restriction map of the *relA*/*cII* gene indicates that there are two *NsiI* sites within it (Fig. 2A). As a result, when this enzyme was used for Southern analysis, three fragments of the wild-type genome with sizes of 3.8, 2.9, and 0.66 kb hybridized with the *relA*/*cII* probe (Fig. 2B). Since the Δ*relA::kan* allele was created by replacing the 0.66-kb *NsiI* internal fragment of the *relA*/*cII* gene, no signal was obtained in this region when the *NsiI*-digested genome of SHK17 was probed with *relA*/*cII* (Fig. 2B). Furthermore, in SHK17, only two *NsiI* bands of 4.4 and 3.3 kb lit up in the autoradiograph (Fig. 2B). These bands can only arise when the Δ*relA::kan* allele replaces the wild-type *relA*/*cII*. The generation of these two new *NsiI* bands in SHK17 could be mapped easily, since the kanamycin resistance gene cassette (1.24 kb) itself contains two *NsiI* sites (GenBank accession number X06404) in very close proximity (only 266 bp from each other), as shown in Fig. 2A. The calculated map distances of the *NsiI* sites present upstream and downstream of the *relA* locus of the El Tor chromosome (reference 23 and this study) and within the kanamycin resistance gene were found to be identical to the sizes of the bands determined from the autoradiogram (Fig. 2B). Hybridization analysis using *EcoRV* also supported the results obtained with the enzymes *HincII* and *NsiI*. The entire *relA* gene of El Tor is located within a 3.8-kb *EcoRV* fragment (Fig. 2A), as obtained from its genome sequence data (23). Thus, the *EcoRV*-digested genome of the wild-type El Tor strain C6709 gave a single band in the 3.8-kb region when a portion of the *relA* gene was used as a probe (Fig. 2B). However, in the case of the mutant strain SHK17, the band was shifted to a 4.4-kb region (Fig. 2B). Here, the increase in band size was ~0.58 kb, which is highly consistent with the result obtained from our *HincII* analysis described above. Altogether, our analysis strongly suggests that the SHK17 mutant strain was generated by replacing the wild-type *relA*/*cII* by a Δ*relA::kan* allele.

The mutant strain SHK17 thus developed was selected for further studies. SHK17 was found to exhibit growth sensitivity to amino acid starvation induced by the addition of SHMT (33) and AT (42) (data not shown). Moreover, following amino acid starvation, SHK17 also did not show any significant (p)pGpp accumulation compared to its isogenic wild-type strain C6709 (Fig. 2). As controls, we used several *E. coli* strains (Table 1), as shown in Fig. 2. These observations are consistent with earlier reports (16, 32), where such inhibition of growth and failure of (p)pGpp accumulation were also exhibited by the *relA* mutants of other organisms upon amino acid starvation. The presence of a basal level of (p)pGpp in the *relA* null mutant may be due to the weak (p)pGpp synthetase activity of the spoT gene product (52), also displayed in other gram-negative organisms (6, 16). This hypothesis was further supported when the extracts of C6709 and its isogenic *relA* mutant SHK17, grown in amino acid-supplemented rich medium (described in Materials and Methods), were used as controls (Fig. 2C, lanes 6 and 7). These controls showed amounts of a basal level of (p)pGpp essentially similar to that of the mutant strain SHK17 grown under amino acid-starved conditions. Although *V. cholerae* possesses the spoT gene (23), whether its product has a weak (p)pGpp synthetase activity remains to be explored.

RelA acts as a positive regulator of virulence gene expression. Since (p)pGpp controls multiple cellular processes in prokaryotes, we were interested to determine whether it has any role in the regulation of expression of the major virulence factors of *V. cholerae*. To explore this possibility, we initially compared the levels of CT production in culture supernatants of both the wild-type El Tor C6709 strain and its *relA* mutant SHK17. Interestingly, the amount of CT in the culture super-
natant of strain SHK17 was found to be significantly lower (~90% reduction) than that of the wild-type strain, C6709, when both strains were grown under similar in vitro conditions favorable for optimal CT production (Fig. 3). To check whether the low level of CT in the culture supernatant of SHK17 was due to a defect in its secretion, we estimated the amount of CT in sonicated whole-cell lysates of both the parental and mutant strains. In both cases, no CT was detected (Fig. 3), indicating that the production and not the secretion of CT was actually affected in the SHK17 mutant strain. To assess whether the decrease of CT production in SHK17 was at the level of transcription, Northern blot analysis was carried out using the ctxAB genes as a probe, where total cellular RNA was prepared from cells of the wild-type and the relA mutant strain grown under AKI conditions. RNA blot analysis revealed that the production of ctxAB transcripts was severely affected in SHK17 compared to the wild-type strain, C6709 (Fig. 3). Consistent with this result, our RT-PCR experiment using RNAs from the isogenic pair C6709 and SHK17 (extracted under conditions similar to those used for the Northern blot experiment) showed that there was a significant decrease in the expression of ctxAB transcripts in the mutant strain compared to the wild type (Fig. 3). The above-mentioned evidence clearly demonstrates that RelA has a role in the regulation of production of CT in V. cholerae.

In addition to CT, TcpA is also an important virulence factor of V. cholerae, and it is well established that the expressions of both of these proteins are coregulated (29). Thus, we expected that TcpA production in SHK17 should also be affected. To test this possibility, RT-PCR was carried out, and we found that there was a significant decrease in production of tcpA transcripts in the mutant compared to the wild-type V. cholerae (Fig. 3). This was further confirmed by our Northern blot experiment using total cellular RNA of the wild-type and mutant strains with the tcpA gene as a probe. Here, also we found a notable reduction in the number of transcripts in SHK17 compared to the wild type (Fig. 3). We also performed an immunoblot experiment using cell extracts prepared from V. cholerae cultures grown under conditions similar to those mentioned above. As expected, the relA mutant SHK17 showed a severe reduction in the production of TcpA compared to its parental strain, C6709 (Fig. 3). Taken together, these data strongly suggest that like CT, expression of TcpA is also RelA dependent.

Since disruption of relA of V. cholerae led to decreased CT and TcpA production, we wanted to explore whether the expression of ToxT, the main regulator of the expression of CT and TcpA (14), is also altered in the mutant strain SHK17. To test this, we performed an RNA blot experiment to check for the expression of toxT transcripts of wild-type and relA mutant strains of V. cholerae. It was found that the toxT transcript level of SHK17 was clearly reduced compared to that of the wild type (Fig. 3). As mentioned previously, the expression of toxT is dependent on two important membrane-bound transcriptional activators, ToxR and TcpP (29). The above-mentioned results led us to question whether disruption of relA could also affect the expression of ToxR and TcpP. However, in the present study, we determined only the status of ToxR, the most important regulator of the virulence cascade (37). RT-PCR was employed again to detect any difference between the transcript levels in the wild-type and SHK17 cells. As shown in Fig. 3, the toxR transcripts of SHK17 cells were barely detectable compared to its strong and significant signal in the wild-type strain, C6709. Therefore, it appears that the reduction in pro-
tein. The anti-OmpU antiserum reacted only with the 38-kDa bands of both the wild type and SHK17, indicating that the band that was down regulated in the mutant SHK17 was indeed that of *V. cholerae* OmpU (Fig. 4). The expression of the OmpT protein was also similarly assessed with rabbit polyclonal antiserum against the OmpT protein of *V. cholerae*. As expected, the OmpT level was increased in the mutant strain SHK17 compared to its parental strain (Fig. 4). Since the expression of ToxR-regulated OmpU and OmpT proteins is also affected upon disruption of the *relA* gene of *V. cholerae*, it is probable that the virulence cascade is affected at the toxR level.

**RelA affects motility of *V. cholerae***. Expression of virulence factors and motility in *V. cholerae* are intimately linked by an as-yet-uncharacterized mechanism (17, 22). Since the production of both CT and TcpA is drastically reduced in the *relA* deletion mutant in *V. cholerae*, it was of interest to compare the motilities of the mutant strain and the wild type. The motility of the *V. cholerae* *relA* mutant was found to be reduced by \( \sim 80\% \) compared to that of the wild type. Thus, it appears that the disruption of *relA* also impairs the motility of *V. cholerae*. Why *relA* mutation leads to the reduction of both the motility and the expression of major virulence factors when previous reports (17) showed that these two mechanisms are reciprocally regulated is unknown and will require further investigation.

**Disruption of relA* VCH affects in vivo CT production**. To examine the effect of *V. cholerae* *relA* mutation on the production of CT in vivo, we used a ligated rabbit ileal loop model (12). This in vivo model has an advantage, as it allows comparative studies of the parental strain and its isogenic mutant concurrently in the same animal and thus avoids variations among individual animals. About 10^6 CFU of *V. cholerae* cells were introduced into each ligated rabbit ileal loop, and the FA ratio was determined as described in Materials and Methods. While the average FA ratio of the wild-type strain, C6709, was 1.5, that of the *relA* mutant SHK17 was only 0.2. The data indicate that, similar to in vitro laboratory conditions, a mutation in *relA* of C6709 has an effect under in vivo situations on the production of the principal virulence factor, CT, since accumulation of a large quantity of fluid in the ligated loop is considered to be due mainly to the enterotoxigenic effect of the toxin (12). To further substantiate our in vivo rabbit ileal loop results, we determined the amount of CT present in the intestinal fluid generated by the wild-type strain, C6709, and its *relA* mutant SHK17 as described in Materials and Methods. As expected, the amount of CT estimated in intestinal fluid accumulated by SHK17 was very small compared to that of its parental strain, C6709 (Fig. 5A). To rule out the possibility that the CT is retained within the cells of the mutant SHK17, we estimated the toxin level in the sonicated cell lysate and compared it with that of the parental strain. No toxin was detected in the lysates of either C6709 or SHK17 (Fig. 5A).

**Disruption of relA* VCH affects intestinal colonization**. To determine whether RelA has any role in intestinal colonization, we performed an in vivo competition experiment in suckling mice (40, 46). The *relA* mutant SHK17 (Kmr) was coinoculated with its isogenic parental strain, C6709-R (C6709 Rif r), into 3- to 5-day-old suckling mice as described in Materials and Methods. In this competition assay, SHK17 was found to be signif-
DISCUSSION

In an attempt to explore the possible involvement of the relA gene in the regulation of expression of the virulence factors in V. cholerae, we cloned and characterized the gene relA<sub>VCH</sub> from the organism. We show that the cloned gene codes for a RelA homolog by the high amino acid sequence similarity of the RelA protein with those of other organisms (38). Furthermore, a deletion-insertion mutant strain of V. cholerae, SHK17, constructed in this study also failed to accumulate (p)pGpp upon amino acid starvation (Fig. 2). All these observations strongly suggest that relA<sub>VCH</sub> functions in a manner similar to that of the relA genes of other gram-negative organisms (6, 16, 43).

Interestingly, our findings in this study demonstrate RelA-dependent regulation of the expression of the prime virulence factors CT and TCP in the El Tor biotype of V. cholerae O1. In vitro expression of CT and TCP in El Tor vibrios has been shown to require AKI conditions (26, 27). Recently, Medrano et al. (34) provided insight into the molecular basis for control of CT and TCP production in El Tor under AKI growth conditions by analyzing the expression of the major virulence determinants toxR, toxT, ctxAB, and tcpA. In the present study, when we assayed the production of CT and TCP in the relA mutant El Tor V. cholerae strain SHK17 grown under AKI conditions, we found, to our surprise, severe defects in the expression of these factors compared to that in the parental strain, C6709 (Fig. 3). Consistent with our in vitro results, we found that the relA mutant strain SHK17 produced significantly less CT in rabbit ileal loops (Fig. 5A) and was severely attenuated in colonizing the infant mouse gut (Fig. 5B). The in vivo defect in colonization shown by SHK17 appears to be due to the low level of production of TcpA (47, 48), the principal colonization factor of V. cholerae (28, 29). Thus, our data from in vivo experiments also support a positive role for RelA-dependent regulation of the expression of the prime virulence factors CT and TCP. The current model for virulence gene regulation in V. cholerae is that of a cascade in which ToxR modulates the expression of another important regulator, ToxT, and ToxT in turn directly controls the expression of several virulence genes of V. cholerae. Thus, ToxR plays a pivotal role in the pathogenesis of V. cholerae. Lee et al. (30) recently highlighted the functional importance of ToxR in virulence gene regulation in El Tor, in which they have shown an absolute requirement of ToxR for the induction of CT during infection. In this study, we provide several pieces of indirect evidence that RelA may affect the virulence cascade at the ToxR level in El Tor; these are (i) very low levels of production of the ToxR-activated major virulence factors CT and TcpA in the relA mutant SHK17 compared to those in the parental strain; (ii) reduced production of the toxT transcripts in the relA mutant, which is directly under the control of ToxR; and (iii) a decreased level of ToxR-activated OmpU and an increased level of ToxR-repressed OmpK in SHK17. At present, it is not known whether RelA, via its effector molecule (p)pGpp, has any direct role in the regulation of transcription of toxR. Proof of this hypothesis will necessitate construction of a relA<sub>VCH</sub> toxR double mutant, and such studies are in progress in our laboratory.

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