Haemagglutinin/protease expression and mucin gel penetration in El Tor biotype *Vibrio cholerae*

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*Vibrio cholerae* of both biotypes produce a soluble Zn^{2+}-dependent metalloprotease: haemagglutinin/protease (Hap), encoded by *hapA*. Hap has been shown to have mucinolytic and cytotoxic activity. These activities are likely to play an important role in the pathogenesis of cholera and the reactogenicity of attenuated vaccine strains. Production of Hap requires transcriptional activation by the HapR regulator and is repressed by glucose. The present study shows that mucin purified from two sources, bile salts, and growth at 37 °C enhanced Hap protease production. Analysis of *hapA* and *hapR* promoter fusions with the *lacZ* gene showed both promoters to be activated in a cell-density-dependent pattern. Glucose repressed and mucin induced the *hapA* promoter by a HapR-independent mechanism. Bile had no effect on either *hapR* or *hapA* promoter activity. Expression of *hapA* was required for vibrios to translocate through a mucin-containing gel. These results suggest Hap to play an important role in cholera pathogenesis by promoting mucin gel penetration, detachment and spreading of infection along the gastrointestinal tract.

**INTRODUCTION**

Microbial metalloproteases display a broad spectrum of pathological activities (Miyoshi & Shinoda, 2000). *Vibrio cholerae* produces a soluble Zn^{2+}-dependent metalloprotease (mucinase): haemagglutinin/protease (Hap), encoded by *hapA* (Booth et al., 1983; Finkelstein & Hanne, 1982). By DNA sequence homology, Hap appears to be closely related to *Pseudomonas aeruginosa* elastase and the metalloproteases secreted by *Legionella pneumophila* and *Vibrio vulnificus* (Miyoshi & Shinoda, 2000). Hap is secreted to the culture medium through the general secretory pathway (Sandkvist et al., 1997). Comparison of the *hapA* ORF with the molecular mass of secreted Hap suggests that its secretion involves sequential cleavage of a signal peptide and a propeptide (Häse & Finkelstein 1991). Very little protease activity is detected in mutant vibrios lacking a functional *hapA* gene, suggesting that *hapA* encodes the major protease activity secreted by *V. cholerae*. Hap can proteolytically activate cholera toxin A subunit (Booth et al., 1984) and the El Tor cytolyisin/haemolysin (Nagamune et al., 1996), and hydrolyse several physiologically important proteins such as mucin, fibronectin and lactoferrin (Finkelstein et al., 1983). Hap perturbs the paracellular barrier of cultured MDCK-1 and T84 intestinal epithelial cells (Wu et al., 1996; Mel et al., 2000) by acting on tight junction-associated proteins (Wu et al., 2000).

In order to cause disease, cholera vibrios must overcome the mucus barrier. The thickness of the human intestinal mucus barrier has been recently estimated to centre around 150 μm (Jordan et al., 1998). Accordingly, infecting vibrios need to overcome a physical barrier of 50 times their length to reach the underlying epithelial cells. It has been reported that *V. cholerae* is attracted to mucus by a CheR-dependent chemotactic mechanism (O’Toole et al., 1999). There is plentiful microscopy evidence indicating that infecting vibrios associate with the protective mucus blanket (Yamamoto & Yokota, 1988). However, very little is known regarding the mechanism by which *V. cholerae* penetrates the mucus gel and much less on how this process is coordinated with the expression of other virulence factors.

We have investigated the role of Hap mucinase in the interaction of cholera vibrios with mucus using cultured HT29-18N2 cells (Benitez et al., 1997), which form a thick meshwork of heterogeneously glycosylated MUC2, MUC3 and MUC5AC mucin (Huet et al., 1987; Phillips & Frisch, 1990; Phillips et al., 1988; Stanley & Phillips, 1999). Inactivation of *hapA* increased adherence to HT29-18N2 cells and diminished detachment of vibrios into the washings (Benitez et al., 1997). In the infant mouse cholera model, inactivation of *hapA* enhanced *V. cholerae* adherence (Robert et al., 1996). The CTXΦ-negative Hap-defective
attenuated strain 638 was well tolerated in volunteers (Benitez et al., 1999). In contrast, V. cholerae 81, the hapA− precursor of strain 638, was strongly reactogenic (presented at the 35th US Japan Cholera and Other Bacterial Enteric Infections Joint Panel Meeting, 3–15 December 1999). V. cholerae produces many potential known reactogenic factors (Hap, haemolysin, RTX toxin, etc.) (Mel et al., 2000; Fullner et al., 2001). Our volunteer studies suggest that Hap could contribute to reactogenicity along with other known or unknown factors. We have postulated that the Hap mucinase activity that mediates detachment of vibrios from the mucin meshwork of HT29-18N2 cells is the same activity that facilitates penetration of the mucus barrier in vivo (Benitez et al., 2001). The diminished reactogenicity of strain 638 could be due its limited capacity to overcome, degrade or penetrate the mucus blanket.

Expression of hapA requires transcriptional activation by HapR (Jobling & Holmes, 1997), an analogue of Vibrio harveyi LuxR. Production of Hap in the stationary phase is enhanced upon nutrient limitation and strongly repressed by glucose and other sugars (Benitez et al., 2001).

In the present work we demonstrate that carbon source limitation, bile salts, mucin and growth at 37°C significantly enhance delivery of enzymically active Hap to the culture medium. Analysis of hapA and hapR–lacZ transcriptional fusions showed that glucose represses and mucin induces hapA promoter activity by a HapR-independent mechanism. Expression of hapA was required for the vibrios to translocate through a mucin-containing gel in a column assay.

**METHODS**

**Strains and media.** V. cholerae C7258 (El Tor, Ogawa) was used to study the effect of glucose, bile, mucin and temperature on the production of Hap. Strains 638 (ΔCTXΦ, hapA::celA) (Benitez et al., 1999) and JBK70 (ΔctxA hapR) were used as negative controls that do not produce Hap. Strain JBK70 is derived from N16961, which contains a natural frame-shift mutation in the celA gene (Zhu et al., 1999). The amplified product extended from the upstream promoter terminator to the hapA translational start (nucleotide 663, accession no. AF001009). The hapR promoter was amplified from plasmid pC1.1 (Jobling & Holmes, 1997) using primers 5′-CTTGATCCGTTTGTACGATCT-3′ and 5′-GCTAAGCTT TTGATGAGGTCCTTA-3′. The amplification product extended from the upstream hpr gene transcribed in the opposite direction to nucleotide 551 of hapR (accession no. AF000716). The strong rntB-T1-T2 transcription terminator was amplified from plasmid pKK3535 (Brosius et al., 1981) using primers 5′-CCCTCTTACAAGTTTTCAAGCTTACAG-3′ and 5′-GAAGCTTGCTCCTTATGATATGAC-3′. PCR was conducted using the Advantage 2 kit (BD Biosciences Clontech) as recommended by the manufacturer.

To make lacZ fusions the 37°C colonies were sequenced using the Advantage 2 kit (BD Biosciences Clontech) as indicated in Fig. 3. Promoter fragments were recovered as KpnI–HindIII fragments and ligated to a HindIII–KpnI fragment of pKrZ1 containing a promoterless lacZ gene, and the pBR322 origin of replication and bla gene (Rothmel et al., 1991). The pUC19 derivative pTT3 containing the amplified rntB-T1-T2 terminator cloned as a Xbal–PstI fragment was modified by digestion with HindIII, Klenow polymerase fill-in and insertion of a KpnI phosphorlated linker (New England BioLabs). The terminator was subsequently inserted as a KpnI fragment upstream from the hapA and hapR promoter sequence to generate plasmids pHaplac11 and pHaplac12 (Fig. 3). Additional transcriptional fusions containing 5′ deletions of the hapA promoter were constructed likewise but varying the upstream primer used to amplify the hapA promoter fragment. The following plasmids harbouring 5′ deletions were constructed using the upstream primer indicated in parentheses: pHaplac4 (5′-ACATGCGATGGTGTCAGGTCTCATGATCAGTAC-3′), pHaplac16 (5′-GGAAATGATCGTGGAACAAATCTAGATCGTAC-3′), pHaplac7 (5′-ACAGCATGCGATGGTGTCAGGTCTCATGATCAGTAC-3′) and pHaplac12 (5′-ACAGCATGCGATGGTGTCAGGTCTCATGATCAGTAC-3′). The extension of each deletion relative to the hapA translational start is provided in Fig. 3. Cloning, subcloning, ligation and transformation were performed by standard procedures. lacZ fusions were introduced into strain AC-V66 by electroporation.

**Immunoblot assay.** Culture supernatents were concentrated by passing through Centricon-10 centrifugal filters (Amicon Bioseparations) and separated by 12% SDS-PAGE. The volume of supernatant applied per lane was standardized to account for differences in OD660 or c.f.u. ml⁻¹. Proteins were electroblotted to a PVDF membrane and sequentially treated with 5% skim milk and rabbit anti-Hap serum in phosphate-buffered saline (pH 7.4) (60 min, 37°C each). The membrane was treated for 60 min at 37°C with anti-rabbit IgG, peroxidase-conjugated (Sigma), and developed with 4-chloro-1-naphthol. Molecular masses were estimated using Bio-Rad SDS-PAGE low-range standards.

**Construction of hapA and hapR–lacZ transcriptional fusions.** The hapA promoter was amplified from plasmid pCH2 (Hase & Finkelstein, 1991) using primers 5′-CAAGGTACCGGGAAAAATGGAAGGCAAC-3′ and 5′-GGCCAGCTCTCTTTTCATCTGAG-3′. The amplification product extended from the upstream ompr promoter terminator to the hapA translational start (nucleotide 663, accession no. AF001009). The hapR promoter was amplified from plasmid pC1.1 (Jobling & Holmes, 1997) using primers 5′-CTTGATCCGTTTGTACGATCT-3′ and 5′-GCTAAGCTTTGGATGAGCTTTGCATACTG-3′. The amplification product extended from the upstream hpr gene transcribed in the opposite direction to nucleotide 551 of hapR (accession no. AF000716). The strong rntB-T1-T2 transcription terminator was amplified from plasmid pKK3535 (Brosius et al., 1981) using primers 5′-CCCTCTTAGAGATTTCAGCCTGATAC-3′ and 5′-GAATCGAATGCTTATGATATGAC-3′. PCR was conducted using the Advantage 2 kit (BD Biosciences Clontech) as recommended by the manufacturer. Annealing temperatures for each pair of primers were set at 5°C below the lowest Tm value. Primers were designed with appropriate restriction sites for subsequent directional cloning in pUC19. Both strands of each insert were sequenced for correctness using the universal and reverse M13 sequencing primers. The hapA and hapR transcriptional fusions were assembled on plasmid pKrZ1 (Rothmel et al., 1991) as indicated in Fig. 3. Promoter fragments were recovered as KpnI–HindIII fragments and ligated to a HindIII–KpnI fragment of pKrZ1 containing a promoterless lacZ gene, and the pBR322 origin of replication and bla gene (Rothmel et al., 1991).

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**Mucin gel penetration assay.** The mucin gel penetration assay used in this work has been described by Sheikh et al. (2002). The procedure is a variation of the invasion chamber method used to study mucus penetration by trichomonads (Lehker & Sweeney, 1999). Briefly, a solution containing 10% PS stomach mucin and 0.3% agarose in reduced strength (0.25 × 1) TSB medium was allowed to settle and form a soft gel at room temperature in a 1 mL tuberculin syringe fitted with a stopcock. Where indicated, d-glucose
(0.4%) or bile (0.02%) was added to the mucin-containing gel. The cells were harvested, reconstituted in phosphate-buffered saline (pH 7.4) and the suspension standardized to OD$_{600}$ 4.5. A 0.1 ml aliquot of cell suspension was layered on top of each column and incubated for 3 h at 30°C or 37°C in a vertical position. At the end of the incubation period the stopcock was removed and 0.1 ml fractions collected from the button by applying gentle pressure. Each fraction was diluted and plated. The number of c.f.u. per fraction was standardized by the total number of c.f.u. per column (relative cell density).

**RESULTS**

**Production of enzymically active Hap is enhanced by bile, mucin and growth at 37°C**

Hap is the major protease secreted by the El Tor biotype strain C7258. When strain C7258 is grown in TSB medium that contains 2.5 g D-glucose l$^{-1}$, protease activity is detected in the supernatant at optical densities higher than 2 when the concentration of D-glucose has dropped below 0.2 g l$^{-1}$. No proteolytic activity is detected if at this stage the culture is supplemented with additional glucose (Benitez et al., 2001). No protease activity could be detected in the supernatant of strain 638, a hapA insertion mutant derived from C7258 (Robert et al., 1996; Benitez et al., 1999). Therefore, we used strain C7258 and an azocasein assay to quantitate production of Hap under different culture conditions. In TSB medium, the presence of bile or growth at 37°C significantly increased the amount of extracellular azocasein-degrading activity (Fig. 1a). Addition of bile immediately prior to cell harvesting or to the enzyme reaction mixture did not have any effect on proteolytic activity (data not shown). This control indicates that bile acts by increasing the amount of Hap released to the culture medium rather than by stimulating its enzyme activity. In order to examine if the presence of a protein substrate in the medium enhances production of Hap, vibrios were grown in protein-free M9 minimal medium with and without added protein. Very little azocasein-degrading activity was produced in M9 minimal medium (Fig. 1b). Addition of mucin – the protein component of mucus – partially purified from porcine stomach (PS) or pure submaxillary gland (SMG) mucin stimulated protease production. Addition of BSA had a much smaller inducing effect, suggesting that vibrios respond specifically to the presence of mucin by making more Hap protease. Stimulation of extracellular protease production by bile or mucins was prevented by addition of glucose, suggesting this protease activity to be Hap. Accordingly, no protease production was stimulated in the hapA mutant strain 638 (data not shown). In order to confirm that the protease activity stimulated by the above factors is the product of hapA we performed the immunoblot experiment shown in Fig. 2. At the protein level, Hap can be identified as a protein band (or a 39-5 38-3 kDa doublet) that specifically reacts with Hap antiserum (Fig. 2, lane A), co-migrates with purified Hap (lane K), is repressed by glucose (lane D), is absent in hapR and hapA mutants (lanes H and I, respectively) and is present in strain 638 containing the hapA$^+$ gene complemented on a plasmid (lane J). More Hap protein was detected in the supernatant of strain C7258 cultivated at 37°C (lane B), or cultivated at 30°C but in the presence of bile (lane C) compared to this strain grown at 30°C (lane A). More Hap protein was detected in the supernatant of C7258 grown in M9 minimal medium supplemented with mucin (lane G) compared to this strain grown in M9 minimal medium containing BSA (lane F) or M9 minimal medium alone (lane E).

**Fig. 1.** Environmental signals controlling protease production in El Tor biotype *V. cholerae*. (a) Effect of bile and temperature on production of azocasein-degrading activity. Strain C7258 was grown for 8 h (OD$_{600}$=1) in TSB medium at 30°C with agitation (230 r.p.m.). The cultures were divided into 10 ml portions and incubated for 4 h with agitation (230 r.p.m.) in: TSB at 30°C; TSB at 30°C supplemented with 0.02% bile; TSB at 30°C supplemented with bile and 0.4% glucose; TSB at 37°C; and TSB at 37°C supplemented with 0.4% glucose. Each value represents the mean of three independent cultures ± SD. (b) Induction of protease production by mucins. Strain C7258 was grown in: M9 minimal medium; M9 plus 0.4% BSA; M9 plus 0.4% pure SMG mucin; M9 plus 0.4% partially purified PS mucin; and M9 plus 0.4% PS mucin, with 0.4% additional glucose added at 12 h. Cultures were incubated in a rotary shaker at 30°C for 16 h. The culture containing PS mucin was divided into halves at 12 h and one half supplemented with 0.4% glucose. Each value represents the mean of three independent cultures ± SD.

**Derepression of the hapA promoter is diminished by glucose**

We examined whether the environmental factors that affect production of Hap act at the level of transcription by affecting the hapA promoter directly or via its...
transcriptional activator HapR. To this end we constructed the hapA and hapR promoter fusions shown in Fig. 3. In order to prevent transcription from the vector, the strong rrnB T T2 transcription terminator was inserted upstream and in the same orientation to the hapA or hapR promoters to create pHaplac11 and pHapRlac2. Plasmid pHaplac11 produced white transformants in *Escherichia coli* to create pHaplac11 and in the same orientation to the hapA and hapR promoters shown in Fig. 5, glucose did not significantly affect derepression of hapA and hapR, respectively, in TSB medium (Fig. 4a). Derepression of hapA and hapR occurred at the end of the exponential phase and increased in a non-linear manner with respect to cell density. Induction of the hapR promoter could be detected earlier (at a lower OD 600) than that of hapA. No azocasein-degrading activity could be detected in the medium prior to induction of hapA (Fig. 4b). As expected, from the results shown in Fig. 1(a), induction of both hapR and hapA was higher at 37 °C than 30 °C (data not shown). Next, we tested whether derepression of hapA (pHaplac11) and hapR (pHapRlac2) at the end of the exponential phase was reduced by addition of D-glucose. As shown in Fig. 5, glucose did not significantly affect hapR promoter function but it significantly reduced derepression of hapA promoter activity (Fig. 5).

### Mucin enhances hapA promoter activity

Addition of PS mucin or pure SMG mucin to M9 minimal medium enhanced production of Hap (Figs 1b and 2); BSA had a smaller effect. Therefore, we tested the effect of mucin and BSA supplementation on hapR and hapA promoter activities. As shown in Table 1, growth in the presence of PS mucin had no effect on the hapR promoter but significantly enhanced hapA promoter activity. Identical results were observed for pure SMG mucin (data not shown). Addition of BSA to M9 minimal medium did not significantly affect the promoter activities studied.

### Bile does not affect hapR and hapA promoter activity

Because bile has been shown to diminish production of cholera toxin (Gupta & Chowdhury, 1997) and HapR represses production of cholera toxin through its effect on TcpP (Zhu *et al.*, 2002), we considered the possibility that bile could enhance production of Hap by increasing expression of HapR. We performed experiments to test the effect of bile on hapR and hapA promoter activities. For instance, strain AC-V66 containing pHapRlac2 (hapR) or pHaplac11 (hapA) was grown in TSB medium for 4 and 6 h, respectively; cultures were divided into halves, one portion was supplemented with 0-02% bile and incubated for 4 h at 37°C and the other portion used as a control. No difference
in $\beta$-galactosidase activity was observed between the pHapRlac2 and pHaplac11 control cultures (3.0 ± 0.0 and 4.98 ± 0.29 nmol ONP min$^{-1}$ per 10$^8$ c.f.u., respectively) and their corresponding bile-supplemented culture (3.14 ± 0.14 and 4.82 ± 0.2 nmol ONP min$^{-1}$10$^8$ c.f.u., respectively). We conclude that the effect of bile on Hap does not involve transcription of either hapR or hapA.

Deletion analysis of the hapA promoter

In order to determine the smallest DNA fragment retaining promoter activity we constructed additional lacZ transcriptional fusions containing 5′ deletions of the hapA promoter (see Fig. 3). Plasmid pHaplac7, containing a DNA sequence extending 201 bp upstream from the hapA translational start, retained promoter activity, while pHaplac12, lacking the 5′ conserved putative CRP-binding pentamer, was inactive (Fig. 3). Plasmid pHaplac7, like all other lacZ fusions tested, produced white colonies on X-Gal medium when transformed into E. coli and blue colonies in V. cholerae AC-V66, confirming its requirement for $\beta$-Galactosidase.

Table 1. Effect of growth in the presence of proteinaceous substrates on the activity of the hapA and hapR promoters

<table>
<thead>
<tr>
<th>Promoter activity</th>
<th>Supplement to M9 minimal medium</th>
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<tr>
<td></td>
<td>None</td>
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<tr>
<td>hapA (pHaplac11)</td>
<td>8.19 ± 0.98</td>
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<tr>
<td>hapR (pHapRlac2)</td>
<td>5.01 ± 2.10</td>
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HapR. Derepression of the lacZ fusions contained in pHaplac7 was also significantly diminished by glucose (data not shown). We conclude that the 201 bp promoter fragment contained in pHaplac7 contains the information required for glucose-sensitive HapR-dependent transcription initiation.

Expression of hapA positively correlates with the capacity of vibrios to translocate through a mucin-containing gel

As previously proposed, Hap could play a major role in cholera pathogenesis by facilitating penetration of the protective mucus barrier. In order to examine the significance of hapA expression to the capacity of vibrios to penetrate mucus we used a simple column penetration assay initially developed for trichomonads (Lehker & Sweeney, 1999) and later applied to enteroaggregative E. coli (Sheikh et al., 2002). As demonstrated in Fig. 6(a), gel penetration required an active hapA gene. Conditions that stimulate Hap production such as incubation at 37°C (Fig. 6b) or bile (Fig. 6c) significantly enhanced column penetration. In contrast, addition of glucose, which represses hapA, prevented mucin gel penetration (Fig. 6d). Bile did not enhance column penetration in strain 638, which lacks a functional hapA gene (Fig. 6c).

DISCUSSION

In order to cause disease, V. cholerae needs to expresses numerous virulence factors in response to host signals in a highly coordinated fashion. Several sensor-response regulator systems have been extensively characterized. However, much less is known about how the production of major proteases like Hap is regulated and coordinated with the expression of other virulence factors. Mucin, bile and intestinal sugars are environmental signals present in the human small bowel that could modulate production of Hap in vivo. In order to understand the mechanism by which glucose, mucin and bile affect Hap production we constructed hapR and hapA–lacZ fusions and tested the effect of these environmental factors on promoter activity. The time-course of hapA and hapR promoter induction showed a cell-density-dependent pattern, with induction of hapR prior to hapA. V. cholerae HapR and LuxO are related to V. harveyi regulators that mediate quorum sensing. It has been recently shown that LuxO represses hapR expression at low cell density (Zhu et al., 2002). Thus, although the autoinducer substances involved in V. cholerae quorum sensing have not been fully characterized, recent reports have provided substantial evidence linking hapR and therefore hapA expression to a quorum-sensing signal (Miller et al., 2002). In a previous work the production of Hap in the stationary phase was accounted for by the depletion of glucose, and experiments using spent medium failed to provide evidence for an autoinducer substance (Benitez et al., 2001). Possible explanations for these results could be addition of insufficient spent culture or insufficient sensitivity of the azocasein assay to detect early production of Hap in cultures supplemented with spent medium. In addition, of the three quorum-sensing systems identified in V. cholerae, system 3 appears to respond to an internal rather than extracellular signal (Miller et al., 2002).

We tested the effect of glucose on derepression of hapR and hapA. Derepression of the hapR promoter was not affected by glucose. In contrast, the activity of the hapA promoter (pHaplac11) was strongly reduced in the presence of glucose. Thus, although the autoinducer substances involved in V. cholerae quorum sensing have not been fully characterized, recent reports have provided substantial evidence linking hapR and therefore hapA expression to a quorum-sensing signal (Miller et al., 2002). In a previous work the production of Hap in the stationary phase was accounted for by the depletion of glucose, and experiments using spent medium failed to provide evidence for an autoinducer substance (Benitez et al., 2001). Possible explanations for these results could be addition of insufficient spent culture or insufficient sensitivity of the azocasein assay to detect early production of Hap in cultures supplemented with spent medium. In addition, of the three quorum-sensing systems identified in V. cholerae, system 3 appears to respond to an internal rather than extracellular signal (Miller et al., 2002).

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Greennough, 1975; Kolb et al., 1993). This condition leads to formation of the cAMP–CRP complex, which binds to responsive promoters to activate transcription (Kolb et al., 1993). The interpretation that hapA expression is controlled by carbon catabolite repression is supported by our previous finding that Hap is not produced in a mutant lacking an active crp gene (Benitez et al., 2001). By constructing several 5′ deletions of the hapA promoter the minimal fragment containing promoter activity was narrowed to the 201 bp fragment contained in pHapac7. This fragment contains a conserved cAMP–CRP-binding TGTGA pentamer separated by 10 bp from the less conserved TCANA pentamer located upstream from the presumptive hapA −35 sequence. A deletion eliminating the upstream conserved pentamer was inactive (Fig. 3, pHaplac12). However, it remains to be established whether CRP actually binds to this site, in which the spacing between the two pentamers is 10 bp rather than the more common 6 or 8 bp. We hypothesize that in vivo, production of Hap is determined by a dual regulation. The onset of hapA expression is controlled by the availability of HapR once a critical cell density is attained. Once HapR is available, the hapA promoter is further activated by carbon source limitation by a mechanism that could involve the cAMP–CRP global regulator. This dual control explains the observation that production of Hap in LB medium occurs at the end of the exponential phase in spite of the fact that this medium does not contain glucose, indicating that glucose limitation does not stimulate hapA expression at a low cell density. Addition of mucin to M9 minimal medium did not affect hapR promoter function but significantly enhanced hapA promoter activity in plasmid pHaplac11. Gastrointestinal mucus has been shown to induce production of the closely related EmpA metalloprotease in the fish pathogen Vibrio anguillarum (Denkin & Nelson, 1999). The finding that mucins isolated from different sources with a different degree of purity enhanced production of Hap and hapA promoter activity strongly suggests mucin, or a component derived from it, to be the active inducing compound. The above results suggest that hapA is transcribed from a complex promoter that integrates multiple (at least two) environmental signals to control protease production. Further studies (already in progress) such as transcript analysis and binding assays are required to understand the mechanism of hapA activation at the molecular level.

In contrast to glucose and mucin, bile had no effect on either hapR or hapA promoter activity. This result suggests that the positive effect of bile on Hap production is not exerted at the level of hapA and hapR transcription. It has been demonstrated that bile affects expression of outer-membrane proteins OmpU and OmpT (Wibenmeyer et al., 2002). It is possible that bile could indirectly affect processing and secretion of Hap through its effect on outer-membrane porin expression and localization. In any case, our results imply that the effect of bile could only occur once conditions for expression of hapA (i.e. high cell density) have been established.

Coordination of protease production with other virulence factors such as cholera toxin and toxin co-regulated pilus (TCP) appears to be exerted at least at two levels: CRP and HapR. It has been shown that Hap is not produced in a crp insertion mutant (Benitez et al., 2001) in which production of cholera toxin and TCP is stimulated (Skorupski & Taylor, 1997a, b). On the other hand, HapR, which activates hapA, was recently shown to repress expression of the TcpP regulator required for expression of cholera toxin and TCP (Zhu et al., 2002). Thus, protease production is coordinated in a reciprocal fashion to cholera toxin and TCP. Expression of Hap appears to be also coordinated with the motility status of the cell, since two classes of hypermotile mutants showed a striking increase in protease production (Gardel & Mekalanos, 1996).

We used a simple column penetration assay to examine the effect of Hap expression on the capacity of vibrios to translocate through a mucin-containing gel. The results (Fig. 6) clearly establish a cause–effect relationship between hapA expression and mucin gel penetration. It is often intuitively accepted that motility plays a role in penetration of the mucus barrier. Since bile also enhances motility in V. cholerae (Gupta & Chowdhury, 1997), we expected it could stimulate mucin gel penetration by increasing both Hap production and flagellar motility. However, bile did not stimulate mucin gel penetration in a hapA mutant background (Fig. 6c, open bars). Flagellar motility in V. cholerae is due to a single polar flagellum (Häse, 2001), and development of hyperflagellated swarming cells has not been observed as it has in other enteric bacteria (Brown & Häse, 2001). Consequently, V. cholerae might not be capable of swimming in highly viscous media. As an example, Vibrio alginolyticus mutants expressing only a polar flagellum (the wild-type makes a polar and a lateral flagellum) are unable to swim at high viscosity (Atsumi et al., 1996). We hypothesize that in the absence of a lateral flagellum or a multi-flagellated stage, production of extracellular proteases could allow V. cholerae to swim by decreasing the viscosity of the medium. The translocation process observed in our assay is clearly different from the surface translocation phenomenon observed by Brown & Häse (2001), which apparently required the production of a proteinaceous wetting agent. This surface translocation was inhibited rather than stimulated by the presence of Hap (Brown & Häse, 2001). It is possible that vibrios use both strategies to disseminate in the gastrointestinal tract. For instance, surface translocation via a wetting agent could allow vibrios to spread at low cell densities when Hap is not produced, while Hap production could increase the efficiency of flagellar motility at high cell densities by breaking down the mucus blanket.

We have proposed that Hap facilitates penetration of the mucus layer by infecting vibrios. The manner in which Hap production is regulated by environmental factors present in the human small bowel is consistent with such a role in pathogenesis. It is likely that at the initial stage of a cholera infection, when a low cell density prevails, hapA is not
expressed, providing a time interval for adherence, microcolony formation and surface translocation. An early wave of TCP expression, known to promote bacterial autoagglutination, could enhance microcolony formation at this stage (Chiang et al., 1995; Lee et al., 1999). As the cell density of infecting vibrios increases (HapR available) and carbon source becomes limited (CRP–cAMP available), in a proteinaceous medium (i.e. mucin) containing bile, production of Hap reaches its maximum. At this stage, degradation of the mucin blanket could allow motile vibrios to either detach or swim toward their target epithelial cells. It remains to be studied if the pattern of expression of Hap in vivo is consistent with this view or if inactivation of hapA perturbs the timely expression of other virulence factors during infection. Finally, since V. cholerae likely interacts with other proteinaceous surfaces outside the human host, regulation of extracellular protease production could have an impact on the ecology of this organism.

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