Cold Shock Response and Major Cold Shock Proteins of *Vibrio cholerae*

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When exponentially growing *Vibrio cholerae* cells were shifted from 37°C to various lower temperatures, it was found that the organism could adapt and grow at temperatures down to 15°C, below which the growth was completely arrested. There was no difference between the patterns of the cold shock responses in toxigenic and nontoxigenic strains of *V. cholerae*. Gel electrophoretic analyses of proteins of cold-exposed cells revealed significant induction of two major cold shock proteins (Csps), whose molecular masses were 7.7 kDa (CspA_{VC}) and 7.5 kDa (CspV), and six other Csps, most of which were much larger. We cloned, sequenced, and analyzed the *cspV* gene encoding the CspV protein of *V. cholerae* O139 strain SG24. Although CspA_{VC} and CspV have similar kinetics of synthesis and down-regulation, the corresponding genes, *cspA* and *cspV*, which are located in the small chromosome, are not located in the same operon. A comparative analysis of the kinetics of synthesis revealed that the CspV protein was synthesized de novo only during cold shock. Although both CspA_{VC} and CspV were stable for several hours in the cold, the CspV protein was degraded rapidly when the culture was shifted back to 37°C, suggesting that this protein is probably necessary for adaptation at lower temperatures. Northern blot analysis confirmed that the *cspV* gene is cold shock inducible and is regulated tightly at the level of transcription. Interestingly, the *cspV* gene has a cold shock-inducible promoter which is only 12 nucleotides from the translational start site, and therefore, it appears that no unusually long 5' untranslated region is present in its mRNA transcript. Thus, this promoter is an exception compared to other promoters of cold shock-inducible genes of different organisms, including *Escherichia coli*. Our results suggest that *V. cholerae* may use an alternative pathway for regulation of gene expression during cold shock.

Most human pathogens have two distinct phases of their life cycle, one when they are within their host and the other when they are in their natural environmental niche (9, 14, 25). Changes in environmental parameters lead to changes in gene expression by microbial pathogens. Thus, pathogens need an exquisite gene regulation network to survive in two such distinct environments. *Vibrio cholerae*, a gram-negative enteric pathogen whose natural environmental niche includes various aquatic bodies (6, 7, 8, 18, 22), is responsible for the severe diarrheal disease cholera and has developed a mechanism to switch gene expression in order to overcome various stressful barriers (16). For example, when *V. cholerae* cells are in the aquatic environment, they have to face fluctuations in various physicochemical parameters. When they are ingested by humans, they have to tolerate a low gastric pH, increased temperature, activities of various intestinal proteases, and other unknown intestinal factors, which profoundly influence the gene expression of the pathogen for combating such stressful stimuli. Currently, little information is available regarding the molecular mechanisms that enable *V. cholerae* to survive fluctuating physical and chemical environmental parameters, such as salinity, oxygen and nutrient availability, osmolarity, pH, atmospheric pressure, and temperature. Among all these parameters environmental temperature plays a very crucial role

in growth and survival. It has recently been shown by using remote sensing data that the occurrence of cholera cases has a high correlation with the annual cycle of sea surface temperature (7, 18).

V. cholerae is remarkable for its pandemic potential, and it can spread globally. The pandemic nature of *V. cholerae* indicates that this organism can adapt and survive in diverse environmental conditions (14). During its spread the pathogen must face drastic changes in environmental temperatures. Moreover, *V. cholerae* often encounters a sudden temperature downshift (i.e., cold shock) as a result of excretion from a human whose body temperature is 37°C. Colwell and Huq (8) showed that among various changes in environmental conditions, a reduction in temperature is important in inducing *V. cholerae* cells to enter a dormant state, termed the viable but nonculturable (VBNC) state, in which metabolically active cells cannot be cultured on microbiological media. Microcosm studies showed that VBNC cells could remain viable in the environment for years and continue to be capable of causing disease (8). Unfortunately, detailed information regarding the molecular basis of the cold shock response in *V. cholerae* is not available. Such information is also important in understanding how this pathogen manages to survive during interepidemic periods.

In *Escherichia coli* a temperature downshift from 37 to 10°C causes transient inhibition of synthesis of most cellular proteins, resulting in a growth lag period called the acclimation phase. During this period at least 16 different cold shock proteins (Csps) are induced (29); one of these, CspA, whose molecular mass is 7.4 kDa, is dramatically induced and is considered essential for cold adaptation (11, 29). The amino acid

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sequence of CspA exhibits 43% identity to the cold shock domain of the eukaryotic Y-box proteins, which interact with RNA and DNA to regulate their functions (17, 26, 34). It has been proposed that CspA of *E. coli* functions as an RNA chaperone to prevent the formation of secondary structures in RNA molecules at low temperatures (29). In addition to CspA, eight other very similar proteins (CspB to -I), constituting the CspA family of proteins, have been identified (29). However, not all of these proteins are cold shock inducible (29). Another unique feature of the cold shock-inducible *cspA* homologues is the presence of an unusually long 5' untranslated region (5'-UTR) in the mRNA transcript that plays an important role in the regulation of cold shock gene expression (29).

In this report, we show that *V. cholerae* has a cold shock response analogous to but distinct from that of *E. coli*. In *V. cholerae* two small Csps, whose molecular masses were 7.7 and 7.5 kDa (CspA_{VC} and CspV, respectively), were induced simultaneously upon sudden exposure to cold. However, only CspV appeared to be a true Csp. The *cspV* gene, located in the small chromosome and encoding the CspV protein, was cloned from strain SG24 of *V. cholerae* O139 and sequenced. Analysis of the sequence indicated that its promoter region does not contain a 5'-UTR, a common feature found in homologous genes characterized from other organisms. Northern blot analysis indicated that expression of the *cspV* gene is tightly regulated at the level of transcription.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. The experiments were performed with the well-characterized, cholera toxin-producing *V. cholerae* O139 Bengal strain SG24 (3, 15, 24). *E. coli* DH5 α (Promega, Madison, Wis.) and plasmid pUC19 (New England Biolabs, Beverly, Mass.) were used for cloning. *E. coli* strain BL21(DE3) and plasmid pET20b(+) (Novagen, Madison, Wis.) were used for expression purposes.

V. cholerae cells were grown in a gyratory shaker at 37°C in nutrient broth (Difco, Detroit, Mich.) containing 0.1 M NaCl (pH 8.0), and for growth at low temperatures, cells were grown aerobically in a rotary-action water bath shaker. Bacterial growth was monitored spectrophotometrically at a wavelength of 585 nm (optical density at 585 nm [OD₅₈₅]). Viable plate counts were determined on nutrient agar plates (22) and were expressed in CFU per milliliter. Cells were maintained at -70°C in nutrient broth with 15% (vol/vol) glycerol. *E. coli* cells were grown in Luria-Bertani medium (1) at 37°C. When necessary, ampicillin was added at a final concentration of 100 μ g ml⁻¹.

Labeling of cellular proteins with [³⁵S]methionine. For labeling of cellular proteins with [³⁵S]methionine (1,000 Ci mmol⁻¹; Amersham, Little Chalfont, United Kingdom), the cells were grown in a modified M9 minimal medium, developed in this study and designated MM9 medium, whose composition was the same as the composition described previously (31) except that the concentration of ammonium chloride was 1.1% instead of 0.1%. *V. cholerae* cells showed optimal growth and efficient labeling when MM9 medium was used (data not shown). *V. cholerae* cells used for cold shock experiments were grown to the mid-exponential phase (OD₅₈₅, 0.55) at 37°C in MM9 medium, and aliquots of the culture were shifted immediately to different low temperatures. The cultivability of a culture at a low temperature was assayed by plate counting (22) at different times after the temperature shift. To analyze Csps, 3 ml of the culture was labeled with [³⁵S]methionine (10 μ Ci ml⁻¹) for 10 min at 37°C, which served as a control, and for 30 min after different periods of exposure to 15°C (see Fig. 2). Sometimes cells were labeled with [³⁵S]methionine for an extended period of time either at 37°C or at 15°C. For pulse-chase experiments cells were labeled with [³⁵S]methionine for different periods of time at 15°C and then chased by adding nonradioactive methionine (Sigma-Aldrich, Bangalore, India) to a final

concentration of 0.2 M (see Fig. 3). In some experiments (see Fig. 3), a *V. cholerae* culture was transferred from 37 to 15°C, labeled with [³⁵S]methionine for 1 h, and then immediately shifted back to 37°C and chased with nonradioactive methionine as described above.

Protein analysis. For better resolution of low-molecular-weight proteins we used the previously described 16.5% Tris-Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (T-SDS-PAGE) system (32). Prestained protein molecular mass standards, including high-molecular-mass standards (14.3 to 200 kDa) and low-molecular-mass standards (3 to 43 kDa) (Gibco-BRL, Gaithersburg, Md.) or SeeBlue prestained standards (210 to 4 kDa; Invitrogen, Carlsbad, Calif.), were used when they were needed. Concentrations of protein samples were determined by the Bradford method (1). For two-dimensional gel electrophoresis (2D-E), whole-cell protein samples were prepared from *V. cholerae* O139 strain SG24 cells by sonication (B. Braun Biotech, Allentown, Pa.), followed by suspension in lysis buffer as described previously (27, 28). Separation in the first dimension was performed by nonequilibrium pH gradient electrophoresis as described previously (27, 28) with a Bio-Rad Protean IIx system by using ampholines (Sigma-Aldrich) in the pH range from 3 to 10. For the second dimension, 16.5% T-SDS-PAGE was performed as described above. For fluorography, gels were treated with the Amplify fluorographic reagent (Amersham) as directed by the manufacturer, and this was followed by drying and autoradiography.

Antibiotic treatment. Chloramphenicol was used at a final concentration of 3 μ g ml⁻¹ as described previously (10), and *V. cholerae* cells were labeled with [³⁵S]methionine before or after addition of chloramphenicol both at 37°C and after cells were shifted to 15°C.

PCR amplification, cloning, and sequencing of the *cspV* gene of *V. cholerae*. The primers used to amplify the *cspV* gene were designed from the available genome sequence data for *V. cholerae* O1 strain N16961 (12). The *cspV* gene was PCR amplified from genomic DNA of *V. cholerae* O139 strain SG24 by using *Taq* DNA polymerase (Invitrogen) and primers VCL1 (5'-GCGAATTCATTCAATTCTGAAGA-3') and VC2 (5'-AAGGATCCGTAAATAATGAGCGGGGAGCAA-3'), which introduced *Eco*RI and *Bam*HI cleavage sites (underlined) for cloning a ~665-bp amplicon into the *Eco*RI-*Bam*HI-digested vector DNA pUC19. The resulting recombinant plasmid containing the *cspV* gene was designated pPD665. The PCR-amplified insert DNA was directly sequenced with an automated DNA sequencer (ABI Prism model 377). A 210-bp fragment spanning the predicted promoter region along with part of the N-terminal coding sequence of the *cspV* open reading frame (ORF) was amplified by PCR by using the 665-bp DNA fragment as the template with primers VCL2 (5'-TGAATTCGATACCCCTTCGTTCAACC-3') and VC1 (5'-ACGGTACCATCGTTGCCACCGTTGCTT-3') and was used as a probe.

Construction of pET20b(+)-*cspV* for overexpression of the *cspV* gene. For overexpression of *cspV*, a 288-bp *Xba*I-*Bam*HI fragment containing the complete ORF plus the intact Shine-Dalgarno sequence of the gene was obtained by digesting pPD665. The gel-purified fragment was ligated to *Xba*I- and *Bam*HI-double-digested pET20b(+) overexpression vector, which contained a T7 promoter under control of the *lac* operator (Novagen). The ligation mixture was transformed into *E. coli* DH5 α cells, and the recombinant plasmid was designated pET20b(+)-*cspV*. Then *cspV* expression plasmid pET20b(+)-*cspV* was transformed into *E. coli* strain BL21(DE3), which contained T7 RNA polymerase. The transformed cells were grown in Luri-Bertani medium supplemented with 0.1% glucose at 37°C with shaking to the mid-logarithmic phase (OD₅₈₅, 0.55). At this cell density, isopropyl- β -D-thiogalactopyranoside (IPTG) (final concentration, 1.0 mM) was added for induction of expression of the *cspV* gene, and incubation was continued for another 2 h; this was followed by harvesting of the cells and analysis of the overexpressed protein by T-SDS-PAGE.

Western blot analysis. Total cellular proteins were separated by T-SDS-PAGE and transferred to nitrocellulose in a transblot apparatus (Bio-Rad) as described previously (1). The blots were incubated for 1 h with rabbit antibody raised against the CspA protein of *E. coli* (kindly provided by M. Inouye) at a dilution of 1:500, and this was followed by treatment with anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Gibco-BRL). Color was developed by using standard methods described previously (1).

Northern blot procedure. Total RNA of *V. cholerae* cells, which were grown at different temperatures for various times, was isolated by using an RNeasy kit (Qiagen GmbH, Hilden, Germany) and the protocol described by the manufacturer. For Northern analysis, the same amount (~20 μ g) of RNA sample was loaded in each lane and separated in a 1.2% agarose gel containing formaldehyde (31); this was followed by staining with ethidium bromide and transfer of the RNA from the gel to a Nytran membrane (Hybond N+; Amersham). The PCR-amplified 210-bp DNA segment of the *cspV* gene of *V. cholerae* (see above) was labeled with [α -³²P]dCTP by using an NEBlot random priming kit (New

FIG. 1. Growth kinetics of *V. cholerae* O139 strain SG24 at various temperatures. The arrow indicates the OD₅₈₅ at which cultures were shifted from 37°C to low temperatures.

England Biolabs) as instructed by the vendor and was used as a probe. Hybridization was carried out at 60°C, and this was followed by washing, exposure to X-ray film, and autoradiography.

Nucleotide sequence and other computer-based analyses. Restriction enzyme recognition sites were determined with the DNASIS program (Hitachi Corporation). A nucleotide similarity search was performed by using the BLASTN program (www.ncbi.nlm.nih.gov). The promoter region was predicted by using the Neural Network Promoter Prediction program (www.fruitfly.org/seq_tools/promoter.html), and the ORF region was predicted by using the ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). All the primers were designed by using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). Theoretical isoelectric points (pI) and molecular weights (M_w) of putative cold shock gene-encoded proteins of *V. cholerae* were determined by using the ExPASy compute pI/ M_w tool (www.expasy.ch/tools/#align).

Nucleotide sequence accession number. The *cspV* gene sequence determined in this study has been deposited in the GenBank database under accession number AF409091.

RESULTS AND DISCUSSION

Minimal growth temperature of *V. cholerae*. When exponentially growing *V. cholerae* O139 cells were transferred from 37 to 10 or 12°C, growth stopped immediately, and the cells exhibited negligible growth for more than 12 h, indicating that these low temperatures are growth nonpermissive for *V. cholerae* (Fig. 1). These results are similar to the results obtained for other *V. cholerae* strains (24) belonging to different serotypes and biotypes (data not shown). There was no significant difference in the cell counts ($\sim 7 \times 10^8$ CFU ml⁻¹) between the cultures before the temperature downshift and the cultures after the temperature downshift, indicating that at the lower temperatures the cells were unable to replicate. Our results are consistent with the microcosm study conducted by Singleton et al. (33). These authors suggested that prolonged survival at 10°C may ultimately induce the VBNC state in *V. cholerae* (33). However, when *V. cholerae* cells were shifted from 37 to 15°C, there was a growth lag of about 4 h, after which growth resumed, and within 6 h of the shift the culture reached the

FIG. 2. Autoradiogram showing the kinetics of induction of CspA_{VC} and CspV. Pulse-labeling at 15°C was performed for the following periods: lane 1, 0 to 30 min; lane 2, 30 to 60 min; lane 3, 60 to 90 min; lane 4, 90 to 120 min; lane 5, 120 to 150 min; lane 6, 150 to 180 min; and lane 7, 270 to 300 min. Lane C contained cells that were labeled for 10 min at 37°C. Lane L contained cells that were labeled at 15°C for 5 h. The solid and open arrowheads indicate the positions of CspA_{VC} and CspV, respectively. Lane M contained standard molecular mass markers, whose sizes (in kilodaltons) are indicated on the left.

stationary phase (Fig. 1). These results suggest that 15°C is the minimal temperature for growth of *V. cholerae* and that below this temperature the cells are unable to grow. In contrast, *E. coli* cells exhibit a similar type of cold shock response (11) at a much lower temperature (10°C), indicating that *V. cholerae* is more sensitive to cold than *E. coli*. When *V. cholerae* cells were shifted from 37 to 22°C, the length of the acclimation phase was reduced drastically, to only 30 min (Fig. 1), indicating that under laboratory conditions a downshift to 22°C has a minor effect on the growth of *V. cholerae*.

Simultaneous induction of a 7.7-kDa Csp (CspA_{VC}) and a 7.5-kDa Csp (CspV) occurs at 15°C. To determine the cold shock-induced proteins of *V. cholerae*, cells were pulse-labeled with [³⁵S]methionine at 15°C and then subjected to T-SDS-PAGE and autoradiography as described in Materials and Methods. The autoradiogram obtained revealed significant induction of two closely migrating protein bands at molecular masses of approximately 7.7 and 7.5 kDa (Fig. 2), which were designated CspA_{VC} and CspV, respectively. This is in contrast to the findings obtained for *E. coli*, in which only one major protein, the 7.4-kDa CspA protein, was induced at a significant level (11). The dramatic increases in the intensities of CspA_{VC} and CspV compared to the intensities of other cellular proteins of *V. cholerae* suggest that these two proteins are likely to be the major Csps of *V. cholerae*. Furthermore, it appeared from the autoradiogram that in addition to CspA_{VC} and CspV another low-molecular-mass protein band in the 8.3-kDa region appeared to be slightly up-regulated at 15°C. However, small amounts of CspA_{VC} were also detected at 37°C (Fig. 2). Thus, it is highly likely that CspV is induced only during exposure of *V. cholerae* cells to a low temperature and is truly a product of a cold shock-inducible gene.

Strains belonging to different serotypes and biotypes, as well as toxinogenic and nontoxinogenic groups (24), had cold shock-induced protein profiles similar to that of *V. cholerae*

FIG. 3. Stability of CspA_{VC} and CspV. (A) Cells were pulse-labeled for the following periods after they were shifted back from 15 to 37°C: lane 1, 0 to 15 min; lane 2, 15 to 30 min; lane 3, 30 to 45 min; lane 4, 45 to 60 min; and lane 5, 0 to 3.5 h. Lanes C1 and C2 contained cells that were labeled for 10 min at 37°C and for 60 to 90 min at 15°C, respectively. Lanes C3 and C4 contained cells that were labeled for 3.5 h at 37°C and at 15°C, respectively. The solid and open arrowheads indicate the positions of CspA_{VC} and CspV, respectively. (B) Lanes C1 and C2 contained cells that were labeled for 10 min at 37°C and for 60 to 90 min at 15°C, respectively. Cells were labeled for 2 h at 15°C and then chased with nonradioactive methionine (see Materials and Methods) for the following periods: lane 1, 1 h; lane 2, 3 h; and lane 3, 12 h. Lanes S1 and S2 contained cells that were labeled at 15°C for 1 h, shifted back to 37°C, and chased with nonradioactive methionine for 15 and 30 min, respectively. The solid and open arrowheads indicate the positions of CspA_{VC} and CspV, respectively.

O139 strain SG24 (data not shown). In contrast, Carroll et al. (5) recently reported induction of only one 8-kDa protein in *V. cholerae* cells upon exposure to cold. The discrepancy between the results of these authors and our results was probably due to poor separation of low-molecular-weight proteins by conventional Tris-glycine SDS-PAGE. Simultaneous induction of significant amounts of two small Csps appears to be typical for *V. cholerae* cells and has not been reported for any other bacterium examined so far. Further analyses of the kinetics of induction revealed that synthesis of both proteins reached the maximum level within 30 to 90 min after the shift (Fig. 2, lanes 1 to 3). The maximal amount of each of the proteins was about 7% of the total cellular proteins, and thus, during cold shock these proteins accounted for about 14% of the total proteins within 1 to 1.5 h. Although the de novo synthesis of both the proteins decreased significantly just after 2 h after the shift, the synthesized proteins were found to be stable for at least 5 h at 15°C (Fig. 2).

Synthesis of CspV is more tightly regulated than synthesis of CspA_{VC}. Initial exposure of *V. cholerae* cells to 15°C for 1 h and then a shift of the culture to 37°C resulted in inhibition of synthesis of CspV within 15 min (Fig. 3A). In contrast, the synthesis of CspA_{VC} decreased only slightly (Fig. 3), indicating that CspV is a true cold shock-induced protein and stable only at low temperatures. The stability of each protein after the cells were shifted from 15 to 37°C was also verified by pulse-chase experiments, as shown in Fig. 3B. These results also indicate that the synthesis of CspV is more tightly regulated than the synthesis of CspA_{VC}.

Role of Csps in cold adaptation. When an exponentially growing culture of *V. cholerae* was preincubated at 15°C for 2 h to allow sufficient expression of the cold stress proteins CspA_{VC} and CspV (as shown in Fig. 2) and then exposed to 10°C, the cells with prior exposure to 15°C were able to grow at 10°C, whereas cells which were shifted directly from 37 to 10°C

FIG. 4. Role of Csps in cold adaptation of *V. cholerae* and status of CspA_{VC} and CspV in the presence of chloramphenicol. (A) Growth of *V. cholerae* SG24 at various temperatures was monitored as described in Materials and Methods. The solid and open arrowheads indicate the OD₅₈₅ values at which *V. cholerae* cells were shifted from 37°C to low temperatures and from 15 to 10°C, respectively. The curved arrow indicates the enhanced growth rate at 10°C after preincubation at 15°C. (B) Lanes 1 and 2 contained *V. cholerae* cells grown at 37°C in absence and presence of chloramphenicol, respectively. [³⁵S]methionine labeling of cellular proteins in the presence of chloramphenicol at 15°C was done for the following periods: lane 3, 0 to 30 min; lane 4, 30 to 60 min; lane 5, 60 to 90 min; lane 6, 150 to 180 min; and lane 7, 5 to 5.5 h. For lanes 8 and 9, chloramphenicol was added 4.5 h after the shift from 37 to 15°C and this was followed by labeling at 5 to 5.5 h and at 6 to 6.5 h, respectively. The solid and open arrowheads indicate the positions of CspA_{VC} and CspV, respectively.

were not able to grow (Fig. 4A). Thus, priming of cells at 15°C probably helped the organism adapt and grow at 10°C, which otherwise is nonpermissive for growth. However, the possibility that other uncharacterized Csps induced during priming of *V. cholerae* cells at 15°C play a role in cold adaptation at 10°C cannot be ruled out.

Status of CspA_{VC} and CspV in the presence of chloramphenicol. When the antibiotic chloramphenicol, an inhibitor of translation, was added at a concentration of 3 μg ml⁻¹ to an actively growing culture of *V. cholerae* at 37°C, the growth of cells was arrested almost immediately. No growth was observed for several hours (data not shown), and there was no induction of any Csps (Fig. 4B, lane 2). These results indicate that inhibition of translation is not the stimulus for induction of Csps during cold shock. However, when chloramphenicol was added just after the shift from 37 to 15°C, although the cells failed to resume growth after a lag period, CspA_{VC} and CspV were found to be synthesized constitutively (Fig. 4B, lanes 3 to 7). Furthermore, constitutive production of both of the pro-

FIG. 5. Autoradiograms of 2D-E gels showing the major Csp of *V. cholerae*. Proteins were labeled and separated as described in Materials and Methods. The spots for proteins that were down-regulated due to a shift from 37 to 15°C and the spots for proteins which were induced due to cold shock are circled in panels A and B, respectively, and are numbered on the basis of size in ascending order in the autoradiograms. The same amount of protein was loaded in each gel.

teins continued for several hours (Fig. 4B), which is significantly different from the results obtained with cells that were not treated with chloramphenicol, as shown in Fig. 2. It was also observed that in the presence of chloramphenicol the expression of CspV was more pronounced than the expression of CspA_{VC} (Fig. 4B, lanes 6 and 7). These results further support the contention that in *V. cholerae* CspV plays a more vital role in cold adaptation than CspA_{VC} plays. Prolonged treatment (>5 h) of cells with chloramphenicol at 15°C showed that there was no further increase in the synthesis of CspA_{VC} and CspV (Fig. 4B, lane 7). Our data are consistent with the results obtained in similar experiments carried out with *E. coli* (29). However, in contrast to the results obtained with *E. coli*, addition of chloramphenicol at 4.5 h after the temperature shift resulted in no significant de novo synthesis of CspA_{VC} or CspV (Fig. 4B, lanes 8 and 9). Together, the data suggest that there is a common mechanism for induction of Csp by chloramphenicol in these two heterogeneous organisms.

2D-E analysis of cold-induced proteins. The number of proteins of *V. cholerae* that were differentially expressed due to cold shock was determined by 2D-E. Comparison of autoradiograms containing the protein spots of cells grown at 37°C and cells grown at 15°C revealed that while the levels of intensity of 40 proteins remained the same, there was down-regulation of at least six proteins (Fig. 5A, spots A1 to A6) and up-regulation of eight proteins of *V. cholerae* during cold shock adaptation (Fig. 5B, spots B1 to B6). Of the eight cold-induced proteins, two basic proteins at about 7.5 and 7.7 kDa were induced significantly (Fig. 5B, spots B1 and B2, respectively), and the sizes of these proteins were similar to those of CspV

and CspA_{VC}, respectively, as determined by one-dimensional T-SDS-PAGE (Fig. 2). These results are consistent with our one-dimensional T-SDS-PAGE results (Fig. 2). An 8.3-kDa small acidic protein was also up-regulated compared to the basal level at 37°C due to cold shock (Fig. 5B, spot B3). It is interesting that while the CspA_{VC} and CspV proteins of *V. cholerae* are highly basic, the major cold-induced protein of *E. coli*, CspA, and its homologues in other organisms are acidic (2, 29).

Identification of genes coding for CspA_{VC} and CspV. By using the sequence data for the whole genome of *V. cholerae* (12) it was found that there are four cold shock-inducible genes coding for ~7-kDa small proteins (Table 1). The *cspD* gene is present in the large chromosome, and the other three genes, *cspA*, a cold shock domain family protein gene, and a cold shock DNA binding domain protein gene (no specific designa-

TABLE 1. Computed pI and *M_w* values of putative cold shock proteins encoded by the predicted cold shock genes of *V. cholerae*^a

Gene (chromosome)	pI	<i>M_w</i>	TIGR accession no. ^b
<i>cspA</i> (small)	9.22	7.629	VCA0166
Cold shock domain family protein coding gene (small) ^c	8.12	7.628	VCA0933
Cold shock DNA binding domain protein coding gene (small)	6.57	7.531	VCA0184
<i>cspD</i> (large)	6.02	8.328	VC1142

^a See reference 12.

^b TIGR, The Institute for Genome Research.

^c Designated *cspV* in this study.

FIG. 7. Northern blot analysis of the *cspV* mRNA transcripts after cold shock. A 210-bp DNA fragment of the *cspV* gene was used as a probe. The time (in minutes) at which an aliquot was removed from a *V. cholerae* culture growing either at 37°C or at 15°C is indicated above each lane. The zero indicates the time of the shift from 37 to 15°C. The same amount of RNA was loaded in each lane. The arrowhead indicates the position of the hybridized band of *cspV* transcripts.

csdA mRNAs of *E. coli* (13, 37), was found to be absent in the upstream region of *cspV*. This analysis indirectly supports the absence of a 5'-UTR in the *cspV* mRNA. It has been shown that the cold box sequence in *E. coli* is able to prolong the production of CspA upon cold shock (21). If these analyses are considered together, it is highly probable that the regulation of expression of *cspV* is different than the *cspA*-like gene regulation in *E. coli*, and further investigation is warranted. On the other hand, the presence of a conserved sequence (5'-ATGA CTGGTCTGTA-3') 12 bases downstream of the initiation codon (nucleotide positions 338 to 352), called the downstream box, which is a characteristic of all true (class I) cold shock-inducible genes examined so far (21, 37), indicates that *cspV* is probably induced during cold shock. This downstream box feature in the cold shock genes has been suggested to be important for the most effective formation of a translation initiation complex (21). Our assumption that the *cspV* gene of *V. cholerae* is a class I cold shock-inducible gene is strongly supported by the recent microarray-based study of Merrell et al. (20), in which they performed a human-shed whole-bacterial-cell transcriptional profiling analysis. These authors showed that it is indeed the *cspV* gene (The Institute for Genomic Research accession number VCA0933) which is induced significantly when the cells are exposed to the environment (apparently low temperatures compared to the human body temperature, 37°C).

Expression of *cspV* in *V. cholerae* is regulated at the transcription level. Northern blot analysis performed with the 210-bp *cspV* gene region as a probe revealed that transcription of the *cspV* gene begins within 15 min after transfer of a culture from 37 to 15°C and reaches a maximum at 60 min and that inhibition occurs within 120 min (Fig. 7). In sharp contrast, no *cspV* transcript was detected in cells maintained at 37°C for various lengths of time (Fig. 7). These results strongly suggest that the *cspV* gene of *V. cholerae* is induced only during cold shock and that its expression is very tightly regulated at the level of transcription. These results are also consistent with the findings of Merrell et al. (20), who showed that there was a high level of transcription of the *cspV* gene (accession number VCA0933) when *V. cholerae* cells were released from the human intestine into the environment.

Overexpression of CspV and recognition of CspV by the CspA antiserum of *E. coli*. To further substantiate that the *cspV* gene does encode a 7.5-kDa protein, we constructed a

FIG. 8. (A) Overexpression of the CspV protein in *E. coli*. Lanes I and U contained cells grown under IPTG-induced and uninduced conditions, respectively. The arrow indicates the position of the overexpressed CspV protein. The solid and open arrowheads indicate the positions of CspA_{VC} and CspV, respectively. Lane M contained protein molecular mass standards, whose sizes (in kilodaltons) are indicated on the left. (B) Immunoblot detection of cold shock-induced CspA_{VC} (solid arrowhead), CspV (open arrowhead), and overexpressed CspV (arrow) by using rabbit polyclonal antiserum against the CspA protein of *E. coli*. Lanes I and U contained cells grown under IPTG-induced and uninduced conditions, respectively. The asterisk indicates the position of a nonspecific protein band of *E. coli* that cross-reacted with the antiserum.

cspV expression plasmid, pET20b(+)-*cspV*, as described in Materials and Methods. When the expression of CspV was induced by IPTG in *E. coli*, there was an additional overexpressed band at ~7.5 kDa in the sample compared to the bands obtained with the cells that were not induced by IPTG (Fig. 8A). To determine the size of the overexpressed protein unequivocally, a protein sample prepared from *V. cholerae* cells exposed to 15°C was also electrophoresed side by side on a gel (Fig. 8A). The migration of the IPTG-induced protein band expressed in *E. coli* was found to be identical to that of the cold-induced CspV protein of wild-type *V. cholerae* (Fig. 8). The results strongly suggested that the *cspV* gene present in plasmid pPD665 coded for the protein CspV. This conclusion was confirmed by an immunoblot experiment in which we used rabbit polyclonal antiserum against the CspA protein of *E. coli*

(kindly provided by M. Inouye, University of Medicine and Dentistry, Newark, N.J.). The anti-CspA antiserum reacted with the 7.5-kDa overexpressed protein of *V. cholerae* in *E. coli*, as well as cold-induced CspA_{VC} and CspV of *V. cholerae* (Fig. 8B and C). These results confirmed that the insert in recombinant plasmid pPD665 indeed coded for the CspV protein of *V. cholerae*.

Conclusion. In this study, we demonstrated the cold shock response in *V. cholerae* and showed that there is significant induction of two major cold shock-related proteins, CspA_{VC} and CspV. It was found that CspV is a class I cold shock-induced protein which is stable only at low temperatures. Our in vitro experimental results are consistent with the results of the recently conducted in vivo study of Merrell et al. (20), in which transcriptional profiling of human-shed *V. cholerae* cells revealed a high level of expression of a cold shock-inducible gene (accession number VCA0933) (Table 1) which is actually the *cspV* gene identified and characterized in the present study. Further research based on these results may contribute to our understanding of the mechanisms of environmental persistence of *V. cholerae* for maintenance of its pathogenic cycle. It is noteworthy that the genes coding for CspA_{VC} and CspV are both located in the small chromosome. Although the evolution and function of the small chromosome in *Vibrio* are currently not known, it has been suggested that the small chromosome might be involved in unique but poorly understood biological properties of *V. cholerae* (12). One such property is the ability of *V. cholerae* cells to enter into the VBNC state, which can be induced by environmental stresses, such as a suboptimal temperature or nutrient starvation (8, 33). Since the molecular basis of initiation and maintenance of the VBNC state has not been determined, it should be interesting to investigate whether the products of the *cspA* and/or *cspV* gene(s) have any role in the transition of *V. cholerae* cells to a dormant phase that leads to survival of the cells in the environment, which appears to be very crucial in maintaining the pathogenic cycle of the organism.

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