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Vibrio cholerae Hcp, a Secreted Protein Coregulated with HlyA

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Hcp is a 28-kDa secreted protein of *Vibrio cholerae* regulated coordinately with the hemolysin, HlyA. Both proteins show a dependence on HlyU for expression, suggesting that Hcp may be secreted by *V. cholerae* in vivo. We have identified and sequenced two genes for Hcp, designated *hcpA* and *hcpB* (hemolysin-coregulated protein). The genes encode identical amino acid sequences. Both express a 28-kDa protein, despite open reading frames with only a 19-kDa capacity, suggesting that the Hcp protein runs aberrantly on polyacrylamide gel electrophoresis. There is no cleavage involved in secretion of Hcp from the cell, suggesting a novel mechanism of secretion. An *hcp* null mutant was constructed, and this strain displayed no deficiency in virulence or colonization in the infant mouse cholera model. From sequence data and primer extension analysis, we predict that the *hcp* promoter is the σ^{54} type, with a candidate integration host factor binding site upstream. Although *hcp* and *hlyA* are coregulated by HlyU, there are no obvious similarities between their promoters. We predict that an intermediate regulator may be involved in the activation of *hcp* by HlyU. This raises the possibility that HlyU is part of a regulatory cascade.

Regulation of virulence genes in *Vibrio cholerae* can involve the ToxR, Fur, or HlyU regulatory system (5). The ToxR, ToxS, and ToxT proteins compose an environmental sensing system controlling expression of the cholera toxin, toxin-coregulated pilus, accessory colonization factor, and other outer membrane proteins (4). This regulatory system is critical for virulence (11). Fur regulates gene expression in response to iron levels, and in addition to controlling IrgA, IrgB (10), and HlyA (21), it affects the expression of more than 20 other proteins in *V. cholerae* (14). The HlyU regulator controls expression of HlyA (26), and an *hlyU* mutation attenuates *V. cholerae* O17 in the infant mouse cholera model (25). The possibility that HlyU controls expression of virulence determinants in addition to HlyA was suggested by the reduced colonizing ability of an *hlyU* mutant compared with that of an *hlyA* mutant (25). Therefore, other HlyU-regulated proteins, such as Hcp, are of interest in the study of *V. cholerae* pathogenesis. Since both HlyA and Hcp are secreted, it is likely that they interact with host tissues, and their coordinate expression suggests that they may be required at similar stages of infection, possibly having complementary activities. To examine these possibilities we have undertaken the characterization of two *hcp* genes from *V. cholerae* O17 and construction of an *hcp* null mutant. Assessment of both the 50% lethal dose and colonizing ability of the *hcp* mutant has been made in the infant mouse model.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *V. cholerae* O17 (El Tor and Ogawa) was obtained from laboratory stocks. *V. cholerae* and *Escherichia coli* were routinely grown in nutrient broth (NB, Oxoid nutrient broth no. 2) consisting of (per liter of distilled H_2O) 10 g of Lab-lemco (Oxoid), 10 g of Bacto Peptone (Oxoid), and 5 g of NaCl. Nutrient agar was NB with the addition of 1.5% BBL technical-grade agar. Ampicillin and kanamycin were used at final concentrations of 50 μg/ml. Plasmids pGEM3Zf(+) and pGEM7Zf(+) (Promega Corporation), pBluescriptIIKS(−) (Stratagene), pSUP301 (20), and pPM1143 (8) and clones in these vectors were maintained in *E. coli* K-12 strain DH5α [F^- ϕ80d *lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *recA1 endA1 hsdR17* ($r_k^-m_k^+$) *supE44* λ− *thiA gyrA relA1*). Clones in the vector pCVD442 (6) were maintained in *E. coli* S17-1 λ*pir* (*pro hsdR* RP4-2-Tc::Mu Km::Tn7 λ*pir*).

Molecular genetic techniques. Standard recombinant DNA techniques described by Sambrook et al. (19) were employed. Degenerate oligonucleotide 546 (Fig. 1) was used as a probe to an O17 cosmid library by 5′ labelling with [γ-^{32}P]ATP by using T4 polynucleotide kinase, and hybridization was detected by autoradiography. Fragments used as probes for Southern analysis and colony hybridization were labelled with digoxigenin by using a Boehringer Mannheim digoxigenin DNA-labelling kit. Probe detection was achieved by using peroxidase or alkaline phosphatase-linked antidigoxigenin Fab fragments and Amersham ECL or Boehringer Mannheim color detection solutions. The DNA sequence was determined by using an Applied Biosystems model 373A automated sequencer with dye-labelled primers. Sequence reactions were carried out on double-stranded plasmid DNA according to the protocols provided by Applied Biosystems. Nested deletion constructs for DNA sequencing were generated by using a Promega Erase-a-Base kit. Transformation, electroporation, and conjugation were done as described previously (25).

SDS-PAGE analysis. Supernatant fractions were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis by precipitation with 12% (final concentration) trichloroacetic acid on ice for 60 min. The pellets were neutralized with NaOH and resuspended in sample buffer. The T7 RNA polymerase system was used as described by Tabor and Richardson (22). Plasmid pGP1-2 provided T7 RNA polymerase after a temperature shift to 42°C. Proteins were electrophoresed on SDS–15% polyacrylamide gels (15) and stained with Coomassie brilliant blue R250. Transfer to a polyvinylidene difluoride membrane was done as recommended by the manufacturer (Bio-Rad).

Hcp antiserum production and immunoblot analysis. To raise anti-Hcp rabbit antiserum, O17 supernatant proteins were separated by SDS-PAGE, and the Hcp band was excised, homogenized with an equal volume of Span 85-paraffin oil (1:5), and used for immunizations. Absorption of the antiserum was carried out against a total of 1.2×10^{11} *E. coli* cells. Western blot (immunoblot) transfer was done according to the method of Towbin et al. (24). The antigen was detected with absorbed rabbit anti-Hcp antiserum (1/2,000) followed by goat anti-rabbit immunoglobulin G coupled with horseradish peroxidase (Nordic Immunology).

Primer extension analysis. RNA was prepared by the hot phenol method of Aiba et al. (1). Transcripts were annealed to synthetic 25-mer oligonucleotide primer 750 (5′-AGAGTCAGCAGTCCATGCACCTGCA-3′) that was 5′ labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Boehringer Mannheim). Primer annealing and extension reactions were carried out as described previously (26). Extension products were separated on 6% acrylamide–8M urea sequencing gels and visualized by autoradiography.

Marker exchange mutagenesis of *hcp*. The use of pCVD442 as a cloning vector for marker exchange mutagenesis was as described by Butterton et al. (3).

Virulence assays in infant mice. The infant mouse cholera model was used as described by Attridge and Rowley (2). Colonization experiments were performed as previously outlined (25).

Sequence analysis. Sequence analysis was performed by using DNAsis and PROsis.

Nucleotide sequence accession number. The sequence determined in this study has been submitted to the EMBL, GenBank, DDBJ nucleotide sequence data libraries under the accession no. 84650.

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Pro Gln Pro Xaa Tyr Ile Ser Ile Glu Gly Gln Thr Gln Gly Leu Ile Thr Ala Gly Ala Xaa Thr Ala
                            ATT GAA GGT CAA ACT CAA GG
                              C   G   C   G   C   G
                              A       A       A
                                      G       G
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FIG. 1. N-terminal amino acid sequence of Hcp. The sequence of degenerate oligonucleotide 546 is shown below the appropriate amino acid sequence. Xaa denotes an unresolved residue.

RESULTS

Cloning and DNA sequence of *hcp* gene. Expression of Hcp is upregulated when plasmids encoding *HlyU* are introduced into *V. cholerae* and downregulated in an *hlyU* mutant, in parallel with HlyA (25). To clone the gene encoding Hcp from O17, a reverse genetics approach was used. Hcp was overexpressed in the supernatant fraction of O17 carrying the *hlyU* clone, pPM3039. The protein band was transferred from a polyacrylamide gel to a polyvinylidene difluoride membrane for N-terminal amino acid sequence analysis. A sequence of 23 amino acids was obtained (A. Gooley, Macquarie University), and on the basis of this sequence a completely degenerate 18-mer oligonucleotide probe (oligonucleotide 546) was designed (Fig. 1) and used to probe an O17 cosmid library. One positive cosmid clone was obtained (pPM3401), and Southern analysis using oligonucleotide 546 as the probe localized the hybridizing sequence to a 1.5-kb *Hin*dIII fragment, which was subcloned into the vector pGEM7Zf(+) to produce pPM3403. A *Pst*I-to-*Sal*I (360-bp) fragment from pPM3403 was used to probe *Hin*dIII-digested O17 chromosomal DNA and detected equally intense bands of 1.5 and 4.7 kb (Fig. 2, lane 1), indicating the presence of a strongly cross-hybridizing sequence in O17. This was also found to be the case in the 10 other *V. cholerae* strains tested, with distinct patterns for the classical and El Tor strains (Fig. 2). To clone the 4.7-kb *Hin*dIII fragment hybridizing with the *Pst*I-*Sal*I probe, a subgenomic library of *V. cholerae* O17 DNA was constructed in the vector pSUP301 and screened with this probe. Several clones carrying the predicted *Hin*dIII fragment were obtained, one of which was pPM3446. Restriction mapping data revealed a cluster of shared sites in pPM3403 and pPM3446 (Fig. 3A), consistent with a region with close sequence similarity.

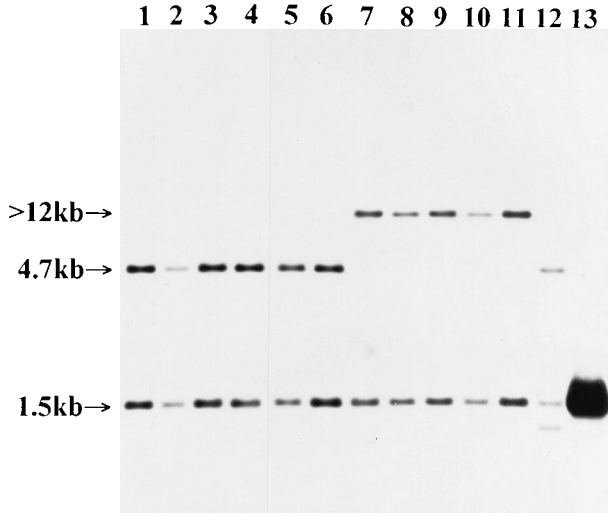

FIG. 2. Southern analysis of *Hin*dIII-digested *V. cholerae* DNA with an *hcp* probe. Lanes: 1 to 6, El Tor strains; 7 to 11 classical strains. Lanes: 1, O17; 2, N16961; 3, BM69; 4, C5; 5, AA14073; 6, C21; 7, AA14041; 8, CA401; 9, CA411; 10, Z17561; 11, 569B; 12, NCV165 (non-O1 strain); 13, pPM3403. The probe was a *Pst*I-*Sal*I fragment of 360 bp. The sizes of the hybridizing bands are shown on the left.

The 1.5-kb *Hin*dIII insert from pPM3403 and a 1.1-kb region from pPM3446 were sequenced and revealed 97% identity from the common *Hin*dIII site to 1,038 nucleotides (nt) upstream (Fig. 3B). Further 5′, the sequences appear to be unrelated. Differences between the two sequences are shown in Fig. 3B. An open reading frame (ORF) of 172 amino acids was identified from both sequences and is shown above the nucleotide sequence, with a potential ribosomal binding site with appropriate spacing underlined. The first 25 amino acids of this ORF, excluding the fMet residue, match the N-terminal amino acid sequence obtained from Hcp, with the exception of the Thr residue at position 3. Apart from this single discrepancy, the agreement between the amino acid and nucleotide sequence data suggests that these ORFs encode the Hcp protein, and they have been designated *hcpA* (pPM3403) and *hcpB* (pPM3446). There is no evidence for cleavage of a signal sequence from Hcp, since the N-terminal amino acid sequence indicates that processing of only the initiating methionine occurs.

Although the amino acid sequences of the *hcpA* and *hcpB* ORFs agreed with that obtained from Hcp, the size of the predicted translation products is only 19 kDa, which did not agree with the 28-kDa Hcp protein band observed on PAGE. To identify the translational products of the two genes, each was cloned downstream of the T7 promoter in either pGEM3Zf+, pGEM7Zf+, or pBluescriptIIKS(−) and overexpressed by T7 RNA polymerase provided by pGP1-2. A protein with a size of ca. 28 kDa was produced from *hcpA*, which comigrated with Hcp from the supernatant fraction of O17, and was detected with anti-Hcp antiserum by Western blot analysis (Fig. 4A). This protein was also produced from *hcpB* clones (Fig. 4B).

The production of a 28-kDa protein from the *hcp* genes suggests that translation may extend past the predicted TAA stop codons, either because of sequence error or by translational readthrough. To examine these possibilities various deletion clones of pPM3403 were overexpressed in the T7 system, and the sizes of their protein products were determined by PAGE. The smallest clone was deleted to 24 nt downstream of the TAA stop codon and still produced a 28-kDa protein after overexpression (Fig. 5). This ruled out sequence error or translational readthrough of the stop codon as likely explanations for the size discrepancy between the Hcp protein and the *hcpA* ORF. It appears more likely that this size discrepancy is the result of aberrant migration of the Hcp protein on PAGE, such that a 19-kDa protein migrates as 28 kDa. Boiling the samples in urea to completely denature them had no effect on migration. Other possible reasons for aberrant migration include high acidity (7) and high proline content, neither of which is predicted from the amino acid sequence of Hcp. Alternatively, the protein may be modified. The possibility of modification of the Cys-5 and Cys-22 residues of Hcp had been suggested from the N-terminal sequencing data, which identified these two residues as novel species. Modification of Cys residues by acylation is known to occur at internal Cys residues in bacteria

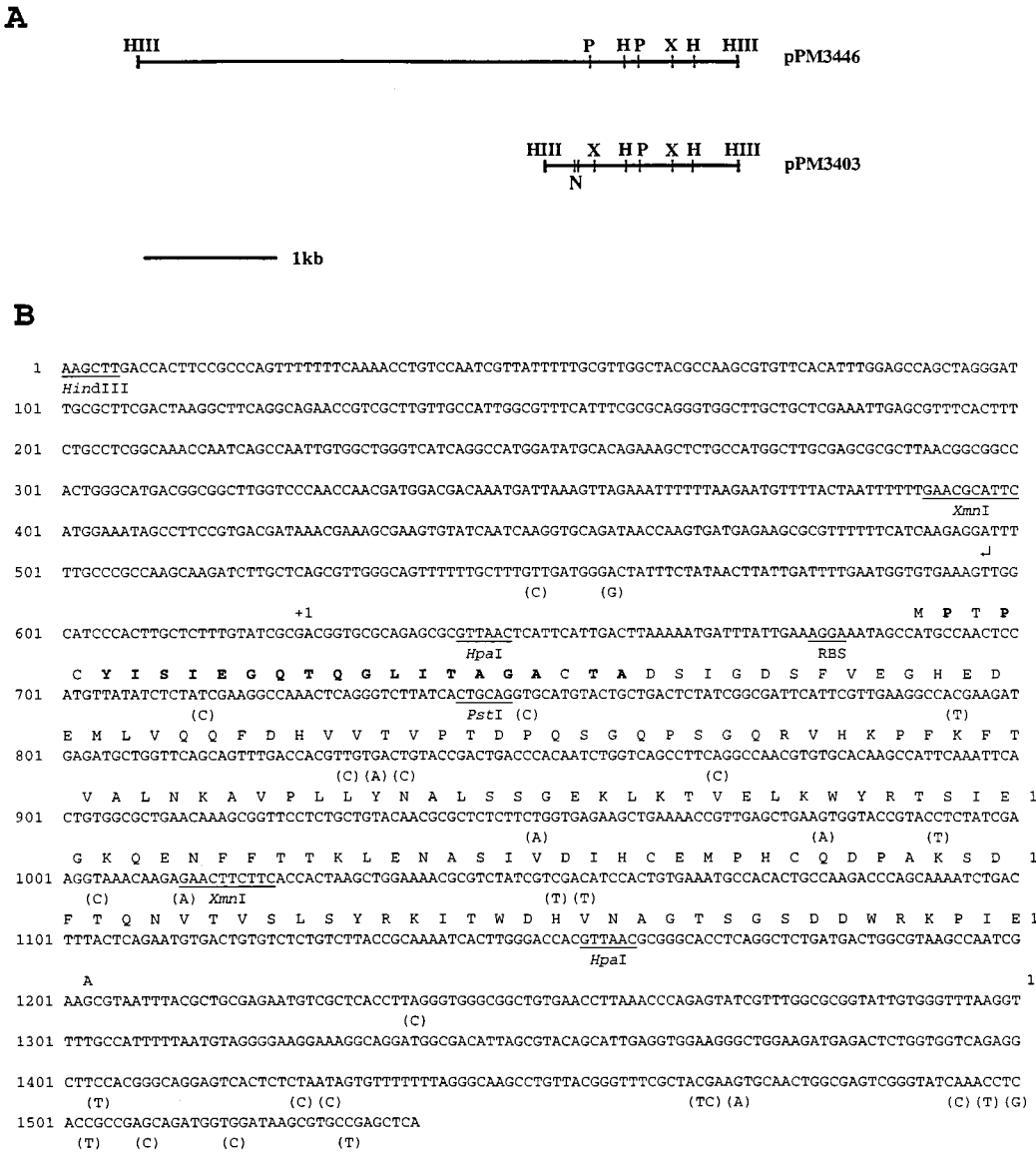

FIG. 3. (A) Restriction maps of *hcp* clones pPM3446 and pPM3403, aligned around a region of common restriction sites. HIII, *Hin*dIII; H, *Hpa*I; N, *Nco*I; X, *Xmn*I; P, *Pst*I. (B) DNA sequence generated from *hcpA* clone pPM3403. The *hcpB* (pPM3446) sequence differs from that of *hcpA* at the positions indicated in parentheses, and 5′ of nt 498 (shown by the leftward arrow) the sequences are unrelated. The 172-amino-acid ORF is shown above the nucleotide sequence, with amino acid number shown on the right. A potential ribosome binding site (RBS) is underlined. Matches between the ORF and the 23 N-terminal amino acids sequenced are shown by bold lettering. Relevant restriction sites have been labelled. The transcriptional start point is at nt 626 and is labelled +1.

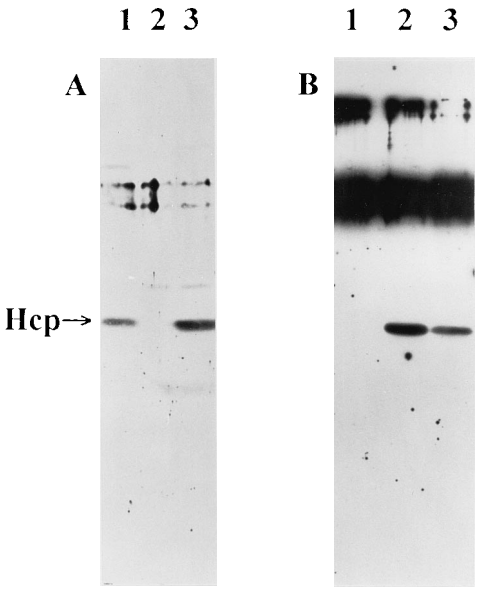

FIG. 4. Western analysis with anti-Hcp antiserum against *hcpA* and *hcpB* translation products. *hcpA* and *hcpB* were expressed from the T7 promoter in pBluescriptKS or pGEM3Zf(+). (A) Lanes: 1, O17 supernatant fraction; 2, pBluescriptKS; 3, pPM3435 (1.5-kb *Hin*dIII *hcpA* fragment in pBluescriptKS). (B) Lanes: 1, pGEM3Zf(+); 2, pPM3435; 3, pPM3474 [1.0-kb *Bgl*II-to-*Hin*dIII *hcpB* fragment in pGEM3Zf(+)].

and leads to altered migration on PAGE. The cytosolic activating protein of *E. coli* transfers an acyl group to the *E. coli* hemolysin, thereby activating it. The cytosolic activating protein runs aberrantly on PAGE (approximately 19 kDa instead of the predicted 9 kDa) because of the acyl group (12). Acylation of cysteine residues often occurs via a thioester linkage, which is sensitive to hydroxylamine treatment (16). However, no effect of hydroxylamine on the migration of Hcp has been observed (data not shown).

The amino acid sequence of Hcp was compared with sequence databases; however, no significant similarities were detected. Kyte and Doolittle (13) hydropathy analysis of Hcp indicates that it is a highly hydrophilic protein, with no significant hydrophobic stretches which could be involved in secretion.

Regulation of Hcp expression. The coregulation of the *hlyA* and *hcp* genes by HlyU suggests that the promoter regions of these genes may show a common motif involved in HlyU binding. To enable a comparison of the *hcp* and *hlyA* promoter sequences, the *hcp* promoter was localized by primer extension analysis. The 5′ end of the *hcp* mRNA was mapped to nt 626 of the DNA sequence (Fig. 3B and 6). No −10 and −35 consensus promoter sequences are apparent in the vicinity of the 5′ end of the *hcp* mRNA; however, a potential σ^{54} consensus sequence (−12/−24) was identified (Fig. 7). The presence of this sequence is consistent with transcriptional initiation in this region, rather than RNA processing, to produce the mapped 5′ end of the *hcp* mRNA. σ^{54} promoters are usually activated by specific environmental signals and are dependent on the binding of an activator to an upstream activator sequence (UAS) (23). The UAS is usually localized 100 to 200 nt upstream of the promoter, and interaction between proteins bound at the UAS and promoter region is often mediated via DNA bending at an integration host factor (IHF) binding site (17). A candidate IHF binding site is present at position −52 relative to the *hcp* transcription start point, with 12 matches to the 13-nt consensus sequence (9) Fig. 7B. On the basis of these features of the putative *hcp* promoter region, it appears that *hcp* and *hlyA* may use different classes of promoters (Fig. 7) (26). Since expression of both genes is ultimately dependent on HlyU, we propose that HlyU acts directly at the *hlyA* promoter but through an intermediate regulator at the *hcp* promoter. The activity or expression of this intermediate regulator would be controlled by HlyU. This is consistent with our observations that an *hlyU* clone (pPM3039) is sufficient to activate an *hlyA*::*cat* (chloramphenicol acetyltransferase gene) transcriptional fusion in *E. coli* DH5α (26), but that this clone is not sufficient to activate an *hcpA*::*cat* transcriptional fusion, judging by growth on chloramphenicol (data not shown). Both *hlyA* and *hcpA* transcriptional fusion constructs were active in *V. cholerae* (data not shown).

Construction of *hcp* mutants in *V. cholerae*. To ascertain whether Hcp plays a role in the pathogenesis of *V. cholerae*, a mutant deficient in both *hcp* genes was constructed. *hcpA* was mutated by replacing the 500-bp *Hpa*I fragment (Fig. 3B and 8A) with a *Hin*cII fragment carrying the Km^r cartridge from pPM1143, thus removing the translational start site and 156

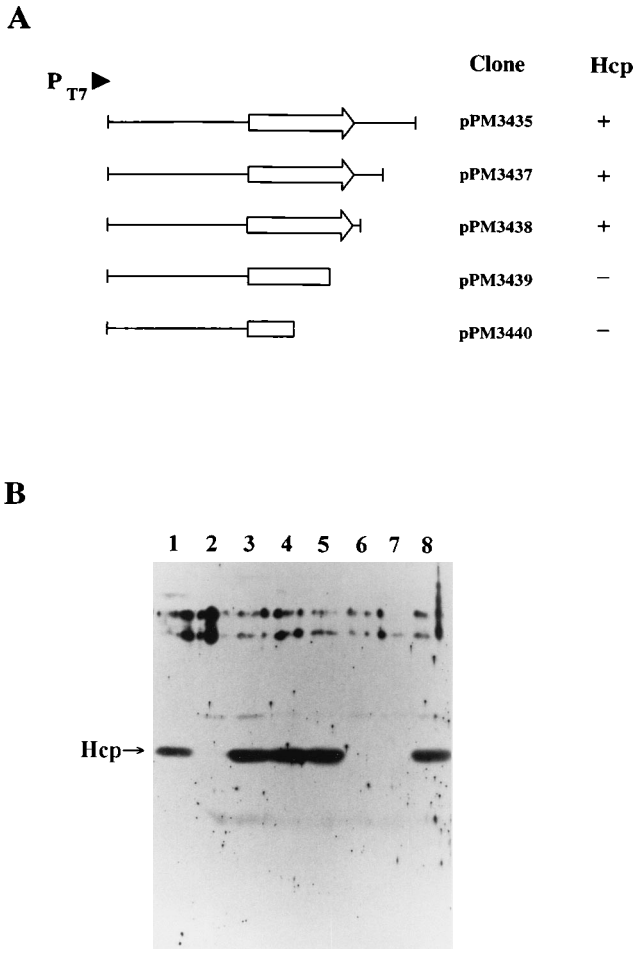

FIG. 5. Production of 28-kDa Hcp protein by 3′ deleted constructs of pPM3403. The 1.5-kb *Hin*dIII insert from pPM3403 was cloned into pBluescriptIIKS in the correct orientation for expression from the T7 promoter (P_{T7}), to create pPM3435. (A) Extent of deletions from distal end of pPM3435; (B) Products of various deletion clones in T7 overexpression system analyzed by Western immunoblot with anti-Hcp antiserum. Lanes: 1, O17 supernatant fraction; 2, pBluescriptKS; 3, pPM3435; 4, pPM3437; 5, pPM3438; 6, pPM3439; 7, pPM3440; 8, pPM3435. The arrow indicates the 28-kDa Hcp.

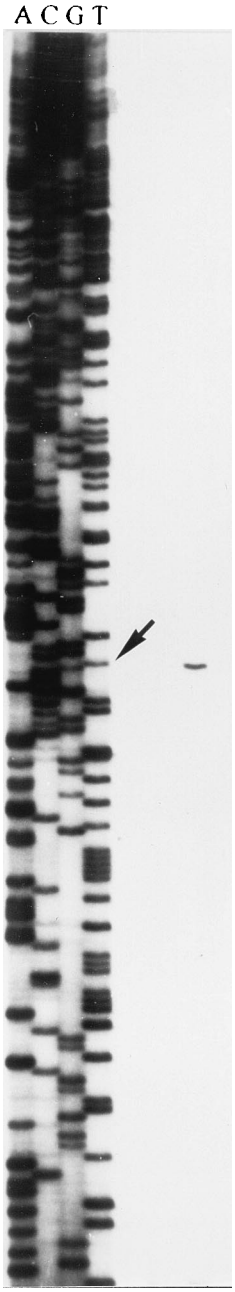

FIG. 6. 5′ end mapping of *hcp* mRNA by primer extension. Total RNA from O17 pPM3039 (*hlyU* clone) was used as a template for primer extension with oligonucleotide no. 750 (nt 743 to 767 inclusive; see Fig. 3B). The extension products were analyzed on a denaturing polyacrylamide gel alongside sequence reaction mixtures employing the oligonucleotide 750 primer and plasmid pPM3403 as the template. The size of the major extension product mapped the 5′ end of the *hcp* mRNA to nt 626 of the sequence shown in Fig. 3B (indicated by the arrow). An extension product with the same size was detected by using O17 RNA, although it required longer exposure (data not shown).

A

σ^{70} TTGACA...(16-18).....TATAAT.....+1
hlyA TTTACATTAGAAACATAAGTGATATTTC.....TA
hcpA GAAAGTTGGCATCCCACTTGCTCTTTGT.....A
σ^{54} CTGGCAC.....TTGCA.....+1

B

IHF consensus binding site TAAGTTATTGATT
hcpA,B putative IHF site (-52) TAACTTATTGATT

FIG. 7. *hlyA* and *hcp* promoter sequence comparison. (A) Predicted promoter regions of *hlyA* and *hcp* aligned from their +1 positions, as defined by primer extension analyses. The consensus sequences for σ^{70} and σ^{54} promoters are shown, with appropriate spacings indicated in parentheses. (B) Putative IHF binding sequence identified at −52 from *hcp* transcription start point (+1) aligned with the IHF consensus sequence (9).

residues of the 172-amino-acid ORF. This defective copy of *hcpA* was introduced into the vector pCVD442 to produce pPM3483. By using the mobilizing strain S17-1 λ*pir*, pPM3483 was introduced into O17 selecting for Ap^r. Since the pCVD442 vector cannot replicate in the absence of the R6K π protein (6), selection on ampicillin selects strains in which the plasmid has integrated into the *V. cholerae* chromosome by homologous recombination. Subsequent selection on Luria broth (LB) plates containing 10% sucrose and kanamycin, but without NaCl, selected for resolved integrants, which had replaced *hcpA* with the *hcpA*::Km^r sequence and lost the plasmid *sacB* marker (which is responsible for sucrose sensitivity). Sucrose-resistant colonies were screened for ampicillin sensitivity, and the alteration to *hcpA* in these isolates was confirmed by Southern analysis (Fig. 8B, lanes 3 and 4). The 1.5-kb *Hin*dIII *hcpA* fragment was replaced by 1.2- and 1.1-kb fragments because of the introduction of a *Hin*dIII site within the Km^r cartridge. One such mutant was called V923.

The *hcpB* gene was inactivated by a deletion of the 500-bp *Hpa*I fragment (Fig. 8A), and the Δ*hcpB* fragment was introduced into pCVD442 to create pPM3485. pPM3485 was introduced into V923 with selection for Ap^r, as described above. Sucrose-resistant Ap^s colonies were subsequently isolated, and these colonies were screened for lack of hybridization to the *Pst*I-*Sal*I probe, which lies completely within the deleted *Hpa*I region of *hcpA* and *hcpB* (Fig. 8A). Between 20 and 50% of the colonies screened were negative and therefore appeared to have lost both copies of *hcp*. Chromosomal DNA from two of these isolates (V939 and V940) showed the expected *hcpA* and *hcpB* alterations on Southern analysis (Fig. 8B). The 4.7-kb *hcpB* fragment was reduced to 4.2 kb.

The phenotype of V939 was confirmed by Western analysis (Fig. 9), which showed Hcp could be detected in supernatant fractions of O17 and *hcpA* mutants but not in V939. The growth rate of V939 was comparable to that of O17 in both NB and minimal medium, and no difference in hemolytic activity could be detected between the two strains.

The 50% lethal dose of V939 in the infant mouse model was 2.6×10^4, compared with 1.9×10^4 for O17. A competition experiment was performed to assess the ability of V939 to colonize the infant mouse gut in the presence of competing O17. This involved feeding seven mice a mixed inoculum of O17 and V939 at an input ratio of 1:1.26. The median output ratio was 1:0.89, indicating no deficiency in colonization by V939. Together these data suggest that the Hcp protein is dispensable for *V. cholerae* infection in the infant mouse model.

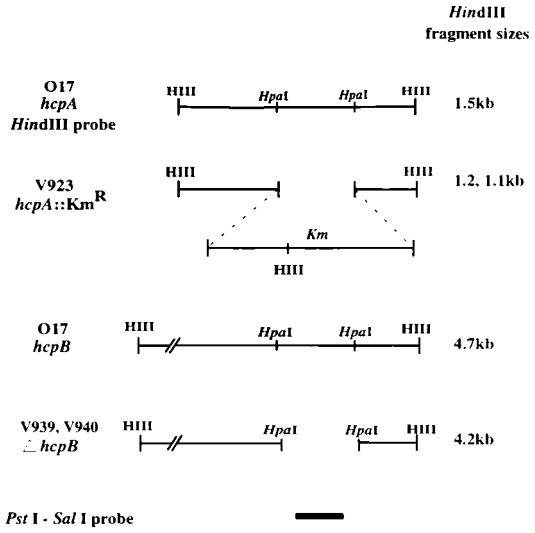

FIG. 8. Southern analysis of *hcpA hcpB* mutants. (A) Changes introduced into *hcpA* and *hcpB*, with *Hin*dIII (HIII) and *Hpa*I sites indicated. The *Pst*I-*Sal*I probe and *Hin*dIII probes used in screening are also shown. (B) Southern analysis of *hcpA* and *hcpA hcpB* mutants using the *Hin*dIII probe. Lanes: 1, V939; 2, V940; 3, O17; 4, V923 (*hcpA*). Fragment sizes are shown on the left.

DISCUSSION

The cloning and sequencing of *hcp* genes have been undertaken as the first steps in the characterization of this secreted protein, which may be expressed in vivo coordinately with HlyA. Two *hcp* genes were identified from Southern analysis, and their sequences show almost complete identity at the nucleotide level and encode identical amino acid sequences. These sequences provided no clues as to a possible role for Hcp, since no significant similarities to database sequences were apparent. When HcpA was expressed from *E. coli*, no activity could be detected against substrates provided in a variety of agar media, including blood (sheep, horse, human, chicken, or rabbit), skim milk, Tween, and DNA. The protein was also tested against HeLa cells, with no evidence of cytotoxic effects.

A mutant defective in both *hcp* genes was constructed and assessed for virulence in the infant mouse cholera model. It was found to have a 50% lethal dose equivalent to that of the wild-type parent strain, O17, suggesting that Hcp is not required for pathogenesis. In addition, the *hcp* mutation had no detectable effect on colonization ability, which is not unexpected for a secreted protein such as Hcp. These results indicate that the deficiency in Hcp production in an *hlyU* mutant is not the reason for the reduced colonizing ability of this mutant (25) and implicate the involvement of another *hlyU*-regulated factor in colonization.

Despite the fact that the role of Hcp has not yet been ascertained, several interesting features that require further investigation have been identified from the amino acid and DNA sequence data. Secretion of the protein is independent of a signal sequence, since the amino acid sequence obtained from secreted Hcp shows that only the initiating methionine is processed. Hcp localizes in the cytoplasm and periplasmic space of *E. coli*, indicating that it can traverse the inner membrane without the need for *V. cholerae*-specific factors; however, such factors are clearly required for secretion through the outer membrane. The *eps* transport system identified by Overbye et al. (18) is responsible for secretion of cholera toxin, soluble hemagglutinin/protease, and chitinase; however, these proteins have signal sequences, and their transport mechanism would therefore be expected to differ from that of Hcp. To date, Hcp is the only known *V. cholerae* protein secreted without a signal sequence.

Hcp appears to migrate aberrantly on PAGE since there is a significant discrepancy between the observed size on PAGE (28 kDa) and the predicted size from the ORF sequence (19 kDa). The limits of the ORF have been confirmed from amino acid sequence data (N terminus) and deletion analysis (C terminus), so we believe that this discrepancy cannot be explained by sequence error or translational readthrough of the TAA stop codons. It may be related to the apparent modification of two cysteine residues at positions 5 and 22, which appeared as

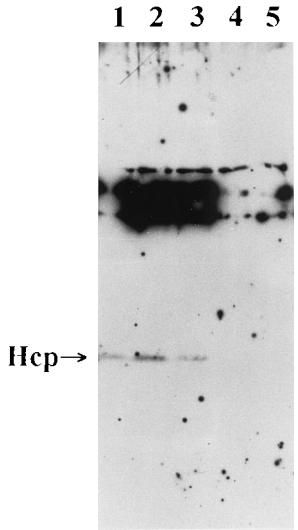

FIG. 9. Confirmation of Hcp− phenotype of *hcpA hcpB* mutants V939 and V940. Trichloroacetic acid-precipitated supernatant fractions were analyzed for Hcp expression with anti-Hcp antiserum in Western immunoblots. Lanes: 1, O17; 2, V922 (*hcpA*); 3, V923 (*hcpA*); 4, V939; 5, V940. The Hcp band is indicated.

novel species in amino acid sequence analysis. Site-directed mutagenesis of these residues is necessary to further investigate this possibility.

Southern analysis indicates that *hcpA* and *hcpB* genes exist in all *V. cholerae* strains examined and that the two biotypes can be differentiated on the basis of their *hcp Hin*dIII restriction fragment sizes. Of 18 *V. cholerae* strains examined for Hcp expression by Western blot analysis, only 4 showed detectable levels of this protein (unpublished data). This suggests that both genes are silent in most strains or that differences in control of expression of these genes exist between strains. Although a similar situation exists with HlyA, there appears to be no obvious correlation between the hemolytic phenotype and the expression of Hcp. These results suggest that factors other than HlyU may be influential in the expression of both HlyA and Hcp.

The *hcp* promoter mapped by primer extension fits the σ^{54} consensus sequence and displays a potential IHF binding site at position −52 relative to the transcription start point. These features are found in *hcpA* and *hcpB* genes; however, since the sequences diverge at position −129, it is possible that binding of an activator to a UAS, and therefore expression of the two genes, may differ. We have proposed the existence of an intermediate activator, to mediate control of *hcp* expression by HlyU. This is necessary to explain how HlyU can regulate both *hlyA* and *hcp* promoters, while these promoters are clearly different at the DNA sequence level. This possibility is supported by the finding that HlyU alone is sufficient to activate the *hlyA* promoter but not the *hcpA* promoter in *E. coli*. Binding of HlyU to *hlyA* and *hcp* promoter fragments is currently being investigated to clarify this possibility. The identification of this predicted intermediate regulator and mutational analysis will reveal its importance to the expression of *hcp* and possibly other *V. cholerae* proteins.

This study has revealed that HlyU may be part of a regulatory cascade, and it will be of interest to investigate the complexity of this system and whether there is interplay with the other characterized regulatory systems in *V. cholerae.*

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