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Initial Studies of the Structural Signal for Extracellular Transport of Cholera Toxin and Other Proteins Recognized by *Vibrio cholerae*

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The specificity of the pathway used by *Vibrio cholerae* for extracellular transport of cholera toxin (CT) and other proteins was examined in several different ways. First, *V. cholerae* was tested for the ability to secrete the B polypeptides of the type II heat-labile enterotoxins of *Escherichia coli*. Genes encoding the B polypeptide of LT-IIb in pBluescriptKS$^-$ phagemids were introduced into *V. cholerae* by electroporation. Culture supernatants and periplasmic extracts were collected from cultures of the *V. cholerae* transformants, and the enterotoxin B subunits were measured by an enzyme-linked immunosorbent assay. Results confirmed that the B polypeptides of both LT-IIa and LT-IIb were secreted by *V. cholerae* with efficiencies comparable to that measured for secretion of CT. Second, the plasmid clones were introduced into strain M14, an *epsE* mutant of *V. cholerae*. M14 failed to transport the B polypeptides of LT-IIa and LT-IIb to the extracellular medium, demonstrating that secretion of type II enterotoxins by *V. cholerae* proceeds by the same pathway used for extracellular transport of CT. These data suggest that an extracellular transport signal recognized by the secretory machinery of *V. cholerae* is present in LT-IIa and LT-IIb. Furthermore, since the B polypeptide of CT has little, if any, primary amino acid sequence homology with the B polypeptide of LT-IIa or LT-IIb, the transport signal is likely to be a conformation-dependent motif. Third, a mutant of the B subunit of CT (CT-B) with lysine substituted for glutamate at amino acid position 11 was shown to be secreted poorly by *V. cholerae*, although it exhibited immunoreactivity and ganglioside G_{M1}-binding activity comparable to that of wild-type CT-B. These findings suggest that Glu-11 may be within or near the extracellular transport motif of CT-B. Finally, the genetic lesion in the *epsE* allele of *V. cholerae* M14 was determined by nucleotide sequence analysis.

Cholera toxin (CT), an oligomeric protein produced by the enteric pathogen *Vibrio cholerae*, is the major virulence factor in eliciting severe diarrheal symptoms associated with cholera. CT consists of a single A polypeptide and five B polypeptides. For maximal biological activity, the A subunit must be cleaved by proteolytic digestion into fragments A1 and A2, and the disulfide bridge that joins them must be reduced. The A1 fragment is an ADP-ribosyltransferase (25), and the A2 fragment links the A subunit in a noncovalent fashion to a pentameric array of CT B subunits (CT-B) (7, 11). The CT-B pentamer binds to ganglioside G_{M1} (G_{M1}) receptors on the surface of the cell membrane (42). Binding of holotoxin triggers uptake of the A1 subunit into the cell, where it catalyzes the ADP-ribosylation of the Gsα regulatory subunit of the adenylate cyclase system, resulting in stimulation of cyclase activity (10, 25).

CT belongs to a family of bacterial proteins designated heat-labile enterotoxins. Members of the family have a variety of phenotypic and genotypic similarities. Each of the holotoxins has a 1:5 ratio of A to B polypeptides, is a potent ADP-ribosyltransferase, and binds to one or more species of ganglioside found on the surface of eukaryotic cells. Serology has established two groups of heat-labile enterotoxins in the *V. cholerae-Escherichia coli* enterotoxin family. Serogroup I (type I toxins) includes CT and LT-I, a heat-labile enterotoxin produced by enterotoxigenic strains of *E. coli*. CT and LT-I have over 77% homology at both the nucleotide and amino acid levels for both A and B subunits (15). Serogroup II (type II toxins) includes the partially cross-reacting antigenic variants LT-IIa and LT-IIb of *E. coli* (33, 34). The A subunits of type II toxins have significant homology with the A subunits of the type I toxins at both the nucleotide and amino acid levels (15). However, the B polypeptides of type II enterotoxins have little, if any, homology with the B polypeptides of the type I enterotoxins (34, 35). The significant differences in amino acid homology between the B polypeptides of the type I and type II toxins are associated with differences in the specificities for binding of these toxins to different gangliosides. CT and LT-I bind preferentially to G_{M1}, while LT-IIa binds best to ganglioside GD_{1b} (GD_{1b}), and LT-IIb binds best to ganglioside GD_{1a} (9).

The pathway for assembly of biologically active holotoxin is conserved in type I and type II enterotoxins (14). The A and B polypeptides are expressed with signal peptides and are exported across the cytoplasmic membrane. After cleavage of the signal peptides, the A and B polypeptides are released into the periplasmic space, where they spontaneously assemble into mature holotoxin. Whereas *E. coli* retains LT-I, LT-IIa, and LT-IIb in the periplasm, *V. cholerae* actively secretes over 95% of CT to the extracellular medium (14, 17). A genetic component of extracellular secretion was revealed in *V. cholerae* by isolation of the mutant strain M14 that is defective in secreting periplasmic CT to the extracellular medium (17). Genetic complementation in M14 was used to identify *epsE*, a gene that is essential for extracellular transport of CT by *V. cholerae* (39). Additional mutagenesis of *V. cholerae* and sequence analysis of

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TABLE 1. List of plasmids and strains

Plasmids or strains	Genotype and/or phenotype	Reference or source
Plasmids		
pBluescriptKS$^+$	Phagemid vector, ColE1 replicon, Amp^r	Stratagene
pBluescriptKS$^-$	Phagemid vector, ColE1 replicon, Amp^r	Stratagene
pBluescriptSK$^-$	Phagemid vector, ColE1 replicon, Amp^r	Stratagene
pK194	Plasmid vector, P15A replicon, Kan^r	21
pK184	Plasmid vector, P15A replicon, Kan^r	21
pTDC400	LT-IIa B-subunit gene in pBluescriptKS$^-$	5
pTDC700	LT-IIb B-subunit gene in pBluescriptKS$^-$	5
pTDC97.1	*ctxB* gene from pMGJ19 in pBluescriptKS$^+$	This study
pTDC411	LT-IIa B-subunit gene from pTDC400 in pK194	This study
pMMB384	*epsE*$^+$ from *V. cholerae* TRH7000	39
pTDC*epsE*	*epsE*$^+$ from pMMB384 in pK184	This study
pTDC-E11K	*ctxB* mutant E11K in pBluescriptKS$^+$	This study
pTDC-E11G	*ctxB* mutant E11G in pBluescriptKS$^+$	This study
pTDC1	*epsE* allele from M14	This study
pTDC3	*epsE* allele from 569B	This study
pMGJ78	*ctxA* in pK184	20a
Strains		
569B	*V. cholerae*, classical biotype	17
M14	*V. cholerae*, *epsE*, derived from 569B	17
JBK70	*V. cholerae*, El Tor biotype, *ctxA ctxB*	24
DH5αF′kan	*E. coli* (F′ *proAB*$^+$ *lacI*^{q} ZΔM15 *zzf*::Tn*5* [Kan^r])	Gibco/Life Technologies; transconjugant with F′ plasmid from *E. coli* XL-1 Blue (Stratagene)
DH5αF′tet	*E. coli* (F′ *proAB lacI*^{q} ZΔM15 Tn*10* [Tet^r])	Bio-Rad
CJ236F′chlor	*E. coli* (F′*cat*) *dut ung*	Bio-Rad

cosmid clones revealed at least 11 additional chromosomal genes (*epsC* to *epsN*) that are genetically linked to *epsE*, all of which are essential for extracellular transport of CT (32). Genes highly homologous to *epsC* to *epsN* are found in the chromosomes of diverse gram-negative species that are capable of extracellular transport (37).

Secretion of CT into the culture medium is not accompanied by release of periplasmic contents in *V. cholerae*. This finding demonstrates that the extracellular transport mechanism of *V. cholerae* is selective and likely involves recognition by the transport machinery of an extracellular transport domain or signal that is present in CT but absent in periplasmic proteins. Results from previous studies suggest that the putative extracellular transport signal is located on CT-B (14). CT-B pentamers and holotoxin are secreted from *V. cholerae* with equal efficiency. CT-A polypeptides are not secreted across the outer membrane by *V. cholerae* unless they are assembled into holotoxin.

Little is known about the structure of the extracellular transport signal(s) in CT. Neill and colleagues (30) demonstrated that *E. coli* enterotoxin LT-I is efficiently secreted by *V. cholerae*. The finding that a functional transport signal is conserved in LT-I was not surprising in view of the high degree of amino acid sequence homology between CT and LT-I. The studies reported here were performed for the following purposes: (i) to determine whether the B subunits of the type II enterotoxins of *E. coli* can also be secreted efficiently by *V. cholerae*, (ii) to initiate a search for specific amino acid residues in the B polypeptides of CT or LT-II that are important for efficient secretion across the outer membrane, and (iii) to define the specific mutation in the *epsE* allele of *V. cholerae* M14.

MATERIALS AND METHODS

Reagents and general methods. Restriction enzymes and other DNA-modifying enzymes were purchased from Gibco/Life Technologies, Inc. (Gaithersburg, Md.) or Boehringer-Mannheim (Indianapolis, Ind.). Alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G was purchased from Sigma Chemical Co. (St. Louis, Mo.). All nonimmunological reagents, including isopropyl-β-D-thiogalactopyranoside (IPTG), were purchased from Sigma and Life Technologies. Oligonucleotides were synthesized with an Applied Biosystems (Foster City, Calif.) model 380A DNA synthesizer or were purchased from Integrated DNA Technologies (Coralville, Iowa). Plasmids were introduced into *E. coli* by osmotic shock (26) and into *V. cholerae* by electroporation with buffer B (27). Unless otherwise specified, strains containing plasmids were cultured on Luria-Bertani (LB) agar at 37°C.

Bacterial strains and plasmids. Plasmids and strains used are listed in Table 1. *E. coli* DH5αF′kan (Bethesda Research Laboratories, Gaithersburg, Md.) or DH5αF′tet (Bio-Rad Laboratories, Hercules, Calif.) were used as hosts in cloning experiments. CJ236F′kan (*dut ung* double mutant) was used to produce uracil-containing single-stranded DNA templates for oligonucleotide-directed mutagenesis. pMGJ19 (22) was used as the source for the *ctxB* gene for engineering pTDC97.1. To construct pTDC97.1, pMGJ19 was digested with *Xba*I, treated with Klenow fragment to produce a blunt-ended DNA fragment, and digested with *Hin*dIII to release a 587-bp DNA fragment containing the wild-type (wt) *ctxB* gene. The 587-bp DNA fragment was inserted into the phagemid pBluescriptKS$^+$ (Stratagene, La Jolla, Calif.) at the *Hin*cII-*Hin*dIII sites. The fragment was oriented in pTDC97.1 such that expression of *ctxB* was under control of the *lac* promoter of the vector. To produce pTDC411, a 792-bp *Hin*dIII-*Eco*RI fragment from pTDC400 (4) was ligated into the *Hin*dIII-*Eco*RI sites of pK194 (21). Expression of the LT-IIa B polypeptide in pTDC411 was under the control of the *lac* promoter in the vector. To produce pTDC*epsE*, a 1.6-kbp *Hin*dIII-*Eco*RI fragment of pMMB384 (39) was ligated into *Hin*dIII-*Eco*RI-digested pK184 (21). Insertion of the fragment into pK184 placed expression of the *epsE* gene under control of the *lac* promoter in the vector. The wt CT-A polypeptide was expressed from pMGJ78 (20a), which was constructed by directionally cloning a *Sac*I-*Hin*cII fragment from pMGJ64 (23) into pK184 such that expression of the *ctxA* gene was under control of the *lac* promoter in the vector.

Preparation of periplasmic extracts. *V. cholerae* 569B and M14 harboring plasmids expressing the B polypeptides of CT, LT-IIa, and LT-IIb were cultured overnight in syncase broth (18) at 37°C, while plasmid-containing JBK70 strains expressing enterotoxin were cultured in YEP broth (19) at 30 or 37°C. Control experiments demonstrated that changing temperatures between 30 and 37°C had a small effect on CT expression in strains 569B and M14, but there was no detectable effect on the efficiency of extracellular secretion of toxin (data not shown). No effect of temperature on expression of B polypeptides from plasmid-encoded genes was observed. Ampicillin to 100 μg/ml, kanamycin to 50 μg/ml, chloramphenicol to 10 μg/ml, and tetracycline to 10 μg/ml were added to the cultures as appropriate to provide selection for the plasmids. To prepare periplasmic extracts, cells from overnight cultures were harvested by centrifugation, washed once with phosphate-buffered saline (PBS), and resuspended to the original volume in PBS. Periplasmic contents were released from the cells by the addition of EDTA and polymyxin B sulfate at final concentrations of 1 mM and

TABLE 2. Nucleotide sequences of the synthetic oligonucleotides used for sequencing, PCR amplification, and site-specific mutagenesis

Oligonucleotide	Sequence[a]	Reference or source
epsE-up	5′-GCGGATCC[1]TATTGAGCAGATGGAAGC-[23]-3′	39
epsE-dn	5′-GCGGATCC[1536]TTAGGACTCCTTAGTCACCC[1517]-3′	39
CT-seq	5′-[1480]GATAGCCATCTCTCTTTTTC[1449]-3′	28
CT-up3	5′-CAAGATAAGCTT[1154]TCAGTAATACTTGCG[1168]-3′	28
CT-dn2	5′-GTAAGAATTC[1431]TTTGGGCAAAACGGTTG[1415]-3′	28
E11X-2	5′-[1395]GTGTTGTGGTA(N)(C/T)(C/G/T)TGCACACAAATC[1370]-3′	28
epsE-1	5′-[370]GAAAGTGAAGATGATGCGCC[390]-3′	39
epsE-2	5′-[640]GCGGTCGATGTGCGGGTTTCA[660]-3′	39
epsE-3	5′-[909]GAATTTGATATTGATGGTATC[929]-3′	39
epsE-4	5′-[1180]GGTGTTCTGGCTCAGCGCTTG[1200]-3′	39
epsE-5	5′-[190]CGGGTGGTCAAGCACGC[207]-3′	39
KS	5′-[774]TCGAGGTCGACGGTATC[758]-3′	Stratagene

[a] The numbers in brackets correspond to the nucleotide positions within the published sequence of the CT-B gene for CT-seq, CT-up3, CT-dn2, and E11X-2, within the published sequence of the *epsE* gene for *epsE*-up, *epsE*-dn, and for *epsE*-1 thru *epsE*-5, and within the published sequence of the phagemid pBluescriptKS$^+$ for KS. The letter N refers to any one of the four nucleotides. Position numbers increase in the 5′ to 3′ direction for sense strand oligonucleotides and decrease in 5′ to 3′ direction for antisense strand oligonucleotides. Nucleotides outside of the brackets were added to incorporate restriction enzyme sites in the DNA fragments amplified by PCR.

2 mg/ml, respectively, by the methods of Connell and Holmes (4, 5). After extraction for 30 min at 37°C, periplasmic extracts were separated from cell debris by centrifugation. To isolate culture supernatant, a volume of overnight culture equal to that used to isolate periplasmic contents was clarified of cells by centrifugation. After discarding the cell pellet, sodium azide was added to the clarified supernatant to a final concentration of 0.02% to inhibit further growth of residual cells. Extracts and supernatants were used immediately or stored at 4°C until needed. Holotoxin and B polypeptides remained stable in the samples for up to 2 months at 4°C.

ELISAs for enterotoxin. Protocols for enzyme-linked immunosorbent assays (ELISAs) were modified from those reported previously for measuring toxin by solid-phase radioimmunoassay (4). ELISAs were performed in 96-well flat-bottom polyvinyl microtiter plates (Gibco/Life Technologies), and all incubations were at 37°C unless otherwise noted. To minimize nonspecific binding, ELISA plates were incubated with PBS containing 10% horse serum for 30 min at room temperature prior to application of samples containing antigen. Samples to be measured for toxin were serially diluted and incubated overnight at room temperature or at 4°C in sensitized ELISA plates, prepared as described below. Unbound antigen was removed by washing with PBS containing 1% horse serum, and the samples were incubated with toxin-specific rabbit antibodies for 60 min. After a second wash to remove unbound antibodies, plates were incubated for 60 min with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Sigma) diluted 1:30,000, as directed by the vendor. Unbound alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin G was removed by washing, and samples were incubated for 60 min at room temperature in substrate buffer (10% diethanolamine, 0.5 mM $MgCl_2$, 0.2% sodium azide, 1 mg of *p*-nitrophenyl phosphate [Gibco/Life Technologies], pH 9.8) for color development. The A_{405} in each well was measured with an automated Titertek Multiskan Plus microtiter plate reader (ICN Biomedicals, Inc., Costa Mesa, Calif.). Purified CT, LT-IIa, and LT-IIb were used as standards in all experiments, and antigen concentrations in samples were calculated from the linear parts of the titration curves. Samples were measured in duplicate for all experiments.

Preparation of hyperimmune goat antisera against CT, LT-IIa, and LT-IIb was reported previously (4, 5, 22). An immunoglobulin fraction of each goat antiserum was prepared by precipitation with half-saturated ammonium sulfate.

Antibody sandwich ELISA (S-ELISA) was used to quantitate the amount of immunoreactive toxin in culture supernatant and extract samples (3). To measure CT, wells of microtiter plates were sensitized with hyperimmune goat anti-CT immunoglobulins at a concentration of 3.2 to 4.0 μg/ml. A 1:2,000 dilution of rabbit antiserum against CT-B was used as the second antibody to detect toxin bound to the surface of the wells (22). S-ELISA was modified to quantitate the amounts of LT-IIa or LT-IIb in samples. To measure LT-IIa by S-ELISA, hyperimmune goat anti-LT-IIa immunoglobulin (3.7 μg/ml) was substituted for goat anti-CT immunoglobulins, and rabbit anti-LT-IIa antiserum diluted to 1:2,000 was substituted for rabbit anti-CT antiserum for the second antibody (4, 5). A combination of hyperimmune goat anti-LT-IIb immunoglobulin (3.0 to 4.0 μg/ml) and a 1:2,000 dilution of rabbit anti-LT-IIb was used in an S-ELISA to measure LT-IIb in extract and supernatant samples (5).

The relative G_{M1}-binding activity of mutant CT-B polypeptides was determined with a G_{M1}-dependent ELISA modified from a protocol previously described by Fukuta et al. (9). Polyvinyl microtiter plates were prepared as described above, with the exception that wells were sensitized with G_{M1} instead of immunoglobulins. G_{M1} was diluted in PBS and applied to wells in a twofold dilution series of from 50 to 2.4 pg of G_{M1} per well. Equivalent amounts of immunoreactive mutant or wt CT-B (0.3 ng) were applied to each well. Plates were incubated overnight and processed for immunoreactive toxin as described above.

Modified G_{M1} ELISAs were used to measure the amounts of pentameric CT-B and holotoxin produced by cells expressing CT-A and mutant or wt CT-B. Microtiter plates sensitized with 10 ng of G_{M1} per well were incubated with periplasmic extracts from *E. coli* expressing from plasmids a combination of wt CT-A and CT-B or a combination of wt CT-A and mutant CT-B. Rabbit anti-CT-B antiserum was used to quantitate the total amount of pentameric CT-B in the samples that was captured by the G_{M1} in the wells. In a second plate containing identical samples of extracts, rabbit anti-CT-A antiserum was used to measure the total amount of CT-A polypeptide that was associated with G_{M1}-bound CT-B, i.e., incorporated into holotoxin. Each rabbit antiserum was diluted to 1:1,000 prior to use in the assays.

Cloning *epsE* genes from chromosomal DNA by PCR. Chromosomal DNA was isolated from overnight cultures of *V. cholerae* cells by a previously described method (1). Briefly, cells were harvested from LB agar plates and immediately resuspended in Tris-EDTA buffer (10 mM Tris, 1 mM EDTA [pH 8.0]). Sodium dodecyl sulfate was added to a final concentration of 10%, and proteinase K was added to a final concentration of 100 μg/ml. The cell suspension was incubated at 56°C until the cells lysed. Proteins, cell wall components, and polysaccharides in the lysate were removed by selective precipitation with cetyl-trimethylammonium bromide (CTAB). The solution was extracted several times with equal volumes of a 1:1 (vol/vol) mixture of chloroform-phenol, and the nucleic acids in the solution were precipitated by the addition of 0.7 volume of isopropanol. After washing with 70% ethanol, the pellet was resuspended in Tris-EDTA, and RNase A was added to a final concentration of 100 μg/ml. After incubation for 60 min at 37°C, the solution was extracted with chloroform-phenol and precipitated as described above. The precipitated chromosomal DNA was dried under mild vacuum, and the resulting pellet was dissolved in Tris-EDTA to a final concentration of between 100 and 500 μg/ml.

Two synthetic oligonucleotides and PCR were used to amplify the wt *epsE* allele from 569B chromosomal DNA and the mutant *epsE* allele from M14 chromosomal DNA. The sequences and positions of the oligonucleotides *epsE*-up and *epsE*-dn are shown in Table 2. Each oligonucleotide incorporated a *Bam*HI site into the 3′ and 5′ termini of the amplified fragment. Amplification reactions were done with a DNA thermal cycler model 480 and a GeneAmp PCR amplification kit (Perkin-Elmer Cetus, Norwalk, Conn.). The final reaction mixture contained 5 ng of each oligonucleotide per ml and 2 μg of chromosomal DNA per ml. Conditions for the reactions were denaturation at 92°C for 2 min, annealing at 50°C for 1 min, and extension at 72°C for 3 min, for 30 cycles. Amplified DNA fragments were resolved by electrophoresis on 0.8% agarose gels and isolated from gel slices with a GeneClean kit (Bio 101, Inc., Vista, Calif.). After digestion with *Bam*HI to create compatible ends, DNA fragments were ligated into the *Bam*HI site of phagemid pBluescriptKS$^+$ or pBluescriptKS$^-$. Plasmid pF8-2 contained the DNA fragment encoding the *epsE*$^+$ allele from wt 569B ligated into pBluescriptKS$^+$, while plasmid pF9-1 contained the DNA fragment encoding the *epsE* allele from M14 ligated into pBluescriptKS$^-$. To facilitate sequencing of wt and mutant alleles of the *epsE* genes by use of *epsE*-specific antisense primers available in the laboratory and to orient the fragments such that expression of the *epsE* genes was under control of the *lac* promoter of the vector, two additional plasmids were derived. To construct pF9-10, a *Ksp*I-*Pst*I fragment of pF9-1 containing the M14 *epsE* allele was ligated into pBluescriptSK$^-$ at the *Ksp*I-*Pst*I sites. Similarly, a *Ksp*I-*Eco*RI fragment of pF8-2 containing the 569B *epsE*$^+$ allele was cloned into pBluescriptSK$^-$ at the *Ksp*I-*Eco*RI sites to derive pTDC3. The nucleotide sequences of pTDC3 and

TABLE 3. Extracellular secretion of the B polypeptides of LT-IIa, the B polypeptides of LT-IIb, and CT from wt and mutant strains of *V. cholerae*[a]

Strain (plasmid)[b]	CT secreted			LT-IIa or LT-IIb[c] secreted			β-Lactamase activity[d]		
	Periplasm[e] (μg/ml)	Sup[e] (μg/ml)	% in Sup	Periplasm (μg/ml)	Sup (μg/ml)	% in Sup	Periplasm (U/ml)	Sup (U/ml)	% in Sup
569B(pBluescriptKS$^+$)	0.01	0.36	98	NA[f]	NA	NA	120.0	9.4	7
M14(pBluescriptKS$^+$)	2.25	0.13	5	NA	NA	NA	275.0	17.8	6
569B(pTDC400)	0.07	2.05	97	0.3	56.3	99	313.0	17.0	5
M14(pTDC400)	0.96	0.36	27	137.7	10.7	7.2	143.3	28.0	16
M14(pTDC400, pTDC*epsE*)	0.02	1.63	99	0.9	166.7	99.5	96.0	13.2	1
569B(pTDC700)	0.02	5.90	99	11.3	85.9	88	60.0	13.0	8
M14(pTDC700)	2.00	0.59	23	167.3	77.6	32	103.0	56.0	35
M14(pTDC700, pTDC*epsE*)	0.03	0.79	96	18.4	153.1	89	90.0	14.4	14

[a] Bacteria were cultured in syncase broth (8) at 37°C to the stationary phase. Immunoreactive B polypeptides of CT, LT-IIa, and LT-IIb were measured by an S-ELISA, as described in Materials and Methods.

[b] Strain 569B is wt *V. cholerae*. Strain M14 is a derivative of 569B with a chromosomal mutation in *epsE* (17, 39).

[c] The B polypeptide of LT-IIa is encoded by pTDC400; the B polypeptide of LT-IIb is encoded by pTDC700.

[d] β-Lactamase activity was measured in the periplasmic extracts and the culture supernatants by the use of PADAC (41). One unit is defined as the amount of β-lactamase activity needed to degrade 1 μmol of PADAC per min.

[e] Periplasm, periplasmic extract; Sup, culture supernatant.

[f] NA, not applicable. The vector pBluescriptKS$^+$ does not encode the B polypeptides of LT-IIa or LT-IIb. Neither anti-LT-IIa nor anti-LT-IIb antisera cross-react with CT (12, 16, 33).

pF9-10 were determined by the dideoxy chain termination method of Sanger et al. (40) with a Sequenase kit (United States Biochemical Co., Cleveland, Ohio) and oligonucleotides *epsE*-1, *epsE*-2, *epsE*-3, *epsE*-4, *epsE*-5, and KS as primers (Table 2). While the sequence of the *epsE*$^+$ allele in pTDC3 was identical to the sequence of a previously reported wt *epsE* gene (39), the sequence of the *epsE* allele in pF9-10 differed at three nucleotide positions (positions 278, 314, and 987). Direct double-stranded DNA sequencing of PCR-amplified DNA from M14 showed that of the three mutations, only the cytosine-to-thymine substitution at nucleotide position 987 (C987T) was present in the *epsE* allele in the M14 chromosome. The mutations at nucleotide positions 278 and 314 were likely *Taq* polymerase-generated errors that occurred during PCR amplification. A *Sca*I site in the *epsE* gene was used to subclone the mutation at nucleotide position 987 away from the two artifactual nucleotide substitution positions 278 and 314. pTDC1 was made by ligating a 2.1-kbp *Sca*I fragment of pF9-10 to a 2.27-kbp *Sca*I fragment of pTDC3. The single cytosine-to-thymine substitution at nucleotide position 987 in pTDC1 was confirmed by dideoxy chain termination sequencing.

Subcloning the *ctxB* E11K allele into pBluescriptKS$^+$. PCR was used to clone the *ctxB* allele encoding the mutant E11K CT-B polypeptide from pMGJ1984 (20a) into the phagemid pBluescriptKS$^+$. Two synthetic oligonucleotides, CT-up3 and CT-dn2, were used to amplify a fragment containing the *ctxB* allele from pMGJ1984 with reaction conditions similar to those used to amplify *epsE* genes from 569B chromosomal DNA. CT-up3 incorporated an *Eco*RI site into the 5′ end of the fragment, while CT-dn3 incorporated a *Hin*dIII site into the 3′ terminus (Table 2). To construct pTDC-E11K, amplified fragments were resolved by agarose gel electrophoresis, purified from agarose gel slices with GeneClean (Bio 101), double-digested with *Eco*RI-*Hin*dIII, and ligated into the *Eco*RI-*Hin*dIII sites of pBluescriptKS$^+$. Single-stranded DNA sequencing was used to confirm the cloned sequence in pTDC-E11K.

Oligonucleotide-directed mutagenesis of CT-B. Oligonucleotide-directed site-specific mutagenesis of pTDC97.1 was used to produce *ctxB* alleles encoding polypeptides that were substituted at amino acid position 11 of mature CT-B. Mutagenesis was performed as directed in the Muta-Gene manual (Bio-Rad, Melville, N.Y.). An antisense synthetic oligonucleotide (E11X-2) that is degenerate at all three nucleotide positions for the codon encoding Glu-11 was used as the mutagenic primer in the reactions (Table 2). *E. coli* CJ236F′chlor containing pTDC97.1 and R408 helper phage was used to prepare uracil-containing single-stranded DNA for use as a template. Products of the reactions were transformed directly into *E. coli* DH5αF′kan, and transformants were sequenced by the dideoxy chain termination methods with the oligonucleotide CT-seq (Table 2) as a primer to screen for CT-B variants with glycine and lysine substitutions for glutamate 11. Plasmid DNA from mutants that encoded E11G and E11K substitutions were introduced into *V. cholerae* JBK70 by electroporation and into DH5αF′tet by osmotic shock with $CaCl_2$ (26).

In vivo assembly of holotoxin from A and B subunits. Mutant CT-B polypeptides and wt CT-A polypeptides were tested for the ability to assemble into holotoxin in vivo in *E. coli* DH5αF′tet by modification of a previously reported method (22) using pMGJ78 as a source of wt CT-A. pMGJ78 and plasmids encoding mutant CT-B polypeptides were cotransformed into DH5αF′tet by osmotic shock with $CaCl_2$. Cotransformants were cultured at 37°C in Superbroth (Stratagene) until the mid-log phase, when IPTG was added to 1 mM to induce expression of the CT-A and CT-B polypeptides. Incubation of the bacterial cultures was continued overnight, and periplasmic extracts were harvested from the *E. coli* cells with polymyxin B sulfate, as described above for extraction of *V. cholerae* (4, 22). Extracts were measured by G_{M1}-dependent ELISA for total amounts of immunoreactive CT-B polypeptide and for total amounts of holotoxin formed from CT-A polypeptide and CT-B polypeptide, as described above.

Assay for β-lactamase activity. β-Lactamase activity in the culture supernatants and periplasmic extracts was measured spectrophotometrically with the chromogenic indicator PADAC (Calbiochem, La Jolla, Calif.) (41). β-Lactamase activities were stable in both periplasmic extracts and culture supernatants for up to 1 month when stored at 4°C.

DNA sequencing. Single-stranded DNA templates used for sequencing by the dideoxy chain termination method of Sanger et al. (40) were isolated from phagemid clones in *E. coli* DH5αF′kan or DH5αF′tet by use of helper phage R408 as described in the Sequenase sequencing kit manual (United States Biochemical). Sequencing of double-stranded DNA from PCR preparations was accomplished by use of the protocol described in the Sequenase manual, with the exception that the PCR-derived DNA fragments used as the template were separated from excess amplimers by gel electrophoresis prior to sequencing. Reactions with single- or double-stranded DNA were done with T7 polymerase (Sequenase) and ^{35}S-deoxyadenosine triphosphate (Amersham, Arlington Heights, Ill.). Reaction products were resolved on 6% polyacrylamide-urea gels and detected by autoradiography.

RESULTS

Secretion of B subunits of LT-II from *V. cholerae* 569B. Extracellular secretion of LT-I was demonstrated by Neill et al. (30) by introducing the enterotoxin plasmid pCG86 into *V. cholerae*. A similar approach was used in these studies to test for secretion of type II enterotoxin B subunits by *V. cholerae*. Plasmid pTDC400 expressing the B polypeptide of LT-IIa and pTDC700 expressing the B polypeptide of LT-IIb were constructed previously in our laboratory (4, 5) (Table 1). Clones that express LT-II holotoxin were not introduced into *V. cholerae* to comply with restrictions in the *NIH Guidelines for Recombinant DNA*, *Appendix F*. Plasmids pTDC400 and pTDC700 were introduced into *V. cholerae* 569B, and strains containing the plasmids were cultured overnight in syncase medium for expression of B polypeptides. The phagemid pBluescriptKS$^+$ was introduced into strain 569B as a control for vector-determined effects. Culture supernatant and cells were separated, periplasmic extracts from cells were prepared, and B subunits of CT and LT-IIa or LT-IIb in each sample were measured by an S-ELISA. β-Lactamase activity expressed from vector-encoded *bla* was used as a convenient marker for a known periplasmic component (13).

Consistent with previous reports on secretion of CT by wt *V. cholerae* (14, 17), over 97% of the CT expressed by 569B

(pTDC400), 569B(pTDC700), and 569B(pBluescriptKS$^+$) was secreted into the culture supernatant (Table 3). When supernatant and periplasmic extracts from broth cultures of these strains were measured for LT-IIa or LT-IIb, a similar pattern of extracellular secretion was found. Ninety-nine percent of the B subunit of LT-IIa produced by 569B(pTDC400) and 88% of the B subunit of LT-IIb produced by 569B(pTDC700) were present in culture supernatants. In contrast, less than 8% of the β-lactamase activity from these strains was present in the supernatants. We conclude that release of CT, LT-IIa, or LT-IIb into the culture supernatants was not due to cellular lysis, increased outer membrane permeability, or other nonspecific factors that would make the cells leak periplasmic contents.

Secretion of LT-II from the *V. cholerae* mutant M14. The B polypeptides of LT-IIa and LT-IIb were expressed at levels in 569B(pTDC400) and 569B(pTDC700), respectively, that far exceeded levels of expression commonly observed for CT in *V. cholerae*. To demonstrate that extracellular secretion of the B polypeptides of LT-IIa and LT-IIb occurred by a specific secretory process and not by passive diffusion of the overexpressed polypeptides across the outer membrane, we introduced pTDC400 and pTDC700 into strain M14 (17), a secretion-negative mutant of *V. cholerae* 569B. Although M14(pTDC400) and M14(pTDC700) produced substantial amounts of LT-IIa and LT-IIb B polypeptide, respectively, both strains were deficient for secretion of the B polypeptides into the supernatant (Table 3). Ninety-three percent of the B polypeptide of LT-IIa remained in the periplasm of M14(pTDC400), and 68% of the B subunit of LT-IIb was present in the periplasm of M14(pTDC700). The partial release of the LT-IIb B polypeptide by M14(pTDC700) and the LT-IIa B polypeptide by M14(pTDC400) was nonspecific and proportional to the release of β-lactamase from these strains into the culture supernatant.

The genetic defect in M14 has been mapped to *epsE*, a gene of the *eps* cluster in the chromosome of *V. cholerae* (39). As further proof that the B polypeptides of LT-IIa and LT-IIb use the same machinery for extracellular secretion as that used by CT, we complemented M14(pTDC400) and M14(pTDC700) with a wt copy of *epsE*. When pTDC*epsE* was introduced into M14(pTDC400), extracellular secretion of the B polypeptide of LT-IIa was restored to levels comparable to those observed when the polypeptides were expressed in 569B(pTDC400) (Table 3). Similar results were obtained for secretion of the B polypeptide of LT-IIb when pTDC*epsE* was introduced into M14(pTDC700). As expected, CT was exported to the supernatant by M14(pTDC*epsE*, pTDC400) and M14(pTDC*epsE*, pTDC700) in a manner that was consistent with previous reports involving the use of a wt *epsE* in complementation experiments (39). These data provide strong evidence that the transport system for extracellular secretion of CT is required for export of the B polypeptides of LT-IIa and LT-IIb by *V. cholerae.*

Effect of single amino acid substitutions on extracellular secretion of CT. The structural features of the B subunits of CT, LT-I, LT-IIa, and LT-IIb that are required for recognition and extracellular transport by the secretory system of *V. cholerae* have not been defined. As a first step towards identifying amino acid residues of CT-B that are important for extracellular secretion, we introduced 19 different plasmids encoding variants of CT-B into JBK70, a strain of *V. cholerae* that is deleted for most of the *ctx* operon. These plasmids encoded the following CT-B polypeptides: A10V, E11K, H13Y, T28I, G33I, K34D, R35D, E36Q, T41I, A46V, E51K, H57Y, A64V, D70N, R73K, W88E, P93S, A95D, and S100N (23a). The variants are designated by the one-letter code for the amino acid present in wt CT-B, its location in the mature polypeptide, and the one-letter code for the amino acid replacement. Transformants were screened for the decreased ability of the mutant CT-B polypeptides to be transported out of the periplasmic space. Extracellular secretion of the mutant CT-B polypeptides was compared with secretion of wt CT-B expressed from plasmid pTDC97.1. Among the 19 mutants screened, only the E11K variant of CT-B produced by pMGJ1984 showed a decrease in secretion to below 60% of the wild-type level (data not shown), but analysis of the data was complicated by the fact that expression of the mutant CT-B by pMGJ1984 was relatively low when compared with expression of the wt CT-B from pTDC97.1. To compare the secretion efficiencies of the mutant and wt CT-B polypeptides under similar conditions, we used PCR to amplify a DNA fragment from pMGJ1984 that was equivalent to the DNA fragment used to construct pTDC97.1, and the amplified DNA fragment from pMGJ1984 was inserted into pBluescriptKS$^+$ to produce pTDC-E11K. JBK70(pTDC-E11K) produced 10-fold-greater amounts of mutant toxin than JBK70(pMGJ1984), and the decrease in secretion of the E11K polypeptide produced by JBK70(pTDC-E11K) was comparable to the reduction in secretion observed with JBK70(pMGJ1984). Whereas 99% of wt CT-B polypeptide was transported to the culture supernatant by JBK70(pTDC97.1), JBK70(pTDC-E11K) secreted only 60% of the E11K mutant B polypeptide (Table 4). Although secretion of the E11K mutant was not absolutely blocked in JBK70, a substantial quantitative decrease in secretion efficiency of mutant E11K was reproducible in replicate experiments. To determine the effect on extracellular secretion of an amino acid residue at position 11 other than lysine, we constructed pTDC-E11G, which encodes the E11G variant CT-B polypeptide. Plasmid pTDC-E11G was introduced into JBK70, and the amount of mutant CT-B was measured in supernatant samples and in periplasmic extracts by S-ELISA. In contrast to E11K, 90% of the E11G variant of CT-B was exported to the culture supernatant (Table 4).

TABLE 4. Extracellular secretion of wt and mutant CT-B polypeptides by *V. cholerae* JBK70

Plasmid	CT-B secreted[a]			β-Lactamase activity[b]		
	Periplasm[c] (μg/ml)	Sup[c] (μg/ml)	% in Sup	Periplasm (U/ml)	Sup (U/ml)	% in Sup
pBluescriptKS$^-$	0	0	0	7.5	1.2	13
pTDC97.1	0.03	1.88	99	2.7	1.2	32
pTDC-E11K	0.81	1.23	60	3.7	1.2	25
pTDC-E11G	0.50	5.70	90	14.0	1.6	10

[a] CT-B in periplasmic extracts and culture supernatants was measured by S-ELISA.
[b] β-Lactamase activity was measured as described in Table 3, footnote *d*.
[c] Periplasmic extracts and culture supernatants were prepared as described in Table 3, footnote *a*. Periplasm, periplasmic extract; Sup, culture supernatant.

To investigate whether the amino acid substitution at E-11 altered protein conformation, we compared the mutant E11K polypeptide with wt CT-B for G_{M1}-binding activity and for ability to assemble with wt CT-A to form holotoxin. Both of these properties depend on proper folding of the CT-B polypeptide. Relative G_{M1}-binding activity of wt CT-B was compared with the binding activity of the E11K mutant by use of a modified G_{M1}-ELISA. To measure holotoxin assembly in vivo, pTDC97.1 expressing wt CT-B and pTDC-E11K expressing mutant CT-B were introduced into *E. coli* DH5αF′tet(pMGJ78). pMGJ78 encodes wt CT-A. The amount of CT-A assembled into holotoxin was measured in periplasmic extracts from *E. coli*

TABLE 5. Effect of wt and mutant *epsE* alleles on extracellular secretion of CT and the B polypeptide of LT-IIa by strain M14

Plasmid(s)	*epsE* genotype	CT secreted			LT-IIa secreted			β-Lactamase activity[a]		
		Periplasm[b] (μg/ml)	Sup[b] (μg/ml)	% in Sup	Periplasm (μg/ml)	Sup (μg/ml)	% in Sup	Periplasm (U/ml)	Sup (U/ml)	% in Sup
pBluescriptKS$^+$	−	0.43	0.13	28	NA[c]	NA	NA	175	35	17
pTDC1	−	0.34	0.08	19	NA	NA	NA	135	33	20
pTDC3	+	0.06	0.28	82	NA	NA	NA	198	10	5
pBluescriptKS$^+$, pTDC411	−	1.6	0.22	12	20.8	0.44	2	286	17	6
pTDC1, pTDC411	−	1.35	0.1	7	6.23	0.55	8	140	0.3	0.2
pTDC3, pTDC411	+	0.55	0.27	33	7.16	19.0	73	275	8.0	3

[a] β-Lactamase activity was determined as described in Table 3, footnote *d*.
[b] Supernatant and periplasmic extracts were prepared as described in Table 3, footnote *a*. CT in samples were measured by S-ELISA using plates sensitized with goat anti-CT immunoglobulins, while LT-IIa in samples was measured by S-ELISA using plates sensitized with goat anti-LT-IIa immunoglobulins. Periplasm, periplasmic extract; Sup, culture supernatant.
[c] NA, not applicable.

DH5αF′tet(pMGJ78, pTDC97.1) and from *E. coli* DH5αF′tet (pMGJ78, pTDC-E11K) with a modified G_{M1}-ELISA. In both G_{M1}-binding activity and ability to assemble with wt CT-A, the mutant E11K B polypeptide was indistinguishable from wt CT-B (data not shown). Within the limitations of these two assays, the findings suggest that the decrease in extracellular secretion of the mutant E11K B polypeptide in JBK70 was a direct effect of substituting Lys-11 for Glu-11 and was not caused by major changes in conformation of the E11K polypeptide.

Identification of the genetic defect in the *epsE* allele of M14. M14 has been used here and in other studies to investigate extracellular secretion in *V. cholerae*. Although the mutation in M14 has been associated with the *epsE* gene of the secretory apparatus (39), the precise nature of the mutation has not been identified. As a first step in identifying the mutation and its effect on extracellular secretion of CT, the wt *epsE* allele of 569B and the mutant *epsE* allele of M14 were amplified by PCR from chromosomal DNAs. The genes were amplified without predicted promoter sequences (39), and DNA fragments containing the promoterless genes were ligated into pBluescriptKS$^-$ and pBluescriptSK$^-$ vectors such that expression was under control of the *lac* promoter. This was done to eliminate effects on extracellular secretion caused by regulatory factors, if any, produced by *V. cholerae* that might influence *epsE* expression. The wt *epsE* allele from 569B is present in pTDC3, whereas the mutant *epsE* allele from M14 is present in pTDC1. Each plasmid was introduced into M14 by electroporation. Analysis of culture supernatants and periplasmic extracts by S-ELISA showed that while pTDC1 did not complement the genetic defect in M14, pTDC3 restored secretion of CT by M14 to a level close to that observed in the wt strain 569B (Table 5). Similar results were obtained for extracellular secretion of the B polypeptide of LT-IIa when pTDC411 was introduced into M14(pTDC1) and into M14(pTDC3) (Table 5).

Sequencing of the *epsE* genes in the plasmids revealed that the nucleotide sequence of the wt allele from 569B in pTDC3 was identical to the sequence of the wt *epsE* allele previously reported from the El Tor strain TRH7000, a *thy* derivative of JBK70, and that the *epsE* allele from M14 in pTDC3 differed at only one nucleotide position from the sequence of the 569B *epsE* gene. A cytosine-to-thymine substitution at nucleotide position 987 of the published sequence of *epsE* (39) encodes an alanine-to-valine conservative substitution at amino acid position 321 in the M14 EpsE polypeptide. To ensure that the single-point mutation at position 987 did not arise as a cloning artifact, double-stranded fragments of DNA containing the *epsE* alleles were amplified from the chromosomes of M14 and 569B by PCR, and the preparations were directly sequenced. The nucleotide sequence from the double-stranded fragments confirmed the mutation in the *epsE* allele of M14 (data not shown).

DISCUSSION

Mechanisms for extracellular secretion of macromolecules are found in a diverse group of numbers of the family *Entero bacteriaceae. Klebsiella oxytoca* (38), *Erwinia chrysanthemi* (13), *Pseudomonas aeruginosa* (2), *Xanthomonas campestris* (6), *Aeromonas hydrophila* (20), and *V. cholerae* (32, 39) each contain a cluster of conserved genes that are essential for extracellular transport. Previous studies of extracellular transport in several of these bacteria showed that the secretory systems were highly specific for their respective extracellular proteins. *E. chrysanthemi* secretes pectate lyase to the extracellular milieu, but pectate lyase was not secreted by *K. oxytoca* nor did *E. chrysanthemi* secrete a pectate lyase cloned from *Erwinia carotovora* to the extracellular medium (13). Recently, Overbye et al. (32) demonstrated that although LT-I enterotoxin was not secreted by *E. chysanthemi*, *Xanthomonas maltophilia*, or *Klebsiella pneumoniae*, seven species of the family *Vibrionaceae* other than *V. cholerae* and two species of the family *Aeromonadaceae* had secretory machineries capable of secreting the toxin to the extracellular milieu. All 10 species of the *Vibrionaceae* and *Aeromonadaceae* that secrete LT-I are closely related on the basis of comparisons between 5S rRNA sequences. Whether LT-II enterotoxins will be secreted by gram-negative bacteria other than *V. cholerae* is not known, but those studies will be facilitated by the availability of the plasmids encoding the B polypeptides of LT-IIa and LT-IIb.

How the various secretory machineries discriminate between proteins in the periplasm is not yet clear. Discrimination likely involves recognition of specific transport signals by one or more components of the transport apparatus. The signals are evidently present on secreted proteins and absent on nonsecreted proteins (37). Many of the bacterial species with extracellular transport mechanisms secrete more than one protein, and those proteins may be very divergent in structure. For example, *E. carotovora* secretes pectate lyase and cellulase, while *V. cholerae* secretes CT, H/A protease, and chitinase. Comparisons of these structurally different proteins have not revealed a common amino acid sequence that might function as the translocation signal (37). The transport signal may be a patch signal, a structure formed by discontinuous amino acid

sequences brought together by folding of the protein (37). This may indeed be the case for proteins secreted by *V. cholerae*. The genetic experiments described here demonstrate that B polypeptides of LT-IIa and LT-IIb are transported across the outer membrane by the same secretory apparatus that transports CT. However, the B polypeptides of CT and LT-IIa and the B polypeptides of CT and LT-IIb have little primary amino acid homology (34, 35). Therefore, it is reasonable to conclude that the structural signals of CT, LT-IIa, and LT-IIb recognized by the secretory machinery are not composed of conserved primary amino acid sequences but are conserved structural motifs formed by secondary or tertiary domains. Why a transport signal would be conserved in LT-II enterotoxins in the absence of a functional secretory system in *E. coli* is not immediately evident. One possibility is that the translocation signal in CT, LT-IIa, and LT-IIb is part of a structural domain that is essential for proper folding of the B polypeptides and may be important for other toxin phenotypes, such as ganglioside binding, pentamer assembly, or holotoxin assembly. Alternatively, it may be that LT-II did not originate in *E. coli* and that the structural domains that are required for secretion of the proteins have been maintained.

Excessive expression of pectate lyase in *E. chrysanthemi* has been shown to saturate the secretion system encoded by the *outC-O* cluster of genes (13). Secretion efficiency is maximal only when the expression level of pectate lyase is decreased to normal levels. This is in contrast to the effect of overexpression of LT-II B polypeptides on secretion efficiency in *V. cholerae*. Although the B polypeptides of LT-IIa and LT-IIb were expressed in molar amounts 50-fold or greater than are typically seen in strain 569B for expression of CT, there was little evidence that the extracellular transport system was saturated for any of the three toxins. There are several possible explanations for why the secretion system of *V. cholerae* is not saturable under the conditions established in these experiments: (i) the cell may respond to increasing concentrations of intracellular or periplasmic toxin by producing additional sites for toxin secretion, (ii) the secretory system of *V. cholerae* may transport toxin at a sufficiently rapid rate such that the large amounts of periplasmic toxin expressed by the strains in these experiments can be easily accommodated, or (iii) sites that are normally used for transport of H/A protease or chitinase may be utilized by toxin so that the total number of sites available is still below the level of saturation for the amount of toxin produced. Further investigation is required to define the factors that determine the capacity of the secretory machinery.

The lack of significant amino acid homology between the B polypeptides of CT and LT-IIa and the B polypeptides of CT and LT-IIb precludes the use of comparative analysis of the primary amino acid sequences to locate the extracellular transport signals in each of the toxins. To initiate studies to locate amino acid residues in CT-B that are important for extracellular secretion, a panel of plasmids encoding mutant CT-B polypeptides with single-point amino acid substitutions was analyzed in *V. cholerae*. E11K, a mutant CT-B polypeptide in which lysine was substituted for glutamate at amino acid position 11, showed a significant reduction in secretion efficiency when expressed in *V. cholerae*, but the lysine substitution did not affect several other properties of CT-B that require proper folding of the CT-B polypeptide. The probability is high, therefore, that the decrease in extracellular transport of the E11K CT-B polypeptide is due to a specific and localized change in a domain that interacts with the secretory apparatus of *V. cholerae.*

Mutational analysis has been extremely valuable in elucidating structure and function of proteins and will be useful in probing EpsE for amino acid residues that are important in extracellular secretion of CT, LT-IIa, and LT-IIb by *V. cholerae*. No mutational or structural analysis has been reported for EpsE, but sequence analysis of the protein has revealed two short tracts of amino acids that are highly homologous to regions (Walker boxes) found in a variety of proteins that have ATP-binding activity (39). Similar Walker sequences are found in the gene products expressed by the secretory genes *pulE*, *xpsE*, and *pilB* (37). The presence of these Walker motifs suggests that the EpsE, PulE, XpsE, and PilB proteins may provide energy for one or more steps in extracellular transport. Mutations within the sequences encoding the Walker regions in the *pulE* gene reduced the efficiency of extracellular secretion of pullulanase by *K. oxytoca* (36, 43). The nature of the effect of the alanine-to-valine substitution at amino acid position 321 on EpsE has not been determined, but the A321V substitution is located outside of the Walker boxes. Whether the A321V substitution makes the M14 EpsE protein more sensitive to degradation or disturbs the conformation of one or more regions of the protein outside of the Walker boxes that are critical for biological function is not known. Future investigations into the effect of the A321V mutation on EpsE structure and function will be simplified by purification of the wt and mutant proteins and by production of EpsE-specific antibodies. An alanine is conserved at the amino acid position corresponding to 321 of EpsE in PulE of *K. oxytoca* (36) and in PilB of *P. aeruginosa* (31). Thus, it is intriguing to speculate that a valine-for-alanine substitution at position 321 in each of these proteins may reduce extracellular secretion of pullulanase in *K. oxytoca* and piliation in *P. aeruginosa*. In *K. oxytoca*, the phenotype of the *pulE* mutant was trans-dominant over the phenotype of the wt *pulE* gene. Now that mutant and wt *epsE* clones are available, similar experiments investigating allelic dominance and its effect on extracellular secretion can be done in *V. cholerae.*

There is no long-term effective vaccine for *V. cholerae*. Until a vaccine is available, alternative strategies to ameliorate the symptoms of the disease are potentially very important. A better understanding of the process of extracellular secretion by *V. cholerae* is a necessary first step in the subsequent design of therapeutic strategies to ameliorate cholera symptoms by inhibiting release of CT.

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ADDENDUM IN PROOF

Sandkvist et al. (EMBO J. **14:**1664–1673, 1995) recently showed by mutational analysis that the Walker A box is required for activity of EpsE and that EpsE is complexed with the product of the *epsL* gene and the cytoplasmic membrane. Furthermore, EpsE was shown to have autophosphorylation activity, suggesting that kinase activity of the protein is likely required for transport of CT out of the periplasm.

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