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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) found in cell walls of fungi and in the integuments of insects and crustaceans. *chiA* has a coding capacity corresponding to a polypeptide of 846 amino acids having a predicted molecular mass of 88.7 kDa. A 52-bp region with promoter activity was found immediately upstream of the *chiA* open reading frame. Insertional inactivation of the chromosomal copy of the gene confirmed that expression of chitinase activity by *V. cholerae* required *chiA*. Fluorescent analogues were used to demonstrate that the enzymatic activity of ChiA was specific for β,1-4 glycosidic bonds located between GlcNAc monomers in chitin. Antibodies against ChiA were obtained by immunization of a rabbit with a MalE-ChiA hybrid protein. Polypeptides with antigenic similarity to ChiA were expressed by classical and El Tor biotypes of *V. cholerae* and by the closely related bacterium *Aeromonas hydrophila*. Immunoblotting experiments using the wild-type strain 569B and the secretion mutant M14 confirmed that ChiA is an extracellular protein which is secreted by the *eps* system. The *eps* system is also responsible for secreting cholera toxin, an oligomeric protein with no amino acid homology to ChiA. These results indicate that ChiA and cholera toxin have functionally similar extracellular transport signals that are essential for *eps*-dependent secretion.

Chitin, a homopolymer of *N*-acetylglucosamine (GlcNAc), is 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, chitobiose, 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 *Vibrio vulnificus* (56, 61), *V. harveyi* (57), and *V. parahemolyticus* (29, 30). Nucleotide sequence analysis indicated that the chitinase of *V. harveyi* is homologous with human hexosamindase, indicating that the two enzymes, as well as other chitinases, are members of a phylogenically related group (56).

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 Luria-Bertani (LB) broth. Classical biotypes of *V. cholerae* were cultured in Syncase broth (26), El Tor biotypes of *V. cholerae* were grown in YEP broth (27), and *Aeromonas hydrophila* was cultured in Tryticase soy broth (Difco Laboratories, Detroit, Mich.). All strains were maintained on LB agar. Chemical reagents were obtained 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 *Sau*3AI, and size fractionated by sucrose gradient centrifugation. DNA fragments 25 to 50 kbp in size were pooled and ligated to *Bam*HI-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 cosmid 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.

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics	Reference or source
Vibrio sp.		
569B	Classical biotype, wild type	23
569B*stp*10*rif*	Spontaneous Str^r Rif^r mutant of 569B	This study
M14	*epsE* mutant of 569B	23
0395	Classical biotype	59
U1	El Tor biotype	40
JBK70	El Tor biotype	31
569B(*chiA*::Kan^r)	Derivative of 569B with a Kan^r gene inserted into *chiA*	This study
A. hydrophila	Undescribed isolate	R. K. Holmes
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 phoA hsdR17* (r_K^- m_K^+) *supE44* λ^- *thi-1 gyrA96 relA1*	Life Technologies
DH5αF′tet	DH5α transconjugant with [F′ *proAB* $lacI^qZ$ΔM15 Tn*10*(Tet^r)]	Life Technologies and Stratagene
LE392	F^- e14$^-$ ($McrA^-$) *hsdR514* (r_K^- m_K^+) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55*	35
S17λ*pir*	Tp^r Sm^r *recA thi pro* $hsdRM^+$ [RP4-1-Tc::Mu:Km^rTn*7*] (λpir)	36
Plasmids		
pCOS5	Cosmid cloning vector, Amp^r Chl^r	10
pBluescriptKS+	Phagemid cloning vector, Amp^r	Stratagene
pBluescriptSKII+	Phagemid cloning vector, Amp^r	Stratagene
pUCK4	Plasmid encoding a Kan^r gene cassette	Pharmacia Biotech
pKAS32	Suicide vector, Amp^r Stp^s	55
pRS415	Promoter probe vector, Amp^r	53
pmal-p2	Expression vector for engineering translational fusions to MalE, Amp^r	New England Biolabs
pTDCC1	Cosmid clone of *V. cholerae* 569B, $chiA^+$	This study
pTDCC2	2.9-kbp *Sau*3AI fragment from pTDCC1 into the *Bam*HI site of pBluescriptKS−	This study
pTDCC2.3	Frameshift mutation at the *Nhe*I site of the insert in pTDCC2	This study
pTDCC2.5	*Bam*HI/*Eco*RI fragment of pTDCC2.4 into *Bam*HI/*Eco*RI site of pBluescriptSKII+	This study
pTDCC2.6	E-tag epitope fused to the 3′ end of *chiA* cloned in pBluescriptSKII+	This study
pJL1	*chiA*/E-tag hybrid gene of pTDCC2.6 fused to the 3′ end of *malE* in pmal-p2	This study
pZ1	Nucleotides 31–465 of the insert of pTDCC2 cloned into pRS415	This study
pZ3	Nucleotides 99–465 of the insert of pTDCC2 cloned into pRS415	This study
pZ13	Nucleotides 18–465 of the insert of pTDCC2 cloned into pRS415	This study
pZ56	Nucleotides 157–465 of the insert of pTDCC2 cloned into pRS415	This study
pAKT-1	Kan^r gene of pUCK4 inserted into the unique *Hpa*I site of pTDCC2	This study
pJPF3	*Kpn*I/*Sst*I fragment of pAKT-1 into the same sites of pKAS32	This study
pTDC*epsE*	Wild-type copy of *epsE* gene of 569B	11

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 using a GeneCleanII kit (Bio 101, Inc., La Jolla, Calif.). Plasmids 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 used to initiate sequencing and for subsequent PCR were purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). The nucleotide sequences of the synthetic oligonucleotides used for sequencing the insert in pTDCC2 were Chi-1 (5′-[−741]GCTGTTCACTGCCCGTTG-3′), Chi-2 (5′-[732]CAACGGGCAGTGACAGC-3′), Chi-3 (5′-[−1504]GGCCAAACCGTTGGTCTA-3′), Chi-4 (5′-[1474]TAGACCAACGGTTTGGCC-3′), Chi-5 (5′-[1936]CAAGCAAGATATGAAAGC-3′), Chi-6 (5′-[−1994]CCTTCAGCGCCACC-3′), Chi-7 (5′-[252]CGGAGTGCCAGTGTG-3′), Chi-8 (5′-[−304]GAACTTTATCACTGCCAA-3′), Chi-9 (5′-[2167]AAAAACATCGGTGGTGAT-3′), Chi-10 (5′-[−2319]ACATGCAGAATATCAAG-3′), Chi-11 (5′-[2253]GTGGAATTTGGGGCGC-3′), Chi-12 (5′-[−2618]CTGCATAATTGGGGTAG-3′), and Chi-13 (5′-[2477]GCCATTGGTTTGCCATCAGG-3′). The numbers in brackets denote the position of the first nucleotide relative to the sequence in Fig. 2. Positive numbers are positions on the sense strand, while negative numbers indicate positions on the antisense strand. The T7 (5′-TAATACGACTCACTATAGGG-3′) and T3 (5′-ATTAACCCTCACTAAAGGGA-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 31 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 silver (37) or colloidal Coomassie brilliant blue (39). Proteins for immunoblotting were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose paper. Blots were blocked for 30 min in a 5% (wt/vol) solution of skim milk in phosphate-buffered saline and incubated in phosphate-buffered saline–5% skim milk to which mouse 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-naphthol (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 (UMAX 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_1 ELISA for CT. Methods to measure CT in samples using ganglioside GM_1-dependent enzyme-linked immunosorbent assay (ELISA) and a rabbit anti-CT antiserum were previously described (11). Purified CT (a gift from R. K. Holmes) was used as a standard.

Biochemical assays for chitinase activity. Chitinase activity was assayed by using chromogenic analogues of chitin (Sigma Biochemicals). For assays using *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (*p*-NAG) (61), fresh colonies cultured overnight on LB agar were transferred by using a sterile toothpick onto small squares of Whatman 3MM filter paper (Schleicher & Schuell, Keene, N.H.). Fifteen microliters of *p*-NAG diluted to 3.6 mg/ml in 0.1 M sodium phosphate buffer (pH 7.4) was pipetted onto each colony. After being wetted with water, filter papers were incubated for 30 min at 37°C and observed for production of a bright yellow color which indicated chitinase (chitobiase) activity. 4-Methylumbelliferyl (4-MU)-*N*-acetyl-α-D-glucosaminide, 4-MU-*N*-acetyl-β-D-glucosaminide, 4-MU-*N*-acetyl-β-D-galactosamide, 4-MU-β-D-*N*,*N*′-diacetylchitobioside, and 4-MU-β-D-*N*,*N*′,*N*″-triacetylchitotrioside were used for detection of chitinase activity (41). Conjugates dissolved initially in a small amount of dimethylformamide were diluted in 0.1 M sodium phosphate buffer (pH 7.4) to a final concentration of 1.43 mM. Fresh colonies from overnight LB agar plates were transferred to squares of filter paper, and 15 μl of a 4-MU conjugate was pipetted to the side of the colony and allowed to diffuse into the cells by capillary transfer. After 15 min of incubation at 37°C, a few drops of an aqueous saturated sodium bicarbonate solution 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 frameshift mutation in *chiA*. *Nhe*I-digested 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 *Nhe*I site was isolated and designated pTDCC2.3.

Construction of promoter probe plasmids. DNA fragments were obtained by PCR from regions upstream of the *chiA* gene in pTDCC2. Synthetic oligonucleotides used as PCR primers were Chi-15 (5′-GGAATT[29]CCGAAAAAGAATTGAAAGC[47]-3′), Chi-17 (5′-GGAATTCC[99]ATTGATTAACATGCA[113]-3′), Chi-18 (5′-GGAA[123]TTCACTCTGGAGTATTAG[140]-3′), Chi-19 (5′GGAAT[157]TCTAATACAGGAGAGAAACA[176]-3′), and Chi-20 (5′-CGGGATC[−465]CTTGGCGTTATTGAGTG[-449]-3′). Brackets denote nucleotide positions, as noted above; restriction sites for *Eco*RI are denoted by single underlining, while the site for *Bam*HI is denoted by a double underline. pTDCC2 was used as a template in PCR using the following reaction conditions: 30 s at 92°C, 45 s at 45°C, and 60 s at 72°C (30 cycles). DNA fragments obtained from the reactions were digested with *Eco*RI and *Bam*HI and ligated into the same sites of the multicloning site of the promoter probe vector pRS415 (53).

Engineering a *chiA* mutant of 569B. The suicide plasmid pKAS32 (55) was used for allelic exchange mutagenesis. The *chiA* gene was insertionally inactivated by ligating an end-repaired *Eco*RI fragment of pUC4K (Pharmacia LKB Biotechnology) into the unique intragenic *Hpa*I site of pTDCC2. This intermediate plasmid was designated pATK-1. A *Kpn*I/*Sst*I fragment of pATK-1 was subsequently ligated into equivalent sites of the suicide plasmid pKAS32 to produce pJPF3. The suicide plasmid was then transformed into *E. coli* S17λ*pir* (36) and conjugated into 569B*stp*10*rif*, a spontaneous streptomycin- and rifampin-resistant mutant of 569B. A single ampicillin-resistant transconjugant having pJPF3 integrated into the genome was grown overnight in LB broth without antibiotic selection at 37°C. Overnight cultures were plated on LB agar containing streptomycin (30 μg/ml) to select for clones in which the plasmid sequences had been deleted from the chromosome by recombination between the wild-type and mutant copies of the *chiA* genes. Streptomycin-resistant clones were screened for loss of plasmid-encoded ampicillin resistance and for loss of chitinase activity on EGC agar. A transconjugant that was ampicillin sensitive and chitinase negative was designated 569B(*chiA*::Kan^r). PCR analysis using the oligonucleotides Chi-2 and Chi-4 and Southern hybridization using pKAS32 and a PCR-derived fragment of *chiA* as hybridization probes were used to confirm the allelic exchange of *chiA* in 569B(*chiA*::Kan^r).

Engineering a *malE-chiA* fusion. PCR was used to facilitate engineering a *malE-chiA* chimera by fusing the *malE* gene to a fragment encoding the entire structural gene of *chiA* excluding the putative signal peptide. A DNA fragment containing the *chiA* gene was amplified from pTDCC2.6, a recombinant plasmid in which sequences encoding a 13-amino-acid E-tag epitope (Pharmacia Biotech) was fused in frame to the 3′ end of *chiA*. Oligonucleotide primers used in the PCR were Chi-Mal (5′-GGTCTAGA[241]TATAACTGTGCCGGAGTG-3′) and Blue-619 (5′-GTAAAACGACGGCCAGTGA-3′). The numbers in brackets denote the position of the first nucleotide in the sense strand relative to the sequence in Fig. 2. Use of Chi-Mal in the PCR incorporated an *Xba*I site (underlined) into the amplified DNA fragment at a position immediately 5′ to the triplet codon for tyrosine-23, the predicted NH_2-terminal amino acid of the mature ChiA protein. Blue-619 is homologous to a region of the multicloning site in pTDCC2.6 downstream of the 3′ ligation joint of the DNA insert. The amplified DNA was digested with the restriction enzymes *Xba*I and *Hin*dIII, and the resulting 2.5-kbp fragment was ligated to the expression vector pmal-p2 in the *Xba*I-*Hin*dIII sites to produce an in-frame hybrid of *malE* to *chiA*. After transformation of the ligation mixture into *E. coli* DH5αF′tet, clones were analyzed by restriction mapping and one clone harboring a plasmid with an appropriate restriction map was screened on EGC agar to confirm a ChiA$^+$ phenotype. The junction sites of plasmid pJL1 were sequenced to confirm the in-frame fusion.

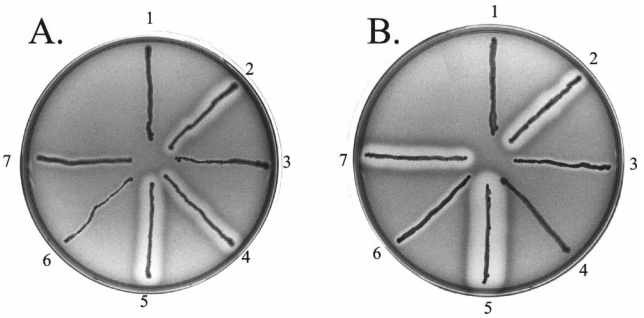

FIG. 1. Detection of chitinase activity produced by *chiA* clones grown on EGC agar. (A) No IPTG induction; (B) induction with 0.1 mM IPTG. Streaks: 1, pCOS5; 2, pTDCC1; 3, pBluescriptKS−; 4, pTDCC2; 5, pTDCC2.6; 6, pmal-p2; 7, pJL1. *E. coli* DH5αF′tet was used as a host strain for all plasmids except pTDCC1, which was expressed in LE392.

Amylose affinity chromatography. The MBP–ChiA–E-tag fusion protein was purified by established methods using sucrose extraction (18, 19) and amylose affinity chromatography (New England Biolabs). The purity of the MBP-ChiA fusion protein was determined by SDS-PAGE and immunoblotting using a rabbit anti-MBP antiserum (New England Biolabs) and an anti-E-tag mouse monoclonal antibody (Pharmacia Biotech).

Preparation of rabbit antiserum. Hyperimmune antiserum to the affinity-purified MBP-ChiA fusion protein was obtained from subcutaneous immunization of a New Zealand White rabbit (5). Immunizations and serum collection were performed by the Monoclonal Antibody Center at the State University of New York at Buffalo.

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, EGC 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 EGC. Three cosmid clones were identified by the ability to elicit a zone of clearing in the EGC agar. To determine if the cosmid clones had common DNA inserts, cosmid DNA prepared from each clone was digested with the restriction enzyme *Bam*HI and the fragments were resolved by agarose gel electrophoresis. All three cosmids had identical *Bam*HI restriction patterns (data not shown). Similar results were obtained when the cosmids were digested with *Sal*I, providing strong evidence that the inserts of the three cosmids were identical. The cosmid from one of the clones was designated pTDCC1 (Fig. 1, streak 2).

Restriction mapping of pTDCC1 identified a DNA insert of 42 kbp which had multiple sites for the restriction enzymes *Nru*I, *Hpa*I, and *Ssp*I. In a first attempt to subclone the chitinase gene, pTDCC1 was digested separately with restriction enzymes *Nru*I, *Hpa*I, and *Ssp*I and the 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 EGC 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 partially digested with *Sau*3AI and fragments from 1.4 to 6.6 kbp 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, streak 4). Nucleotide sequencing of the 2,915-bp *Sau*3AI insert revealed an open reading frame (ORF) of 2,538 bp

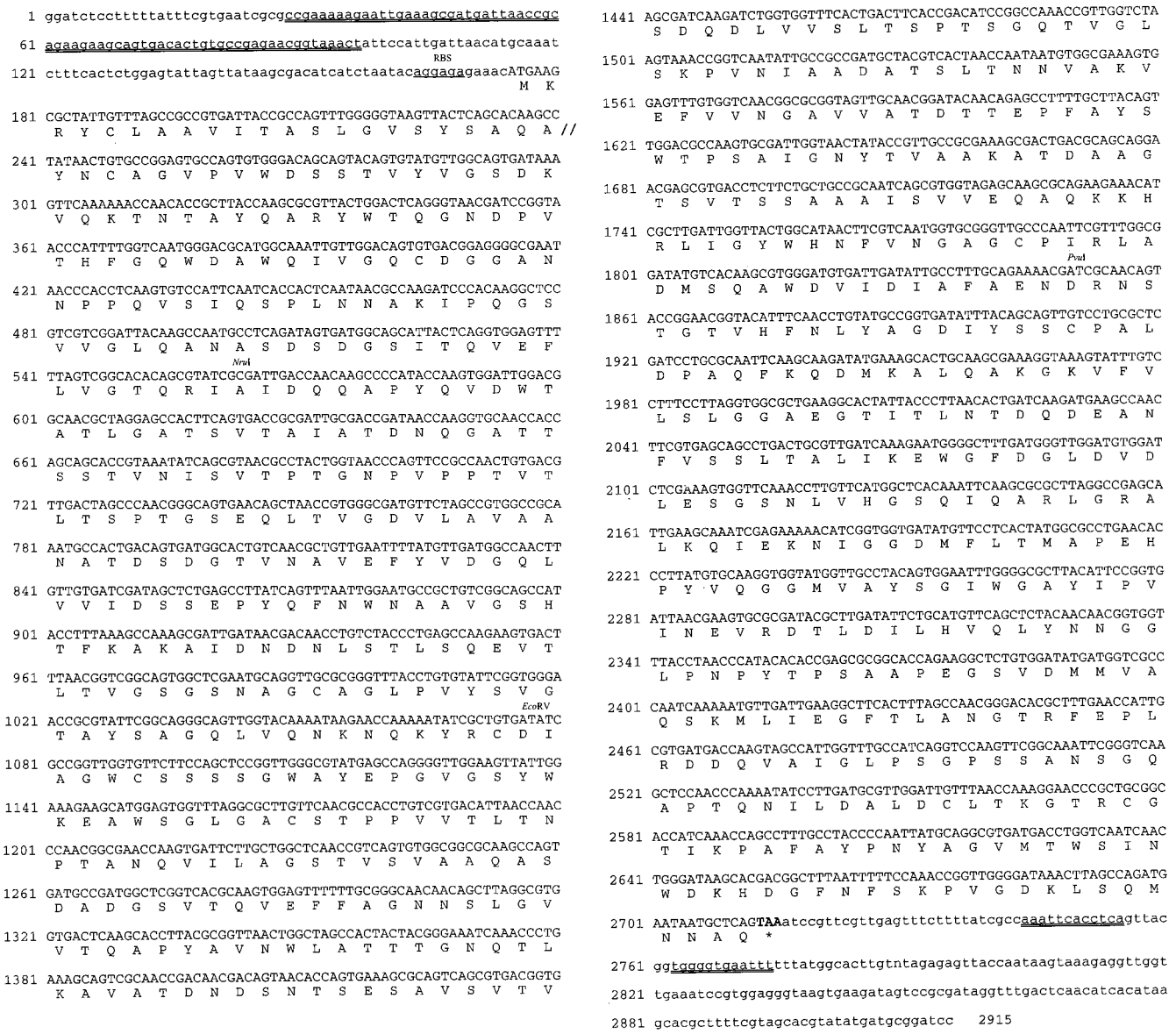

FIG. 2. Nucleotide sequence of the DNA insert in pTDCC2. A promoter region at positions 29 to 97 is denoted by double underlining; single underlining indicates a putative ribosomal binding site (RBS). A potential signal peptidase I cleavage site is marked by //. A putative transcription termination sequence located at 2744 to 2774 is double underlined. Nucleotides comprising the ORF of *chiA* are in uppercase letters; flanking regions are in lowercase letters. The predicted amino acid sequence of ChiA is designated in single-letter amino acid code.

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 *Aeromonas* 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 *Nhe*I site to engineer a −1 frameshift mutation in the putative gene (Fig. 2). Chitinase activity was not produced by *E. coli* DH5αF′tet cells which had been transformed with pTDCC2.3, 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

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

Strain or clone	Reaction					
		Substrate bound covalently to 4-MU[b]				
	p-NAG[a]	*N*-acetyl-α-D-glucosaminide	*N*-acetyl-β-D-glucosaminide	*N*-acetyl-β-D-galactosaminide	β-D-*N*,*N*′-diacetyl-chitobioside	β-D-*N*,*N*′,*N*″-triacetyl-chitotrioside
V. cholerae 569B	+	−	+	−	+	+
E. coli DH5αF′tet(pTDCC2)	−	−	−	−	+	+
E. coli DH5αF′tet(pTDCC2.3)	ND[c]	−	−	−	−	−
E. coli DH5αF′tet(pBluescriptKS+)	ND	−	−	−	−	−

[a] Hydrolysis of the substrate releases a yellow pigment (61). +, yellow; −, no color change.
[b] Hydrolysis of the substrate releases 4-methylumbelliferone, which fluoresces when exposed to UV (366 nm) light (41). +, fluorescent; −, not fluorescent.
[c] ND, not done.

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 endochitiase, 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 σ^{70}-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.

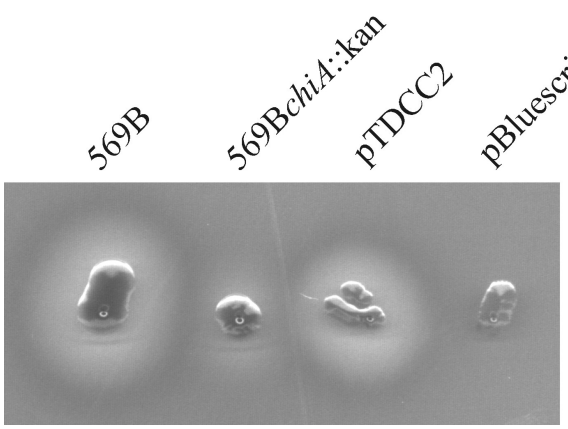

FIG. 3. An insertional mutation in *chiA* abolishes endochitinase activity in *V. cholerae* 569B. Production of chitinase activity was measured by culturing wild-type (569B) and mutant [569B(*chiA*::Kan^r)] *V. cholerae* strains on EGC agar. *E. coli* DH5αF′tet cells containing pTDCC2 and the phagemid vector pBluescriptKS− served as positive and negative controls, respectively.

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 fluorescences 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*′-diacetylchitobiose, and 4-MU-β-D-*N*,*N*′,*N*″-triacetylchitotriose. When expressed in DHαF′tet, pTDCC2 directed the hydrolysis of 4-MU-β-D-*N*,*N*′-diacetylchitobiose and 4-MU-β-D-*N*,*N*′,*N*″-triacetylchitotriose (Table 2). DH5αF′tet(pTDCC2) did not react with 4-MU-*N*-acetyl-α-D-glucosaminide, 4-MU-*N*-acetyl-β-D-glucosaminide, or 4-MU-*N*-acetyl-β-D-galactosaminide. DH5αF′tet(pTDCC2) also did not hydrolyze *p*-NAG, 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 *malE-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

TABLE 3. Mapping the *chiA* promoter in *V. cholerae* 569B

Plasmid	Segment of pTDCC2 inserted[a]	Promoter activity[b] (mean ± SD)
pRS415		34.1 ± 0.5
pZ1	31–465	191.6 ± 17.4
pZ3	99–465	70.8 ± 1.2
pZ13	123–465	48.6 ± 1.6
pZ56	157–465	44.1 ± 2.6

[a] Numbering of nucleotides is consistent with that used for the sequence of the insert of pTDCC2 in Fig. 2.
[b] Units of β-galactosidase activity, determined by the method of Miller and Mekalanos (36).

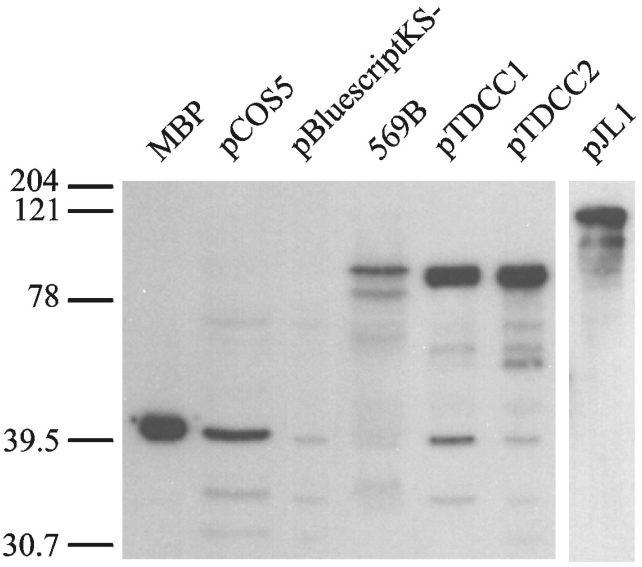

FIG. 4. Expression of recombinant ChiA. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune antiserum. All plasmids were expressed in *E. coli* DH5αF′tet with the exception that pTDCC1 was expressed in *E. coli* LE392. Molecular mass standards are in kilodaltons.

the vector such that an in-frame fusion to the 3′ end of *malE* fusion was produced. Introduction of the fusion plasmid pJL1 into DH5αF′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 DH5αF′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 DH5αF′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 likely the endogenous MalE of *E. coli*. 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

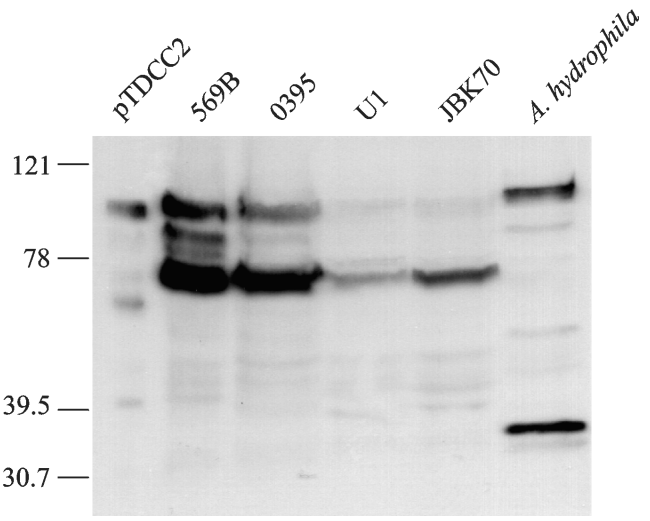

FIG. 5. Expression of chitinase by classical (569B and 0395) and El Tor (JBK70 and U1) strains of *V. cholerae* and by *A. hydrophila*. Bacterial cultures comprised cells and culture supernatants were used for the immunoblotting analysis. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune antiserum. The antiserum did not react with proteins in fresh culture media (data not shown). pTDCC2 was expressed in *E. coli* DH5αF′tet. Molecular mass standards are in kilodaltons.

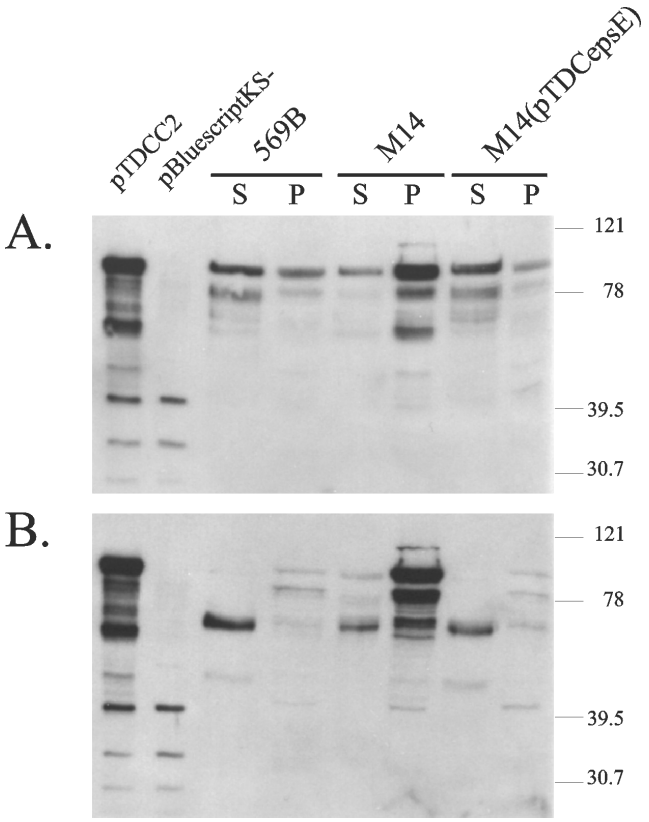

FIG. 6. Secretion of ChiA by *V. cholerae* depends on *epsE*. (A) Late-log-phase culture; (B) stationary-phase culture. M14 is an *epsE* mutant derived from 569B. pTDC*epsE* is a clone of the wild-type *epsE* gene from 569B. pTDCC2 and pBluescriptKS− were expressed in *E. coli* DH5αF′tet. Proteins in the immunoblot were detected with a rabbit anti-ChiA hyperimmune serum. S, culture supernatant; P, periplasmic extract. Molecular mass standards are in kilodaltons.

due solely to the mutation in *epsE*, M14 was complemented with pTDC*epsE*, a plasmid encoding a wild-type copy of the *epsE* gene of 569B. Complementation of the mutant with pTDC*epsE* (11) restored extracellular secretion of ChiA. Over 82% of total immunoreactive protein was located in the culture supernatant M14(pTDC*epsE*) 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 GM_1 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 pTDC*epsE* 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. chysanthemi*, *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 those 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 *pul* 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(pTDCC2) 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 chitinases of *Serratia mar-*

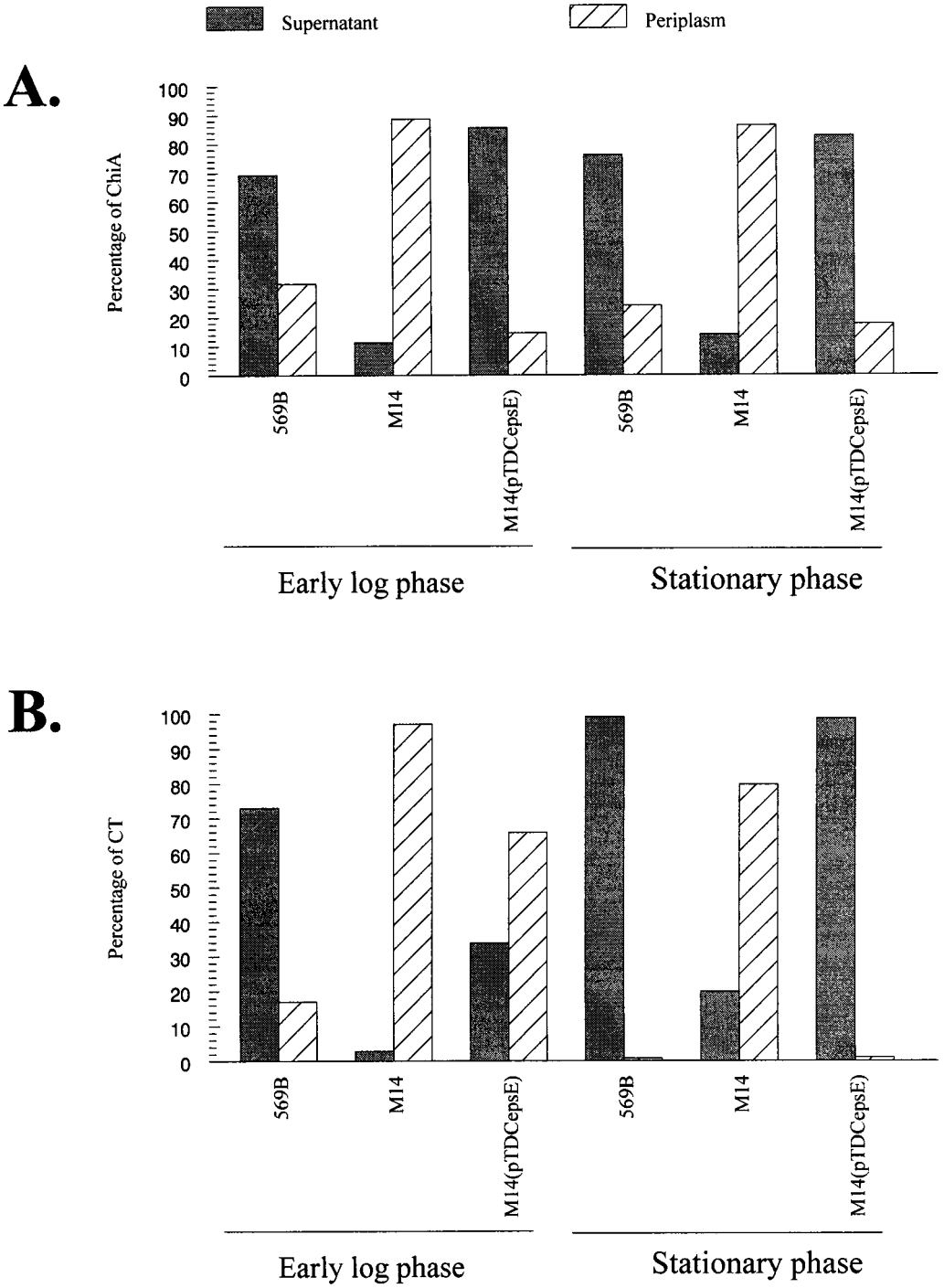

FIG. 7. Localization of ChiA and CT in wild-type and mutant *V. cholerae*. Percentage values represent the relative amounts of ChiA and CT in the respective samples. (A) Total immunoreactive ChiA in culture supernatants and periplasmic extracts. Semiquantitative data were obtained from densitometric 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 GM_1 ELISA (4, 11).

cescens (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-*

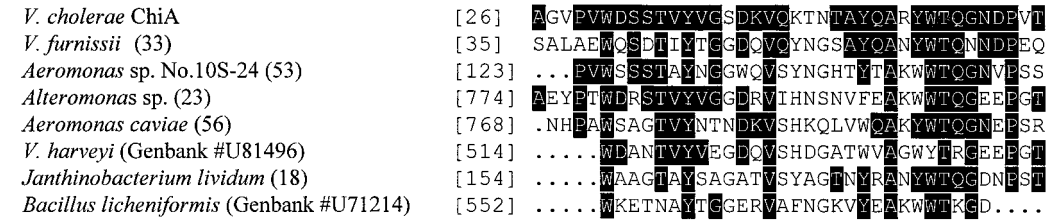

FIG. 8. Homology of ChiA to chitinases of other bacterial species. Amino acids are provided in single-letter code. The position of the first amino acid in the sequence of the respective protein is denoted in brackets. Amino acids of ChiA which are conserved in the other proteins are indicated (white letters on a black background).

cescens 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 purposely 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), *Aeromonas* 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 of *Aeromonas* 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*, *Aeromonas* 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 homopolymer 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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