Endochitinase Is Transported to the Extracellular Milieu by the eps-Encoded General Secretory Pathway of Vibrio cholerae

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The chiA gene of Vibrio cholerae encodes a polypeptide which degrades chitin, a homopolymer of N-acetylglucosamine (GlcNAc), in a major component of the cell walls of fungi and the integuments of crustaceans and insects (38). The molecule is one of the most abundant biopolymers in nature and is used by many microorganisms as a source of carbon. Utilization of chitin as a nutrient usually requires degradation of the molecule to GlcNAc monomers. Complete degradation of chitin in both prokaryotes and eukaryotes is a two-step process which involves successive hydrolysis of the β-1,4 glycosidic bonds linking the GlcNAc subunits. In the first stage, endochitinase binds and degrades tetrameric and longer polymeric forms of GlcNAc to produce the disaccharide chitobiose. In the second step, chitobiase hydrolyzes chitobiose to GlcNAc monomers. The enzymes for chitin degradation are usually coordinately regulated and in several organisms are induced by chitosan, chitobiase, GlcNAc, or glucosamine (2, 7, 44).

Members of the family Vibrionaceae thrive in marine environments where chitin is abundant. It is not surprising that many Vibrionaceae evolved systems for utilizing chitin as a nutrient source. Chitinases have been identified in many prokaryotes and eukaryotes is a two-step process which involves successive hydrolysis of the β-1,4 glycosidic bonds linking the GlcNAc subunits. In the first stage, endochitinase binds and degrades tetrameric and longer polymeric forms of GlcNAc to produce the disaccharide chitobiose. In the second step, chitobiase hydrolyzes chitobiose to GlcNAc monomers. The enzymes for chitin degradation are usually coordinately regulated and in several organisms are induced by chitosan, chitobiase, GlcNAc, or glucosamine (2, 7, 44).

V. cholerae is a human intestinal pathogen that resides in brackish and marine waters. In vitro experiments established that V. cholerae has the potential to use chitin as a sole source of carbon for growth (15). It is likely, therefore, that production of chitinase (29, 30, 42) by V. cholerae provides the bacterium with a readily available nutrient source in aquatic environments. Hydrolysis of chitin by V. cholerae is an extracellular process that requires expression of epsE, one of a cluster of genes in the eps locus (43, 46–48). Several proteins of V. cholerae are dependent on the eps system for extracellular transport, including cholera toxin (CT), an undefined protease, and a chitinase activity (43, 48). Although expression of chitinase activity has been reported for V. cholerae, the enzyme responsible for the activity has not been identified. To further characterize the extracellular chitinase of V. cholerae, we cloned a gene encoding chitinase activity. Here we report the nucleotide sequence of a cloned endochitinase gene and establish that the protein encoded by that gene is secreted to the extracellular environment by an eps-dependent mechanism.

Materials and Methods

Bacterial strains, plasmids, and reagents. Bacterial strains and plasmids used in this investigation are listed in Table 1. Escherichia coli strains were cultured in LB broth. Classical biotypes of V. cholerae were cultured in trypticase soy broth (Difco Laboratories, Detroit, Mich.). All strains were maintained on LB agar. Chemical reagents were purchased from Sigma Biochemicals (St. Louis, Mo.), Life Technologies, Inc. (Gaithersburg, Md.), and Fisher Scientific (Springfield, N.J.). Unless otherwise noted, ampicillin was used at 150 μg/ml, chloramphenicol was used at 10 μg/ml, tetracycline was used at 10 μg/ml, and kanamycin was used at 50 μg/ml. All antibiotics were purchased from Sigma Biochemicals.

Preparation of a genomic library of V. cholerae 569B. Preparation of a genomic library of 569B was previously reported (10). Chromosomal DNA from V. cholerae 569B was prepared by standard methods, partially digested with the restriction enzyme Sau3AI, and size fractionated by sucrose gradient centrifugation. DNA fragments 25 to 50 kbp in size were pooled and ligated to BamHI-digested cosmid vector pCOS5 (10). Ligated DNA was packaged into bacteriophage lambda capsids, using a Gigapack packaging extract (Stratagene Cloning Systems, La Jolla, Calif.), which were transfected into E. coli LE392. The average insert size of the cosmids clones was 37 kbp.

Preparation of EGC agar. LB agar medium containing ethylene glycol chitin (EGC) was prepared by mixing 600 μl of an aqueous solution of EGC (10 mg/ml) Sigma Biochemicals) and 160 μl of 1% aqueous solution of trypan blue with 16 ml of molten (56°C) LB agar. Antibiotics were added, as appropriate. EGC plates inoculated with chitinase-producing strains were incubated at 37°C until clear halos surrounding the colonies were detected.
Acrylamide and bisacrylamide were purchased from Pharmacia Biotechnology. Flanking the multicloning site (Stratagene).

9
CTATAGGG-3

A. hydrophila
Undescribed isolate
R. K. Holmes

**TABLE 1.** Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibrio sp.</td>
<td>Classical biotype, wild type</td>
<td>23</td>
</tr>
<tr>
<td>569B</td>
<td></td>
<td>Life Technologies</td>
</tr>
<tr>
<td>569B(palp1036)</td>
<td>Spontaneous StrR R17 mutant of 569B</td>
<td>This study</td>
</tr>
<tr>
<td>M54</td>
<td>epsE mutant of 569B</td>
<td>23</td>
</tr>
<tr>
<td>0395</td>
<td>Classical biotype</td>
<td>59</td>
</tr>
<tr>
<td>U1</td>
<td>El Tor biotype</td>
<td>40</td>
</tr>
<tr>
<td>JBK70</td>
<td>El Tor biotype</td>
<td>31</td>
</tr>
<tr>
<td>569B(chiA::Kan')</td>
<td>Derivative of 569B with a Kan' gene inserted into chiA</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli</td>
<td>DH5α</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>DH5αF′tet</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>LE392</td>
<td>-</td>
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<tr>
<td></td>
<td>S17×pir</td>
<td>-</td>
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<tr>
<td>Plasmids</td>
<td>pCO5</td>
<td>Cosmid cloning vector, Amp' Chi'</td>
</tr>
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<td></td>
<td>pBlueScriptKS+</td>
<td>Phagemid cloning vector, Amp+</td>
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<td>pBlueScriptSKII+</td>
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<td>pUCK4</td>
<td>Plasmid encoding a Kan' gene cassette</td>
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<td>pKAS32</td>
<td>Suicide vector, Amp' R1</td>
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<td>pRS415</td>
<td>Promoter probe vector, Amp'</td>
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<td>pmal-p2</td>
<td>Expression vector for engineering translational fusions to MalE, Amp'</td>
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<td>pTDCC1</td>
<td>Cosmid clone of V. cholerae 569B, chiA</td>
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<td>pTDCC2</td>
<td>2.9-kbp Sau3AI fragment from pTDCC1 into the BamHI site of pBluescriptKS+</td>
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<td>pTDCC2.3</td>
<td>Frameshift mutation at the Nhel site of the insert in pTDCC2</td>
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<td>pTDCC2.5</td>
<td>BamHI/EcoRI fragment of pTDCC2 cloned into the BamHI site of pBluescriptSKII+</td>
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<td>pTDCC2.6</td>
<td>E-tag epitope fused to the 3' end of chiA cloned in pBlueScriptSKII+</td>
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<td>pJL1</td>
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<td>Nucleotides 157–465 of the insert of pTDCC2 cloned into pRS415</td>
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<td>pAKT-1</td>
<td>Kan' gene of pUCK4 inserted into the unique HpaI site of pTDCC2</td>
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<tr>
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<td>pJPF3</td>
<td>KpnI/SstI fragment of pAKT-1 into the same sites of pKAS32</td>
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<td></td>
<td>pTDCCepsE</td>
<td>Wild-type copy of epsE gene of 569B</td>
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</table>

Isolation and manipulation of plasmid DNA. Plasmids were obtained from E. coli by using a modified alkaline lysis method (25) and ethidium bromide extraction (58) or by use of PlasmidPure spin filters (Sigma Biochemicals). Restriction enzymes and DNA-modifying enzymes were purchased from Life Technologies. Agarose was purchased from J. T. Baker (Phillipsburg, N.J.). DNA fragments used for subcloning were isolated from agarose gel slices by extraction (58) or by use of PlasmidPure spin filters (Sigma Biochemicals).

E. coli DH5α were transformed into E. coli by osmotic shock (35), and transformants were selected on LB agar containing appropriate antibiotics.

**DNA sequencing.** Double-stranded DNA sequencing was performed at the Sequencing Facility of the Center for Advanced Molecular Biology and Immunology at the State University of New York at Buffalo, using fluorescent dye primer or dye terminator chemistry. Synthetic single-stranded oligonucleotide primers were used to initiate sequencing and for subsequent PCR were purchased from Integrated DNA Technologies, Inc. (Corvalle, Iowa). The nucleotide sequences of the synthetic oligonucleotides used for sequencing the insert in pTDCC2 were Chi-1 (5′-[5′]-741[CTGTTCACTGCGTGGTGG-3′], Chi-2 (5′-[732]CACACCGGACGCAGC-3′), Chi-3 (5′-[1504]GGCCAAACCGT TGCGCT-3′), Chi-4 (5′-[2167]AAACTCGTTGAGCC-3′), Chi-5 (5′-[370]GGTGCCACA-3′), Chi-6 (5′-[1994]CTCTAGCCGACC-3′), Chi-7 (5′-[252]CCCAAGCTGCGAGGTTG-3′), Chi-8 (5′-[304]GAAC TTTTAATCTGCAAC-3′), Chi-9 (5′-[2167]AAACTCGTTGAGCC-3′), Chi-10 (5′-[2319]ACATGCAGATATCAAG-3′), Chi-11 (5′-[2253]GTGGA ATTTGGCCGCC-3′), Chi-12 (5′-[2403]CTGAATATGCGGATAC-3′), and Chi-13 (5′-[2477]GCCATGCGGAGGATCG-3′). The numbers in brackets denote the position of the first nucleotide relative to the sequence in Fig. 2. Positive numbers are on the sense strand, while negative numbers are located on the antisense strand. The T7 (5′-TAATACGACTCACTATAGGG-3′) and T3 (5′-ATTAAACCTCTAGATAGGG-3′) primers are homologous to 5′ and 3′ regions of pBlueScriptKS+ and pBlueScriptSKII+ flanking the multicloning site (Stratagene).

**SDS-PAGE and Western (immunoblot) assays.** Isoelectric focusing-grade acrylamide and bisacrylamide were purchased from Pharmacia Biotechnology (Piscataway, N.J.). Unless otherwise noted, samples were prepared by solubilizing at 100°C for 10 min in a buffer containing 3 mM Tris (pH 6.8), 1% sodium dodecyl sulfate (SDS), 2.5% 2-mercaptoethanol, and 5% glycerol. Proteins in the solubilized samples were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 8.75% gels (33) which were stained for proteins with Coomassie brilliant blue (39). For immunoblotting, proteins were transferred to nitrocellulose paper. Blots were blocked for 30 min in a 5% (vol/vol) solution of skim milk in phosphate-buffered saline and incubated in phosphate-buffered saline–5% skim milk to which monoclonal antibody to the E-tag epitope (1:200; Pharmacia Biotech), rabbit anti-maltose binding protein (MBP) antiserum (1:2,000; New England Biolabs, Inc., La Jolla, Calif.), or rabbit anti-MBP-ChiA-E-tag antiserum (1:15,000) had been added. Affinity-purified rabbit anti-mouse immunoglobulin G antibodies (1:2,000; Sigma Biochemicals) were used as a second antibody when immunoblots had been initially probed with the mouse anti-E-tag monoclonal antibody. To detect antibody-bound polypeptides, immunoblots were probed with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:10,000; Sigma Biochemicals) and developed with 4-chloro-1-naphtol (20). In some cases, immunoblots were developed with a luminol reagent kit (DuPont/New England Nuclear, Wilmington, Del.). Fluorescent signals from the luminol blots were detected by exposure on Biomar Blue-sensitive autoradiographic film (Marsh Biomedical Products, Inc., Rochester, N.Y.). A Vista-S6E scanner (UXMAX Technologies, Inc., Fremont, Calif.) and Molecular Analyst/PC software (Bio-Rad Laboratories, Hercules, Calif.) were used for semiquantitative densitometric analysis of the exposed films.

Preparation of culture supernatants and periplasmic extracts. Culture supernatants and periplasmic extracts of bacterial cultures having an optical density at 600 nm of between 4.1 and 5.6 for late-log-phase cultures and between 4.5 and 5.4 for stationary-phase cultures were obtained as previously described (9, 11).

GM, ELISA for CT. Methods to measure CT in samples using ganglioside GM1-dependent enzyme-linked immunosorbent assay (ELISA) and a rabbit anti-CT antisera were previously described (11). Purified CT (a gift from R. K. Holmes) was used as a standard.

The numbers in brackets indicate positions on the antisense strand.

The numbers in brackets indicate positions on the sense strand.
Biochemical assays for chitinase activity. Chitinase activity was assayed by using chromogenic analogues of chitin (Sigma Biochemicals). For assays using p-nitrophenyl-N-acetyl-b-D-glucosaminide (p-NAG) (61), fresh colonies cultured overnight on LB agar were transferred by using a sterile toothpick onto small squares of filter paper, and 15 μl of a 4-MU conjugate was pipetted onto the colony to enhance fluorescence. Colonies were observed under 366-nm UV light for blue fluorescence which indicated chitinase-mediated hydrolysis of the conjugates to 4-methylumbelliferone. 

Construction of a帧shift mutation of chiA. Nelh-disrupted and Klenow-repaired pTDCC2 was self-ligated under conditions that favored recircularization of the linearized, blunt-ended plasmid. The ligation mixture was transformed into E. coli DH5αF′tet, and transformants were selected by plating onto LB agar containing ampicillin and tetracycline. A plasmid that had lost the NheI site was isolated and designated pTDCC2.3.

RESULTS

Cloning of chiA. Chitinases are commonly produced by members of the family Vibrionaceae (61). To determine if chitinase was produced by V. cholerae 569B, EGAC agar was stab inoculated with the strain. After 24 h of incubation, the site around the colony was surrounded by a large zone of clearing, indicating hydrolysis of the EGC (data not shown). To isolate the gene or genes that encoded the chitinase activity, approximately 800 cosmid clones of a 569B genomic library were screened for the ability to hydrolyze EGAC. Three cosmid clones were identified by the ability to elicit a zone of clearing in the EGAC agar. To determine if the cosmid clones had common DNA inserts, cosmid DNA prepared from each clone was digested with the restriction enzyme BanHI and the fragments were resolved by agarose gel electrophoresis. All three cosmids had identical BanHI restriction patterns (data not shown). Similar results were obtained when the cosmids were digested with SalI, providing strong evidence that the inserts of the three cosmid clones were identical. The cosmid from one of the clones was designated pTDCC1 (Fig. 1, strain 2).

Restriction mapping of pTDCC1 identified a DNA insert of 42 kb which had multiple sites for the restriction enzymes NruI, HpaI, and SspI. In a first attempt to subclone the chitinase gene, pTDCC1 was digested separately with restriction enzymes NruI, HpaI, and SspI, and fragments from each digestion were independently ligated into the expression vector pBluescriptKS+. None of the transformants from the ligations produced a chitinase-positive clone on EGAC agar, which suggested that the chitinase gene in pTDCC1 contained sites for each of the three restriction enzymes. As an alternative strategy to subclone the chitinase gene in pTDCC1, the cosmid was digested with SalI and fragments from 1.4 to 6.6 kb in size were ligated to pBluescriptKS+. Transformation of the ligation reaction into DH5αF′tet produced one chitinase-positive clone having a plasmid which was designated pTDCC2 (Fig. 1, strain 4).

Nucleotide sequencing of the 2,915-bp Sau3AI insert revealed an open reading frame (ORF) of 2,538 bp.
having the capacity to encode a polypeptide of 846 amino acids. A ribosomal binding site was found upstream of the ORF which was terminated by a TAA translation termination codon. Downstream of the translation termination codon was a nucleotide sequence having characteristics of a transcription terminator. A potential signal peptidase I cleavage site (Ala-X-Ala) (60) was found proximal to a series of hydrophobic amino acids at the N-terminal end of the predicted polypeptide. The arrangement of amino acids was consistent with a signal sequence that likely mediates transport of the encoded polypeptide across the cytoplasmic membrane.

The amino acid sequence encoded by the ORF in pTDCC2 had limited homology to the chitodextrinase of \( V. \) \( furnissii \) (33), the chitinase precursor of \( A. \) \( sp. \) strain 10S-24 (52), and chitinases produced by several other bacterial species (see Fig. 8).

**Confirming the ORF by frameshift mutagenesis.** To confirm that the ORF in pTDCC2 encoded the chitinase activity observed on EGC agar, we used a unique \( NheI \) site to engineer a 2,538-bp ORF in pTDCC2. The plasmid encoding the mutated ORF (Table 2). Based on the analysis of the nucleotide sequence and phenotypic characterization of the mutated plasmid, the 2,538-bp ORF in pTDCC2 was designated \( chiA \).

**Use of allelic exchange to engineer a \( chiA \) mutant of 569B.** To demonstrate that the \( chiA \) gene encoded the major chitinase activity of 569B, the wild-type copy of the gene in the chromosome was replaced by an insertionally inactivated copy of \( chiA \). The mutant, 569B(\( chiA \)::Kan r), exhibited little or no endochitinase activity on EGC agar (Fig. 3). In addition, Western blotting of 569B(\( chiA \)::Kan r) with the anti-ChiA antiserum showed a lack of the ChiA protein.
confirmed that the mutant did not produce immunoreactive protein (data not shown). We surmise that the slight residual chitinolytic activity evident in the EGC agar assay was due to nonspecific chitin degradation. An alternative explanation is that V. cholerae likely expresses, in addition to endochitinase, a chitobiase which degrades chitobiose, an intermediate molecule in the degradative pathway of chitin. It is possible that chitobiase has some minor reactivity against EGC.

**Mapping the chiA promoter.** Expression of chiA was likely a result of the activity of an endogenous promoter within the 3.0-kbp insert of pTDCC2, since nucleotide sequence analysis of the pTDCC2 showed that chiA was positioned in the orientation opposite that of the lac promoter in the vector. Rather than having no effect on expression, addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to cultures of E. coli DH5αF’tet(pTDCC2) caused a decrease in chitinase activity (Fig. 1, streak 4). This observation suggested that the promoter was present in the 174 nucleotides that preceded the ATG initiation codon of the gene. To map the promoter of chiA more precisely, four DNA fragments isolated by PCR from the 5’ end of the insert of pTDCC2 (nucleotides 31 to 465, 99 to 465, 123 to 465, and 157 to 465) were cloned into pRS415 (53), a vector engineered for promoter analysis. When introduced into 569B, only pZ1 expressed high levels of β-galactosidase activity; pZ3 containing nucleotides 98 to 465 expressed very little β-galactosidase, as did plasmids containing shorter segments of the DNA upstream of chiA (Table 3). These results strongly suggested that promoter activity for chiA was located within a 69-bp region bounded by nucleotides 29 and 97 (Fig. 2). Sequences homologous to consensus –35 and –10 hexamers which are components of most σ70-like promoters were not evident in the 68-bp region. It should be noted that 569B exhibited a low level of endogenous β-galactosidase activity (pRS415 [Table 3]), but this background did not interfere with the measurements of promoter activity.

**Defining the substrate specificity ChiA.** To determine the substrate specificity of the enzyme encoded by chiA, we employed a rapid test method that used a series of 4-MU conjugates (41). Hydrolysis of the glycosidic bonds of the conjugates releases 4-umbelliferone, which fluoresces with a strong blue light when illuminated with UV irradiation. Five different 4-MU conjugates were used in these experiments: 4-MU-N-acetyl-α-D-glucosaminide, 4-MU-N-acetyl-β-D-glucosaminide, 4-MU-N-acetyl-β-D-galactosaminide, 4-MU-β-D-N,N',N'-triacytlychitotribose, and 4-MU-β-D-N,N',N'-triacetylchitotriose. When expressed in DH5αF’tet(pTDCC2) cells, it was likely a conjugate that releases a yellow pigment when the glycosidic bonds are cleaved (61). A minor amount of hydrolytic activity for 4-MU-N-acetyl-β-D-glucosaminide was evident after prolonged incubation of the substrate on DH5αF’tet(pTDCC2) cells. It was concluded from the pattern of degradation of the synthetic substrates that ChiA encoded by pTDCC2 was likely an endochitinase capable of hydrolyzing β,1-4 glycosidic bonds.

**Production of anti-ChiA hyperimmune serum.** To expedite the process of obtaining purified ChiA for raising antibodies, a maltE-chiA fusion was constructed by using the expression vector pmal-p2 (New England Biolabs) and a PCR-amplified fragment of pTDCC2.6. Ligation of the amplified fragment into pmal-p2 by directional cloning positioned the chiA gene into pBluescriptKS+.

**TABLE 2. Use of synthetic chitin analogues to determine the substrate specificities of wild-type and recombinant ChiA**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>p-NAG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N-acetyl-α-D-glucosaminide</th>
<th>N-acetyl-β-D-glucosaminide</th>
<th>N-acetyl-β-D-galactosaminide</th>
<th>β-β-N,N'-diacytlychitobiose</th>
<th>β-β-N,N',N'-triacetylchitotriose</th>
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</thead>
<tbody>
<tr>
<td>V. cholerae 569B</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. coli DH5αF’tet(pTDCC2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>E. coli DH5αF’tet(pTDCC2.3)</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>E. coli DH5αF’tet(pBluescriptKS+&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>ND</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup> Hydrolysis of the substrate releases a yellow pigment (61). +, yellow; –, no color change.

<sup>b</sup> Hydrolysis of the substrate releases 4-methylumbelliferone, which fluoresces when exposed to UV (366 nm) light (41). +, fluorescent; –, not fluorescent.

<sup>c</sup> ND, not done.

**FIG. 3. An insertion mutation in chiA abolishes endochitinase activity in V. cholerae 569B.** Production of chitinase activity was measured by culturing wild-type (569B) and mutant [569B(chiA::Kan)] V. cholerae strains on EGC agar. E. coli DH5αF’tet cells containing pTDCC2 and the phagemid vector pBluescriptKS+ served as positive and negative controls, respectively.
E. coli expressed in DH5a with the exception that pTDCC1 was expressed in E. coli LE392. Molecular mass standards are in kilodaltons.

FIG. 4. Expression of recombinant ChiA. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune antiserum. All plasmids were expressed in E. coli DH5α F′ tet with the exception that pTDCC1 was expressed in E. coli LE392. Molecular mass standards are in kilodaltons.

the vector such that an in-frame fusion to the 3′ end of malE fusion was produced. Introduction of the fusion plasmid pJL1 into DH5α F′ tet produced a strain that expressed strong IPTG-inducible chitinase activity on EGC agar (Fig. 1, streak 7).

Chromatography using an amylose affinity matrix was used to purify the hybrid MBP-ChiA polypeptide from DH5α F′ tet (pJL1) (data not shown). Immunization of a rabbit with the affinity-purified preparation produced a high-titer antiserum that reacted in immunoblots with a 90-kDa polypeptide encoded by pTDCC1 and pTDCC2 (Fig. 4) and with an immunoreactive polypeptide of similar size in 569B. The antiserum also reacted with a polypeptide of 127 kDa in DH5α F′ tet (pJL1), which was consistent with the predicted size of the MBP-ChiA fusion protein. A smaller polypeptide of 42 kDa in the immunoblots that reacted with the anti-MBP-ChiA antiserum was slightly larger in size to recombinant MalE. No immunoreactive polypeptide of similar size was detected in 569B, indicating that V. cholerae did not express a protein with antigenic similarity to MalE. Immunoblotting of whole cells of V. cholerae showed that the preimmune rabbit serum did not react with ChiA (data not shown).

Expression of ChiA in classical and El Tor biotypes of V. cholerae and in A. hydrophila. Bacterial cultures of two classical biotypes (569B and 0395) and two El Tor biotypes (U1 and JBK70) of V. cholerae were analyzed for reactivity to the anti-ChiA antiserum. All four strains synthesized immunoreactive polypeptides that were similar in size to the recombinant polypeptide encoded by pTDCC2 (Fig. 5). A smaller immunoreactive polypeptide having an apparent molecular mass of 65 kDa was also present in all four strains. No protein of V. cholerae corresponding to the 42-kDa MalE of E. coli was detected in the anti-ChiA immunoblots.

Two anti-ChiA reactive proteins were found in A. hydrophila, a bacterium that is taxonomically related to V. cholerae (Fig. 5, lane 6). The slower-migrating polypeptide had an apparent molecular mass of ~96 kDa, which was slightly larger than the molecular mass of recombinant ChiA. The faster-migrating polypeptide had an apparent molecular mass of 37 kDa, which was similar to the molecular mass of the E. coli MalE.

Extracellular transport of ChiA. Chitinases in other species are typically extracellular proteins. To establish whether ChiA was transported to the extracellular medium by V. cholerae, culture supernatants and periplasmic extracts isolated from late-log-phase and stationary-phase cultures of 569B were analyzed by SDS-PAGE and immunoblotting for ChiA (Fig. 6A). In late-log-phase cultures of 569B, two immunoreactive polypeptides were found in the culture supernatant. The major polypeptide had an apparent molecular mass of 90 kDa, which was equivalent in size to recombinant ChiA. A minor, faster-migrating polypeptide of 76 kDa was also evident. Periplasmic extracts of 569B cultures contained only the 90-kDa polypeptide. Semiquantitative densitometric analysis of the immunoblots demonstrated that the majority of the total immunoreactive protein (69.4%) of 569B was located in the culture supernatant of late-log-phase cultures (Fig. 6).

Immunoblots were also used to measure the amount of ChiA polypeptides in the culture supernatants and periplasmic extracts of stationary-phase cultures of 569B. In stationary-phase cultures, the major immunoreactive polypeptide was a smaller 65-kDa polypeptide (Fig. 6B). The 90-kDa polypeptide was found in the periplasmic extracts but not in the culture supernatants. Semiquantitative densitometric analysis showed that most (76%) of the total immunoreactive protein from stationary-phase cultures of 569B was located in the culture supernatant (Fig. 7). These data are consistent with a model where during later phases of growth ChiA is either processed or degraded from 90 kDa in size to 65 kDa prior to release of the protein into the culture medium.

Previous data supported the contention that chitinase activity was dependent on the eps system for extracellular transport. To establish whether ChiA was secreted by the eps system, immunoblot experiments were done with M14, a derivative of 569B having a mutation in epsE (11, 48). In contrast to 569B, less than 14% of the total immunoreactive protein was associated with the culture supernatant in both late-log-phase and stationary-phase cultures of M14 (Fig. 6 and 7). To determine whether the defect in extracellular transport of ChiA was

FIG. 5. Expression of chitinase by classical (569B and 0395) and El Tor (JBK70 and U1) strains of V. cholerae and by A. hydrophila. Bacterial cultures comprised cells and culture supernatants were used for the immunoblotting analysis. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune antiserum. The antiserum did not react with proteins in fresh culture media (data not shown). pTDCC2 was expressed in E. coli DH5α F′ tet. Molecular mass standards are in kilodaltons.
due solely to the mutation in epsE, M14 was complemented with pTDCepsE, a plasmid encoding a wild-type copy of the epsE gene of S. marcescens. Complementation of the mutant with pTDCepsE (11) restored extracellular secretion of ChiA. Over 82% of total immunoreactive protein was located in the culture supernatant M14(pTDCepsE) in both late-log-phase and stationary-phase cultures (Fig. 6 and Fig. 7).

Since CT is known to be secreted by the eps pathway, culture supernatants and periplasmic extracts were also measured for CT by ganglioside GM1 ELISA (5, 12). While CT was transported into the supernatant by 569B, M14 was unable to transport CT from the periplasm. Complementation of M14 with plasmid pTDCepsE restored extracellular transport of CT (Fig. 6 and 7).

These data provided strong evidence that the eps-encoded pathway is required for extracellular transport of both CT and ChiA.

DISCUSSION

Mechanisms for extracellular transport of proteins have been identified in a diverse number of bacteria. Klebsiella oxytoca (44), Erwinia chrysanthemi (22), Erwinia carotovora (34), Pseudomonas aeruginosa (1), Xanthomonas campestris (13), and A. hydrophila (3) contain a cluster of genes that encode the main terminal branch of the general secretory pathway (GSP). Recently, it was determined that V. cholerae has a cluster of genes that are homologous to the secretory clusters in those bacteria. At least 12 genes are encoded by the eps cluster of V. cholerae (43, 47, 48). Extracellular transport of CT, an oligomeric enterotoxin produced by the bacterium, is dependent on expression of the eps genes (11, 43, 47, 48). Here we provide strong evidence that the eps system of V. cholerae is also involved in extracellular transport of ChiA.

The degree of shared homology observed among the secretory systems of the diverse bacteria does not enable the systems to be interchanged. In most cases, extracellular proteins are secreted only by their cognate secretory systems. For example, E. chrysanthemi secretes a pectate lyase, but the pectate lyase of E. chrysanthemi is not secreted by K. oxytoca (22). The type I heat-labile enterotoxin LT-I, a protein that is highly homologous to CT, is secreted by V. cholerae and seven species of the family Vibrionaceae but not by E. chrysanthemi, Xanthomonas maltophilia, or K. pneumoniae (43). Of the secretory systems that have been well described, it appears that the eps system of V. cholerae is the most promiscuous. Previous investigations established that the eps system of V. cholerae promotes secretion not only of CT and LT-I but also of the B polypeptides of LT-IIa and LT-IIb, two members of the type II heat-labile enterotoxins of enterotoxigenic E. coli (11). ChiA can now be added to the list of extracellular proteins secreted by the eps system. The ability of CT, LT-I, LT-IIa, LT-IIb, and ChiA to traffic through the same secretory pathway suggests that each of the proteins has an extracellular transport signal that is recognized by the eps system. The molecular structures of the signals in the five proteins have not been identified, but there is growing evidence that the signals are not composed of a linear array of conserved amino acids. CT and the type II enterotoxins have little if any amino acid homology yet are secreted with equal efficiency by V. cholerae (11). From these observations, it was hypothesized that the transport signals in these three proteins were likely conserved conformation-dependent motifs. Since ChiA is not homologous to CT or to the type II enterotoxins, it is likely that the extracellular transport signal in ChiA is also a conformation-dependent motif. A similar situation was observed in K. oxytoca, where two nonadjacent regions of pullulanase were required to promote translocation of a β-lactamase fusion protein across the outer membrane (50, 51). It is possible that many if not all extracellular transport signals in proteins transported by eps-like secretory systems are comprised of conformation-dependent domains. If that is indeed the case, experiments to define the structures of the transport signals may require high-resolution crystal structures of the proteins.

When expressed in E. coli, CT and the type II heat-labile enterotoxins LT-IIa and LT-IIb accumulate in the periplasm of the cell. A similar pattern of periplasmic accumulation of extracellular proteins was observed when the genes for amylase (17), aerolysin (24), and protease (45) of A. hydrophila and the gene for pullulanase of K. pneumoniae (12) were expressed in E. coli. For pullulanase, mobilization of the proteins out of the periplasm of E. coli required coexpression of the pil cluster, the cognate secretory system (12). However, when the genes for CT, LT-IIa, and LT-IIb were expressed in E. coli, low but significant amounts of the proteins were found in the extracellular medium (11). Hydrolysis of EGC by DH5α(pTDCCC2) suggested that recombinant ChiA was also released to the extracellular medium. Using β-lactamase as a marker for periplasmic proteins, we found that recombinant ChiA, CT, LT-IIa, and LT-IIb in the culture supernatants of E. coli could be attributed to passive release by natural autolysis of the cells (data not shown). Results from these control experiments did not rule out the possibility that small amounts of ChiA are actively transported in E. coli. The chininases of Serratia mar-

FIG. 6. Secretion of ChiA by V. cholerae depends on epsE. (A) Late-log-phase culture; (B) stationary-phase culture. M14 is an epsE mutant derived from S. marcescens. pTDCepsE is a clone of the wild-type epsE gene from S. marcescens. pTDCepsE and pBluecriptKS were expressed in E. coli DH5αF′tet. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune serum. S, culture supernatant; P, periplasmic extract. Molecular mass standards are in kilodaltons.
(28) and _A. hydrophila_ (6) were mostly found in the extracellular medium when the genes for these proteins were cloned in _E. coli_. The mechanism by which these chitinases were transported to the medium has not been elucidated. An intriguing possibility is that _E. coli_ transports the chitinases across the outer membrane by an intrinsic secretory system. Genes homologous to those encoding the main terminal branch of the GSP have been identified in _E. coli_ (14). Complementation experiments using the _pul_ system of _K. oxytoca_ showed that at least two of the _E. coli_ genes, _gspO_ and _gspG_, were functional (14). Although it has not been demonstrated that the _gsp_ secretory system of _E. coli_ is expressed under most laboratory conditions, it is an interesting proposition that _gsp_ genes may be involved in transport of the chitinases of _S. mar-

**FIG. 7.** Localization of ChiA and CT in wild-type and mutant _V. cholerae_. Percentage values represent the relative amounts of ChiA and CT in the respective samples. (A) Total immunoreactive ChiA in culture supernatants and periplasmic extracts. Semiquantitative data were obtained from densitometric analysis of the immunoblots in Fig. 6. (B) Total CT in the culture supernatants and periplasmic extracts used for the immunoblot shown in Fig. 6. CT in the samples was measured by GM₁ ELISA (4, 11).
cascens and A. hydrophila across the E. coli outer membrane. Low-level transport of ChiA by the gsp system could be responsible for the extracellular chitinase activity of E. coli harboring chiA genes. Expression of chiA in E. coli gsp mutants will be required to test this rather speculative hypothesis.

The predicted molecular mass of the recombinant ChiA was in good agreement with the size of the largest immunoreactive protein detected in the anti-ChiA immunoblots of 569B. Time course experiments demonstrated that while a 90-kDa immunoreactive protein was present in the culture supernatants from late-log-phase cultures of 569B, little if any 90-kDa polypeptide was evident in culture supernatants from stationary-phase cultures. In those culture supernatants, the predominant immunoreactive molecule was a 65-kDa polypeptide. Results from preliminary experiments using EGC zymograms indicated that the 65-kDa polypeptide was enzymatically active (data not shown). While it is possible that the 65-kDa polypeptide was simply a degradative product of the 90-kDa polypeptide, it is equally possible that ChiA is purposefully processed to the smaller polypeptide by factors that are expressed only when the cells enter stationary phase.

Amino acid sequence analysis showed that ChiA has homology to chitinases produced by the soil bacteria Bacillus licheniformis (GenBank entry U71214), Janthinobacterium lividum (16), and Alteromonas sp. (21), as well as the marine species V. harveyi (GenBank entry U81496), Vibrio furnissii (32), Alteromonas sp. strain 10S-24 (52), and Aeromonas caviae (54) (Fig. 8). Significant homology to ChiA was confined to a 38-amino-acid domain. The degree of sequence similarity observed among the chitinases suggests an importance for the domain in enzymatic activity. Structure-function studies of the chitinase from Alteromonas sp. strain 10S-24 (52) and of related chitinases indicated that this domain may have chitin-binding activity. Although conserved in structure, the location of the putative chitin-binding domain is divergent. In ChiA of V. cholerae and the chitinases of V. furnissii, Alteromonas sp. strain 10S-24, and J. lividum, the domain is located in the amino-terminal third of the proteins. In the chitinases of V. harveyi, A. caviae, and B. licheniformis, the domain is located in the carboxyl-terminal third of the proteins. Mutational analysis will be needed to confirm the role of the domain in enzymatic activity of ChiA.

Degradation of chitin by free-living V. cholerae is thought to begin with binding of the bacterium to the homopolypeptide by a chitin-binding surface receptor. In vivo experiments demonstrated that the chitin-binding receptor also has affinity for ligands on the surfaces of rabbit epithelial cells and chicken erythrocytes (49). Binding to the cell surface was inhibited by GlcNAc, the monomeric unit of chitin. β,1-4-linked glycosidic bonds occur frequently in glycosylated molecules found on the surfaces of many epithelial cells. It is tempting to speculate that V. cholerae may bind to intestinal cells by interaction between the chitin-binding receptor and an unidentified glycosylated cell surface molecule. Furthermore, it is conceivable that the β,1-4-linked glycosidic bonds in these cell surface molecules are cleaved by chitinases produced by V. cholerae. It will be interesting to determine if ChiA has hydrolytic activity for glycosylated molecules on the intestinal epithelial cell surface and whether mutations in chiA reduce the virulence of the pathogen.

Translocation of proteins across membranes is a fundamental property of prokaryotic and eukaryotic cells. To better understand the process of protein translocation, it will be necessary to elucidate the mechanisms by which the secretory systems recognize and transport extracellular proteins. Investigations into extracellular secretion of ChiA and other proteins by V. cholerae will facilitate experiments to discover the cognate transport signals and how those signals interact with components of the secretory machinery. Current experiments are focused on the use of genetics to delimit the regions of CT and ChiA that are required for extracellular transport.

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