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A 14-Kilodalton Inner Membrane Protein of *Vibrio cholerae* Biotype El Tor Confers Resistance to Group IV Choleraphage Infection to Classical Vibrios

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Choleraphage ϕ149 differentiates the two biotypes, classical and el tor, of *Vibrio cholerae*. This phage cannot replicate in *V. cholerae* biotype el tor cells because the concatemeric DNA intermediates produced are unstable and cannot be chased to mature phage DNA. A *V. cholerae* biotype el tor gene coding for a 14,000-Da inner membrane protein which destabilizes the concatemeric DNA intermediates by hindering their binding to the cell membrane has been identified. Presumably, a 22,000-Da *V. cholerae* biotype el tor protein might also have a role in conferring phage ϕ149 resistance to cells belonging to the biotype el tor. A nucleotide sequence homologous to the 1.2-kb *V. cholerae* biotype el tor DNA coding for both the 14,000- and 22,000-Da proteins is present in all strains of classical vibrios but is not transcribed. The nucleotide sequence of the gene coding for the 14,000-Da protein has been determined.

Vibrio cholerae strains of serotype O1 comprise two different biotypes, classical and el tor. Of the seven pandemics of cholera recorded in recent times, *V. cholerae* biotype el tor has been identified as the causative agent of the seventh pandemic, while the previous ones were due to the classical strains. Recently, there has been a resurgence of classical vibrios in certain areas of endemicity (33). In spite of broad similarities in the nature of infection by the classical and el tor vibrios, important differences exist in the epidemiology of el tor compared with that of classical vibrios. The ratio of asymptomatic infection to clinical illness is greater with the el tor biotype than with the classical vibrios (2), and the el tor vibrios survive longer under unfavorable conditions. Both the classical and the el tor strains of *V. cholerae* react with *V. cholerae* antisera, and taxonomic studies have shown that these two biotypes are the same species (12). They are isogenic, and tests to distinguish them often give ambiguous results (16). One of the most reliable criteria differentiating between these two biotypes is their susceptibility to group IV choleraphages. This group of choleraphages can infect and lyse all strains of classical vibrios but none of the el tor biotype (24).

The process of infection by phage ϕ149, a representative strain of group IV choleraphages, has recently been examined for classical vibrios. Phage ϕ149 is a large virus and contains a linear double-stranded DNA molecule with a size of 102 kb (36). The DNA molecules are a limited set of circular permutation of the phage genome and have several single-strand interruptions along their length that are repairable by DNA ligase (36). The phage DNA codes for 26 early and 23 late proteins (31). The intracellular replication of phage ϕ149 DNA involves a concatemeric DNA replicative intermediate serving as the substrate for the synthesis of mature phage DNA, which is eventually packaged by a headful mechanism (11). The growth of this phage is extremely sensitive to the concentration of phosphate ions, and no phage growth occurs in medium containing more than 0.1% phosphate (31). After infection under high-phosphate conditions, concatemeric DNA structures are not formed, although synthesis of monomeric molecules is unaffected (11).

Phage ϕ149 adsorbs irreversibly to the biotype el tor cells, and 50% of the injected phage DNA binds to the cell membrane. Synthesis of monomeric phage DNA continued, similar to that observed for the permissive host. However, the concatemeric DNA intermediates produced were unstable and could not be chased to mature phage DNA (9). Although most of the early proteins are made, only some of the late proteins were transiently synthesized for infection in el tor cells. Formation and stabilization of concatemeric DNA structures essential for the synthesis of mature phage DNA and their packaging into phage heads are not hindered in *V. cholerae* biotype el tor cells for the replication of choleraphage ϕ138 belonging to serological group II. This phage also contains a linear double-stranded circularly permuted DNA molecule and can replicate in both *V. cholerae* classical and biotype el tor cells (10). Thus, some function present in the el tor biotype and not in the classical vibrios might be specifically involved in the destabilization of the concatemeric DNA during replication of group IV choleraphages in *V. cholerae* biotype el tor cells. The present report describes experiments to identify the relevant host gene(s). The results presented here show that a 14,000-Da inner membrane protein of *V. cholerae* biotype el tor causes destabilization of ϕ149 concatemeric DNA. When the gene coding for this protein was introduced into *V. cholerae* cells, the concatemeric DNA synthesized following ϕ149 infection was unstable and could not be chased to mature phage DNA.

MATERIALS AND METHODS

Bacterial strains, phages, media, and buffer. The bacteria, bacteriophages, and plasmids used in this study are listed in Table 1. *V. cholerae* Ogawa 154, the universal host for the propagation of choleraphages, was used for phage propagation. *V. cholerae* 569B and *V. cholerae* biotype el tor strain MAK757 were used as hosts for phage infection study. Cells were grown and maintained as described previously (18, 21,

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TABLE 1. Bacterial strains, phages, and plasmids used

Strain, plasmid, or phage	Characteristic(s)	Source or reference
Strains		
V. cholerae MAK757	Biotype el tor, serotype Ogawa, mildly toxigenic	
V. cholerae 569B	Biotype classical, serotype Inaba, prototroph, hypertoxigenic	
E. coli JM101	SupE *thi* (*lac proAB*) F' *traD36 proAB lacI*^{q}Z M15	38
E. coli CSR603	*thr-1 ara-14 leuB64 (gpt-proA)62 lacY1 tax-33 supE44 phr-1 galK2 rac gyrA78 recA1 rpsL31 kdgK51 xyl-5 mtl-1 uvrA-6*	34
E. coli S17-1	*rec hsdR* Pro RP4 -2TC::Mu Km::Tn*7*	30
Plasmids		
pUC18	Amp^r	38
pSUP106	Can^r Tet^r, conjugative	30
pDB350	pSUP106 carrying 3.5-kb *Eco*RI *V. cholerae* biotype el tor DNA fragment, Tet^r	Present study
pDB180	pSUP106 carrying 1.8-kb *Eco*RI-*Pst*I *V. cholerae* biotype el tor DNA fragment	Present study
pDB120	pSUP106 carrying 1.2-kb *Eco*RI-*Hin*cII *V. cholerae* biotype el tor DNA fragment	Present study
pSB120	pUC18 carrying 1.2-kb *Eco*RI-*Hin*cII *V. cholerae* biotype el tor DNA fragment, Amp^r	Present study
pRC110	pUC18 carrying the 1.1-kb *Bam*HI-*Hin*cII fragment of the 1.2-kb *Eco*RI-*Hin*cII *V. cholerae* biotype el tor DNA fragment	Present study
pRC80	pUC18 carrying the 0.8-kb *Eco*RI-*Bcl*I fragment of the 1.2-kb *Eco*RI-*Hin*cII *V. cholerae* biotype el tor DNA fragment	Present study
Phages		
ϕ149	Group IV choleraphage, circularly permuted linear double-stranded DNA (molecular weight, 69 × 10^6; 102 kb)	36
ϕ138	Group II choleraphage, circularly permuted linear double-stranded DNA (molecular weight, 30 × 10^6; 45 kb)	10

32). High-titered phage stocks and ^{32}P-labeled phages were prepared as described previously (31). TCBS agar (Difco) plates (pH 8.6) were used for selecting *V. cholerae* cells. The Tris-Casamino Acids-glucose medium containing 0.04% K_2HPO_4, used for phage growth, is referred to as low-phosphate medium, whereas the same medium containing 0.4% K_2HPO_4 used for cell growth is referred to as high-phosphate medium. Label termination buffer contained 50 mM Tris-hydrochloride (pH 7.5), 5 mM $MgCl_2$, 2 mM NaN_3, and 3 mg of thymidine per ml.

Preparation of infected cell lysates. Cells in the logarithmic phase of growth (2 × 10^8 to 3 × 10^8 CFU/ml) in high-phosphate medium were infected with phage ϕ149 at a multiplicity of infection of 10. Two minutes was allowed for adsorption, after which cells were washed and suspended in low-phosphate medium. At different times during infection, samples of infected culture were withdrawn and, whenever required, labeled with 20 μCi of [^{3}H]thymidine (specific activity, 18.8 Ci/mmol; Bhabha Atomic Research Centre, Bombay, India) per ml for the desired lengths of time. Labeling was terminated by adding an equal volume of ice-cold label termination buffer, and the cells were sedimented (10,000 × *g*, 10 min, 4°C) and lysed by suspension in 1/20 of the culture volume of 0.01 M Tris (pH 8.8)–0.001 M EDTA buffer containing 2% Sarkosyl NL97. The lysate was incubated at 37°C for 15 min before sedimentation analysis.

In vitro mutagenesis of plasmid DNA. Plasmid pDB350 was mutagenized in vitro by hydroxylamine (3). Plasmid DNA was dissolved in 1 M sodium acetate buffer (pH 4.9) to give a final concentration of 10 μg/50 μl. To 50 μl of the plasmid

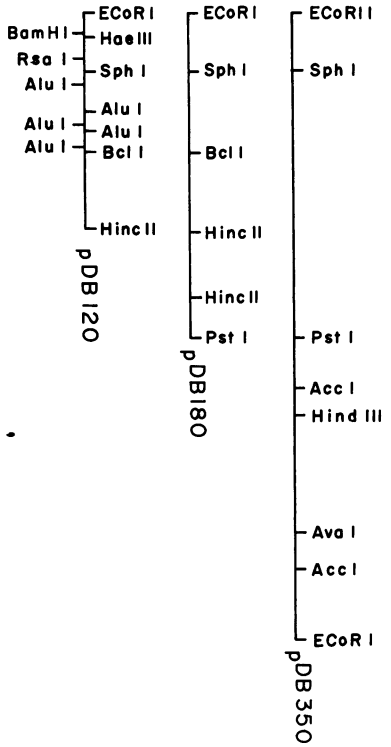

FIG. 1. Restriction map of the 3.5-, 1.8-, and 1.2-kb DNA segments of *V. cholerae* biotype el tor of plasmids pDB350, pDB180, and pDB120, respectively.

solution, 150 μl of 2 M hydroxylamine in sodium acetate buffer was added, and the mixture was incubated at 55°C for 1 h. Then, the pH of the suspension was adjusted to 7.6 by adding 1 M Tris-HCl–20 mM EDTA (pH 8.0). The plasmid DNA was precipitated, washed, dissolved in TE buffer (10 mM Tris, 1 mM EDTA [pH 7.6]), and used for transformation. Ampicillin (50 μg/ml) was used for selection of resistant clones.

Sucrose gradient centrifugation. Infected cell lysates were analyzed by velocity sedimentation in neutral 5 to 20% (wt/vol) sucrose gradients with a Sorvall AH650 rotor at 30,000 rpm for 150 min at 15°C, as described previously (8). For analysis of fast-sedimentation complex, infected cells were lysed gently by 3 to 4 cycles of freezing in liquid nitrogen and thawing at 37°C, layered on a 5 to 20% sucrose gradient formed over a shelf of 1.3 g of CsCl per ml in 40% sucrose, and centrifuged for 2 h at 30,000 rpm in a Beckman model L7-55 ultracentrifuge with an SW50.1 rotor (11).

Isolation of DNA and RNA. Cellular DNA was isolated from 500 ml of stationary-phase culture, purified by treatment with DNase-free RNase and pronase, and stored at 4°C in 10 mM Tris-HCl (pH 8.0)–1 mM EDTA buffer (27). Plasmid DNA was prepared by the method of Birnboim and Doly (6). Total cellular RNA was isolated from cells in the logarithmic phase of growth by the method of Chomczynski and Sacchi (8).

Transformation and conjugation. Transformations in *Escherichia coli* were performed by using standard methods (22). The transformation of *V. cholerae* cells by plasmid DNA was carried out by using the recently described method of Panda et al. (27). The plasmid pSUP106 and its derivatives pDB350, pDB180, and pDB120 were conjugally transferred to *V. cholerae* 569B cells as described previously (18).

Nuclease digestion and Southern and Northern blotting. Digestion of DNA with different endonucleases was carried out according to the instructions of the manufacturers. Whenever required, DNA fragments were recovered from low-melting-point agarose gels. For construction of physical maps, linkages between the restriction fragments were determined either from an analysis of partial-digestion products or from restriction fragments produced by a mixture of the enzymes. Digestion of restriction fragments with *Bal* 31 was used to confirm the linkages.

For Southern blot experiments, approximately 5 μg of DNA to be analyzed was digested with the desired restriction enzymes and electrophoresed on agarose (0.8%) slab gels at 3 V/cm of gel. The gels were stained with ethidium bromide, irradiated with UV light to nick the DNA, denatured, and blotted to nitrocellulose (37). Hybridization was carried out at 60°C without formamide. The filters were washed, dried, and exposed to X-ray films.

For Northern (RNA) blotting, approximately 10 μg of total RNA was electrophoresed on 1% formaldehyde-agarose gels and transferred to nitrocellulose (22). Hybridization was carried out at 37°C in 50% formamide with Denhardt buffer (22). The filters were washed at room temperature, dried, and exposed to Kodak XR-5 film with an intensifying screen.

Nick translation. Approximately 1 μg of the desired DNA fragments was nick translated by using [α-^{32}P]ATP (Amersham International, Amersham, United Kingdom) and DNA polymerase I (22). The reaction was carried out at 16°C for 1 h, and the nick-translated DNA was separated from the unincorporated [α-^{32}P]ATP by being passed through a Sephadex G50 column.

Gel electrophoresis. Analysis of DNA restriction fragments was done with agarose (0.8%) horizontal slab gels or polyacrylamide vertical slab gels as described previously (28). For electrophoresis of RNA, formaldehyde-agarose gels were used (22). Proteins were analyzed in 12% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) by the method of Laemmli (20).

Protein labeling in maxicells. Plasmid-coded proteins were examined by the maxicell method (34). *E. coli* CSR603 carrying either pUC18 or pUC18 containing the 3.5- or 1.2-kb *V. cholerae* biotype el tor DNA fragment was grown (2×10^8 CFU/ml) in minimal medium containing 1% Casamino Acids. The cells were irradiated with UV light (50 J/m) and incubated at 37°C for 1 h. At the end of the period, 200 μg of D-cycloserine was added, and the incubation was continued for 16 h at 37°C. The cells were then harvested, suspended in fresh sulfur-depleted medium, incubated at 37°C for 1 h, and labeled with 5 μCi of [^{35}S]methionine (Amersham International, Amersham, United Kingdom) per ml for another hour. The labeled cells were harvested and washed, and when required, membrane samples were prepared by sonication and centrifugation (15). Either whole-cell lysates or membrane preparations were suspended in electrophoresis sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) followed by autoradiography of the dried gels as described previously (31).

DNA sequencing. The DNA sequence was determined by the dideoxynucleotide chain termination method (35). The 0.78-kb *Eco*RI-*Bcl*I fragment and 0.79-kb *Sph*I-*Hin*cII fragment of the 1.2-kb *V. cholerae* biotype el tor DNA were cloned in pUC18. The 1.2-kb DNA was also digested with *Sau*3A1, and the resulting fragments were cloned into pUC18.

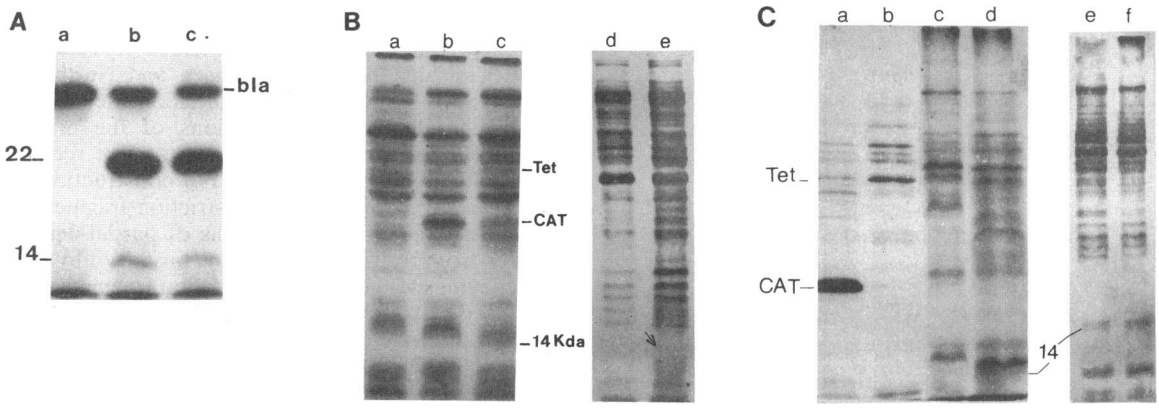

FIG. 2. Identification of the products encoded by the 3.5- and 1.2-kb DNA segments of *V. cholerae* biotype el tor. UV-irradiated cells carrying the plasmid or cells carrying plasmid with the insert were labeled with [^{35}S]methionine for 1 h, and soluble extracts of proteins were analyzed by SDS-PAGE followed by autoradiography as described in the text. (A) *E. coli* CSR603 cells carrying plasmid pUC18 either alone (lane a) or containing the 3.5-kb (lane b) or 1.2-kb (lane c) fragment. (B) UV-irradiated *V. cholerae* 569B proteins (lane a), proteins encoded by plasmid pSUP106 (lane b) or pDB120 (lane c) in UV-irradiated *V. cholerae* 569B, and *V. cholerae* 569B cells carrying plasmid pRC110 (lane d) or pSB120 (lane e). The arrow indicates the 14,000-Da protein. (C) Intracellular localization of the 14-kDa protein in *V. cholerae* cells carrying plasmid pDB120. *V. cholerae* 569B cells carrying plasmid pSUP106 or pDB120 were UV irradiated and labeled with [^{35}S]methionine for 1 h. Inner membrane, outer membrane, and cytoplasmic proteins were isolated and analyzed by SDS-PAGE. Cytoplasmic proteins of *V. cholerae* 569B cells carrying plasmid pSUP106 (lane a) or pDB120 (lane b) and inner membrane proteins of *V. cholerae* 569B cells carrying plasmid pSUP106 (lane c), pDB120 (lane d), pSB120 (lane e), or pRC110 (lane f) are shown. The numbers indicate the molecular masses of the polypeptides in kilodaltons. The β-lactamase (bla), tetracycline (Tet), and chloramphenicol acetyltransferase (CAT) gene products are indicated.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences of the *V. cholerae* biotype el tor gene coding for the 14,000-Da protein have been reported to the EMBL data bank under accession number X-56017.

RESULTS

***V. cholerae* biotype el tor gene function conferring resistance of *V. cholerae* cells to phage ϕ149 infection.** The concatemeric DNA replicative intermediates synthesized following infection of *V. cholerae* biotype el tor cells by phage ϕ149 are unstable and cannot be chased to mature phage DNA (9). To identify the *V. cholerae* biotype el tor gene function which might be responsible for the destabilization of the concatemeric DNA intermediate, a genomic library of *V. cholerae* biotype el tor was constructed by cloning *Eco*RI restriction fragments into the conjugative plasmid pSUP106 and was maintained in *E. coli* S17.1 in LB plates or slants containing tetracycline. The gene bank was conjugally transferred to *V. cholerae* 569B, the permissive host for phage ϕ149, and from several thousand tetracycline-resistant colonies, two colonies which were resistant to ϕ149 infection were recovered. When the plasmid, designated as pDB350, isolated from the phage-resistant *V. cholerae* cells was transferred to phage-sensitive cells, all transformed cells exhibited resistance to ϕ149 infection. The recombinant plasmid pDB350 contained a 3.5-kb *V. cholerae* biotype el tor DNA segment.

A cleavage map of the 3.5-kb DNA segment was constructed by using several restriction enzymes (Fig. 1). The enzyme *Pst*I has a single site in the 3.5-kb DNA fragment and produced two fragments with sizes of 1.8 and 1.7 kb. Both these fragments were cloned in plasmid pSUP106 and conjugally transferred to *V. cholerae* 569B. Cells harboring the recombinant plasmid pDB180 carrying the 1.8-kb DNA fragment (Fig. 1) conferred resistance of *V. cholerae* cells to ϕ149 infection. Cells carrying the plasmid with the 1.7-kb insert were sensitive to ϕ149 infection. The 1.8-kb DNA fragment was further digested with the enzyme *Hin*cII (Fig. 1), and the 1.2-kb *Eco*RI-*Hin*cII fragment was cloned in plasmid pSUP106. *V. cholerae* cells harboring the recombinant plasmid (pDB120) carrying the 1.2-kb *V. cholerae* biotype el tor DNA fragment conferred resistance of *V. cholerae* cells to ϕ149 infection. To confirm that the product(s) of the 1.2-kb *V. cholerae* biotype el tor DNA fragment indeed conferred resistance of *V. cholerae* cells to phage ϕ149 infection, plasmid pDB120 was mutagenized in vitro, transferred to *E. coli* cells, and conjugally introduced into *V. cholerae* cells. Several transformed cells which were sensitive to ϕ149 infection were isolated. The 1.2-kb *V. cholerae* el tor DNA fragment in the plasmids isolated from these sensitive cells was cloned into plasmid pSUP106 and conjugally transferred into *V. cholerae* cells. All transformed cells were sensitive to phage ϕ149 infection. These results eliminate the possibility that mutation in the plasmid vector produced the observed effect.

Product(s) of the 1.2-kb DNA fragment. The protein(s) encoded by the 1.2-kb *V. cholerae* biotype el tor DNA segment was examined by the maxicell technique with *E. coli* CSR603. Since plasmid pDB120 could not be stably maintained in CSR603 cells, the 1.2-kb DNA segment was cloned into the *Eco*RI-*Hin*cII site of the plasmid pUC18, and the recombinant plasmid was transformed into *E. coli* CSR603. The transformed cells were irradiated with UV light and labeled with [^{35}S]methionine for 1 h, and the soluble extracts of proteins were analyzed by SDS-PAGE and autoradiography. The 1.2-kb *V. cholerae* biotype el tor DNA fragment codes for two proteins with molecular weights of 22,000 and 14,000 (Fig. 2A, lane c). The 3.5-kb DNA of the plasmid pDB350 also coded for these two proteins (Fig. 2A, lane b). The level of expression of the 14,000-Da protein was about 10-fold less than that of the 22,000-Da protein.

To examine the expression of the 1.2-kb *V. cholerae* biotype el tor DNA fragment in *V. cholerae* cells, cells carrying plasmid pDB120 were irradiated with UV light and labeled with [^{35}S]methionine, and the soluble extracts of proteins were analyzed by SDS-PAGE and autoradiography. Surprisingly, only the 14,000-Da protein could be detected in *V. cholerae* cells carrying plasmid pDB120 (Fig. 2B, lane c). Neither the 22,000-Da nor the 14,000-Da protein was detected in *V. cholerae* cells carrying plasmid pSUP106 (Fig. 2B, lane b) or in cells of strain 569B (Fig. 2B, lane a).

The 22,000-Da protein encoded by the 1.2-kb *V. cholerae* biotype el tor DNA fragment in *E. coli* CSR603 cells could not be detected in *V. cholerae* cells carrying the cloned 1.2-kb DNA fragment. It has been demonstrated that the 22,000-Da protein of *V. cholerae* biotype el tor represents the *sulA* gene product, an inhibitor of cell division, and is cleaved following the activation of *recA* protease (unpublished observation). As opposed to *E. coli* CSR603 cells, which have mutations in the *recA*, *gyrA*, and *phr* genes, the *V. cholerae* cells used were wild type for these gene functions. Under the maxicell experimental conditions, the *recA* protease of *V. cholerae* is activated and cleaves the 22,000-Da protein encoded by plasmid pDB120. This explains why the 22,000-Da protein could not be detected in *V. cholerae* cells carrying plasmid pDB120 (Fig. 2B, lane c). Furthermore, when plasmid pDB120 was cloned in *E. coli* JM101, which is also *recA*$^+$, and the plasmid-coded proteins were examined, the 22,000-Da protein could not be detected.

V. cholerae cells carrying plasmid pDB120 were resistant to phage ϕ149 infection. Even after UV irradiation when the 22,000-Da protein could not be detected, the cells were resistant to phage infection. These results apparently suggested that the 14,000-Da protein may be sufficient to confer resistance of *V. cholerae* cells to phage ϕ149 infection, although a role of the 22,000-Da protein could not be ruled out in this process because the cleavage of this protein by activated *recA* protease might not be complete. To investigate the relative roles of these two proteins in conferring phage ϕ149 resistance, deletions were made in the coding region of either the 14,000- or 22,000-Da protein and the effect of such deletions on phage infection was examined. Deletion of the *Eco*RI-*Bam*HI fragment of the 1.2-kb *V. cholerae* biotype el tor DNA segment (Fig. 1) removed the first 28 amino acids from the N-terminal end of the 14,000-Da protein. The remaining *Bam*HI-*Hin*cII segment of the 1.2-kb DNA was cloned in the pUC vectors. Deletion of the *Hin*cII-*Bcl*I fragment of the 1.2-kb DNA (Fig. 1) eliminated the first 50 amino acids from the N-terminal end of the 22,000-Da protein, keeping the coding region of the 14,000-Da protein intact. The *Eco*RI-*Bcl*I fragment was cloned in a pUC vector. The recombinant plasmids pRC110 and pRC80, respectively, were separately transformed into *E. coli* CSR603 to analyze the plasmid-coded gene products and into *V. cholerae* 569B cells to examine the effect of the deletions on phage infection. *V. cholerae* 569B cells carrying plasmid pRC110 or pRC80 were sensitive to phage ϕ149 infection. Surprisingly, neither the 14,000- nor the 22,000-Da protein could be detected in the maxicell experiment with *E. coli* CSR603 carrying either plasmid pRC110 or pRC80. Thus, whether the 14,000-Da protein alone is sufficient to confer resistance of *V. cholerae* cells to ϕ149 infection or whether both the 22,000- and 14,000-Da proteins are required is not clear.

The 14,000-Da protein is located in the inner membrane. To determine the intracellular location of the 14,000-Da protein, the outer membrane, inner membrane, and cytoplasmic proteins of UV-irradiated *V. cholerae* 569B cells carrying plasmid pDB120 were analyzed by SDS-PAGE. The 14,000-Da protein was recovered from the inner membrane (Fig. 2C, lane d). This protein was not detected either in the outer membrane preparation or in the cytoplasmic fraction (Fig. 2C, lane b) of cells carrying plasmid pDB120. When these experiments were repeated with *V. cholerae* 569B cells carrying plasmid pSUP106 without the insert, the 14,000-Da protein could not be detected in the inner membrane (Fig. 2C, lane c), the outer membrane, or the cytoplasmic fractions (Fig. 2C, lane a). When the 1.2-kb DNA fragment was cloned in the chloramphenicol acetyltransferase site of the plasmid pSUP106, there was no synthesis of the chloramphenicol acetyltransferase protein in cells carrying the recombinant plasmid (Fig. 2C, lane b). When the inner and outer membrane and the cytoplasmic proteins of *V. cholerae* 569B cells carrying plasmid pRC110 were analyzed by SDS-PAGE, the 14,000-Da protein could not be detected in any of the subcellular fractions (Fig. 2C, lane e).

Replication of ϕ149 in *V. cholerae* cells carrying the plasmid pDB120. To investigate whether presence of the 14,000-Da protein in *V. cholerae* cells produces an effect on ϕ149 replication similar to that reported for *V. cholerae* biotype el tor cells (9), the intracellular replication of phage ϕ149 in the permissive host carrying the plasmid pDB120 was examined. *V. cholerae* 569B carrying the plasmid pDB120 was infected with phage ϕ149 at a multiplicity of infection of 10, and the pulse-labeled intracellular DNA was analyzed by neutral sucrose gradients at different times during infection. Up to approximately 15 min after infection, most of the pulse-labeled DNA cosedimented with the ^{32}P-labeled ϕ149 DNA used as a marker. Phage DNA synthesized 30 min after infection sedimented faster than the monomeric DNA and was distributed in a wider peak (Fig. 3Aa), similar to that reported for infection in *V. cholerae* biotype el tor cells (9). Up to 60 min of infection, the synthesis of this concatemeric DNA continued at a reduced rate (Fig. 3Aa) and none of the newly synthesized DNA molecules were resolved as monomeric units in the gradients (Fig. 3Aa).

At a time during infection when synthesis of the high-molecular-weight DNA intermediate predominates, phage ϕ149-infected *V. cholerae* cells carrying the plasmid pDB120 were pulse-labeled for 5 min with [^{3}H]thymidine and the pulse-labeled DNA was chased by suspending infected cells in unlabeled medium (Fig. 3Ab). During the 45-min chase, no conversion of the high-molecular-weight DNA to monomers occurred and the acid-precipitable radioactivity in the high-molecular-weight DNA intermediate was reduced gradually. Thus, similar to that reported for infection of *V. cholerae* biotype el tor cells by ϕ149 (14), the high-molecular-weight DNA intermediate produced in classical vibrios carrying the 1.2-kb *V. cholerae* biotype el tor genome segment was unstable and could not serve as a substrate for the synthesis of mature phage DNA.

During replication of phage ϕ149 DNA in the permissive host, the concatemeric DNA intermediate binds to the cell membrane and packaging of mature phage DNA into phage heads occurs at the membrane site (11). For infection in biotype el tor, at no time during infection was membrane attachment of the concatemeric DNA observed (9). To examine whether the high-molecular-weight DNA produced during ϕ149 infection of *V. cholerae* cells carrying plasmid pDB120 attaches to the membrane at any time during infection, cells were infected with ^{32}P-labeled phage and, at different times, pulse-labeled with [^{3}H]thymidine to monitor the newly synthesized DNA. The infected cells were lysed

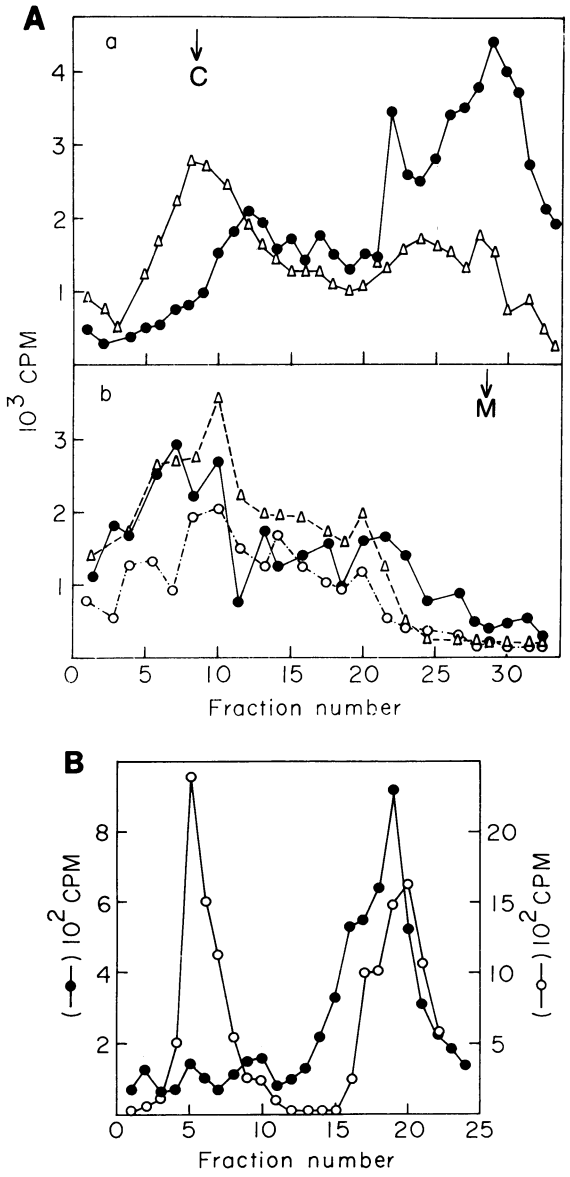

FIG. 3. (A) Velocity sedimentation analysis of ϕ149 DNA replicative intermediates during intracellular replication in *V. cholerae* 569B cells carrying the plasmid pDB120. (a) Cells in the logarithmic phase of growth were infected with ϕ149 at a multiplicity of infection of 10 and pulse-labeled for 5 min with 20 μCi of [^{3}H]thymidine per ml at 15 (●) and 35 (△) min after infection. The cell lysates were analyzed on 5 to 20% sucrose gradients. (b) Pulse-labeled DNA at 35 min after infection (△) was chased in unlabeled medium for 15 (●) and 30 (○) min. The positions of the monomeric and concatemeric ϕ149 DNA in the gradient are indicated by the letters M and C, respectively. The results of sedimentation are indicated from right to left. (B) Distribution of ϕ149 DNA in fast- and free-sedimenting components at 35 min after infection. Cells in the logarithmic phase of growth were infected with ^{32}P-labeled phage ϕ149 (○) at a multiplicity of infection of 10 and pulse-labeled for 5 min with 20 μCi of [^{3}H]thymidine per ml (●). The cells were lysed by freezing and thawing and analyzed on 5 to 20% sucrose gradients formed over a CsCl shelf as described in the text. Sedimentation results are indicated from right to left.

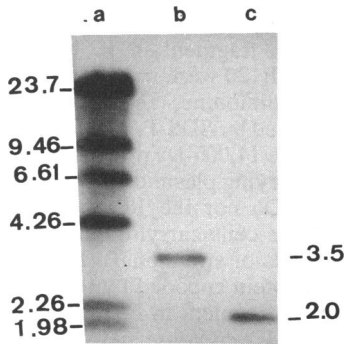

FIG. 4. Southern blot hybridization of *Eco*RI-digested *V. cholerae* 569B (lane c) and *V. cholerae* biotype el tor strain MAK757 (lane b) by using the nick-translated 1.2-kb *V. cholerae* biotype el tor DNA fragment as a probe. Lane a: DNA digested with *Hin*dIII as a molecular weight marker. The numbers represent the sizes of the fragments in kilobases.

by freezing and thawing, and the lysates were analyzed by sedimentation through neutral sucrose gradients to detect membrane-associated DNA as described in Materials and Methods. About 60% of the input parental label was associated with the cell membrane (Fig. 3B). However, unlike that observed for infection in the permissive host, at no time during infection was the newly synthesized DNA associated with the cell membrane. The presence of the 14,000-Da protein in the inner membrane presumably hinders membrane association of the ϕ149 concatemeric DNA. This experiment was repeated with phage ϕ138 which also contains a linear double-stranded circularly permuted DNA molecule (10). The presence of the 14,000-Da protein had no effect on the membrane binding of the concatemeric DNA replicative intermediate produced during ϕ138 replication. Thus, the 14,000-Da protein selectively affects the membrane association of the ϕ149 concatemeric DNA.

The 1.2-kb DNA sequence is present in classical vibrios. Dot blot hybridization of several strains of classical vibrios with the nick-translated 1.2-kb DNA as a probe showed that all strains of classical vibrios examined hybridized with the 1.2-kb *V. cholerae* biotype el tor DNA fragment. Several *E. coli* strains used as controls did not hybridize with the 1.2-kb DNA sequence. By using one representative strain of each of the classical and el tor biotypes, Southern blot hybridization was carried out by digesting the cell DNA with the enzyme *Eco*RI, using the 1.2-kb DNA as a probe. As expected, the 1.2-kb DNA probe hybridized with the 3.5-kb fragment of *Eco*RI-digested *V. cholerae* biotype el tor DNA (Fig. 4, lane b). Although classical vibrios are reported to be isogenic with the biotype el tor, the 1.2-kb DNA probe hybridized with 2-kb fragments of *Eco*RI-digested *V. cholerae* 569B DNA (Fig. 4, lane c). To examine whether the observed restriction fragment length polymorphism can be used to differentiate classical vibrios from the biotype el tor by using the 1.2-kb DNA as a probe, several strains of classical vibrios and the biotype el tor were digested with the enzyme *Eco*RI and Southern blot hybridization was performed with the 1.2-kb DNA as a probe. The polymorphism was restricted to only the hypertoxigenic strain 569B of classical vibrios. When mild or nontoxigenic strains were used, the 1.2-kb DNA fragment hybridized with 3.5-kb DNA fragments. *V. cholerae* 569B being an atypical hypertoxigenic strain, an examination of other hypertoxigenic strains

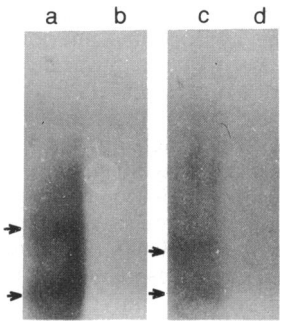

FIG. 5. Northern blot hybridization of total RNA isolated from el tor strain MAK757 (lane a), *V. cholerae* 569B (lane b), *V. cholerae* 569B carrying plasmid pDB120 (lane c), and *E. coli* HB101 (lane d), by using the nick-translated 1.2-kb *V. cholerae* biotype el tor DNA fragment as the probe. The amount (10 μg) of RNA loaded on the gel, as estimated from the intensity of bands after ethidium bromide staining, was similar in all cases. The arrows indicate the positions of the hybridized transcripts.

is necessary before any link between the observed polymorphism and the hypertoxigenic phenotype can be established.

The facts that a DNA sequence homologous to the 1.2-kb *V. cholerae* biotype el tor DNA is present in *V. cholerae* cells and that the 14,000-Da protein was not synthesized in the classical vibrios led us to examine whether this sequence is at all transcribed in the classical vibrios. Total RNA was isolated from cells of the classical strain 569B and the el tor strain MAK757 and from cells of strain 569B carrying plasmid pDB120. The RNA was electrophoresed, transferred to a nitrocellulose filter, and hybridized with the nick-translated 1.2-kb DNA fragment. The RNA from *V. cholerae* biotype el tor cells and *V. cholerae* cells carrying the plasmid pDB120 hybridized with the nick-translated probe (Fig. 5, lanes a and c). The two bands with sizes of 370 and 630 bases correspond to transcripts of the 14,000- and 22,000-Da proteins. RNA from *V. cholerae* cells (Fig. 5, lane b), cells carrying plasmid pSUP106, and *E. coli* cells (Fig. 5, lane d) used as controls did not hybridize with the probe. Thus, sequences homologous to 1.2-kb DNA in *V. cholerae* cells are not transcribed. Nucleotide sequencing of the 1.2-kb DNA showed that these two proteins are synthesized from the opposite strands with a 70-bp overlap at the 3′ end.

Nucleotide sequence of the 1.2-kb DNA. The nucleotide sequence of 1.2-kb *V. cholerae* biotype el tor DNA has been determined. There are two open reading frames of 125 and 205 amino acids that can code for the 14,000- and 22,000-Da proteins. The nucleotide sequence for the 14,000-Da protein is shown in Fig. 6. That the identified open reading frame produces the 14,000-Da protein (Fig. 2C) was confirmed by deleting the first 100 bp of the 1.2-kb DNA fragment and examining the protein produced in maxicell experiments. Following deletion of these base pairs, the 14,000-Da protein could not be detected (Fig. 2B, lane d; Fig. 2C, lane e). The Shine-Dalgarno sequence of the type -G-G-G-A-G-G-G-A-, -A-G-G-, -G-G-A-, or -G-A-G- 5 to 9 nucleotides upstream of the initiator codon is not present in the DNA sequence. The absence of a strong ribosome binding site might be responsible for the low level of expression of the gene product (Fig. 2A, lane c). The amino acid sequence of the 14,000-Da protein derived from the nucleotide sequence of the gene coding for the protein shows that about 50% of the amino acids constituting the protein are hydrophobic in nature. The hydrophobicity of the 14,000-Da protein along its amino acid

```
-20        -10                          10                  20
AATTCGGCATTGGTCAACACGG ATG TTG GGG CAC AAA GGT TAT AAG
                          L   G   H   K   G   Y   K

      30          40          50          60
GAC GCG TTT GAC GCT GTT GTT TCA AGC AGA AAA AGG ATG CGC GCA
  D   A   F   D   A   V   V   S   S   R   K   R   M   R   A

70          80          90          100         110
CCA TAT CCT GCA TAT GGA TCC AAG GCA TGT ATT GTT GCC CAT CTC
  P   Y   P   A   Y   G   S   K   A   C   I   V   A   H   L

      120         130         140         150
CAA TCG GCC CTC CTA ACC CGA GTC GAT AAG GGG GAA GCA TTT TTT
  Q   S   A   L   L   T   R   V   D   K   G   E   A   F   F

160         170         180         190         200
TCA GTG CCC CAC CTT CCG GTG CTA GCA CGA TTC CTG TAC GTA ATA
  S   V   P   H   L   P   V   L   A   R   F   L   Y   V   I

      210         220         230         240
AAC AGA CCC GCG CGT CTG TTC AGA TTG TGC TTT GAG GGC GCG CTG
  N   R   P   A   R   L   F   R   L   C   F   E   G   A   L

250         260         270         280         290
TTC CAT GTT GGC AAA TTG GTG AGA AAA TGG TCA TTC TTA CTG AAG
  F   H   V   G   K   L   V   R   K   W   S   F   L   L   K

      300         310         320         330
GCT CTC ATC ACA GGC ATG CTG CTT GTT GGT CGC ATA AAA ACC AGG
  A   L   I   T   G   M   L   L   V   G   R   I   K   T   R

340         350         360         370         380
CAG ATC CAC TCA AAA ATA CGG CGG GTG GGT GAG CGC TAGCTCGAA
  Q   I   H   S   K   I   R   R   V   G   E   R

     390         400         410         420         430         440
TTTTCTCGACTAATTGTTCCGTGATGTCTAGGCGACTACGGGCAATACCTGCTTTT

          450         460         470         480         490
GGCTTTTCCTCAGCGTTTATCGGCAATCGGTTGTCCGGTCGAAGATTGATGATGGC

  500         510         520
ATCTATCCCGTTGAAATCAGAGAG
```

FIG. 6. Nucleotide and deduced amino acid sequences of the *V. cholerae* biotype el tor gene coding for the 14,000-Da protein.

sequence was evaluated (Fig. 7) by the method of Kyte and Doolittle (19). Several stretches of such hydrophobic amino acids in the primary sequence suggest the possibility of intercalation of part of the protein in the hydrophobic region of the inner membrane.

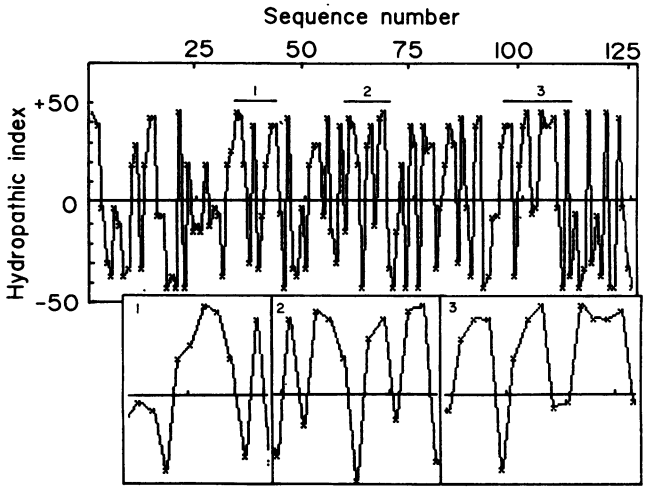

FIG. 7. Hydropathicity profile of the 14,000-Da protein determined according to the method of Kyte and Doolittle (19). Hydrophobic and hydrophilic regions of the protein are displayed above and below the middle line, respectively, of the upper panel. The bars marked 1, 2, and 3 are possible membrane-spanning domains shown in an expanded scale in the lower panel.

DISCUSSION

The results presented in this report show that a 14,000-Da *V. cholerae* biotype el tor protein and presumably a 22,000-Da protein are responsible for conferring to the cells resistance to phage ϕ149 infection. The genes coding for these two proteins are transcribed from the opposite strands of a 1.2-kb *V. cholerae* biotype el tor DNA segment with a 70-bp overlap at the 3′ end. When the 1.2-kb DNA fragment was transformed into the permissive host, the classical biotype, the transformed cells were resistant to phage infection and the concatemeric DNA replicative intermediates produced in these cells following phage ϕ149 infection were unstable and could not be chased to mature phage DNA (Fig. 3A) as was observed for infection in the el tor biotype (9).

Several phage functions responsible for the synthesis and stabilization of concatemeric DNA intermediates produced during replication of circularly permuted or terminally redundant DNA phages have been identified (9, 17, 23, 29). One of the host functions known to influence the stability of concatemeric DNA intermediates during intracellular replication of bacteriophage λ *gam* mutants (14), T4 gene 2 mutants (25), and phages P1 and P2 (39) is the RecBC nuclease. Although the status of the *recBC* genes in either of the biotypes of *V. cholerae* has not been examined in detail, from the studies of DNA repair mechanisms operative in these organisms (13, 26), from sensitivity to mitomycin, and from an examination of homologous recombination (4, 5), it seems likely that both the biotypes lack the RecBC enzyme. Furthermore, both the 14,000- and 22,000-Da proteins are located in the inner membrane and are not likely to function as RecBC nuclease, which, at least in *E. coli*, is a cytoplasmic protein (1). *lit* mutants of *E. coli* are unable to support the late gene expression of phage T4, but neither DNA replication nor DNA encapsidation into phage heads is significantly altered (7). However, while the absence of the Lit protein in *E. coli* inhibits growth of phage T4, the presence of the 14,000- and 22,000-Da *V. cholerae* biotype el tor proteins in the classical biotype hinders phage DNA replication and packaging.

While assigning the roles of the 14,000- and 22,000-Da proteins coded by the 1.2-kb biotype el tor DNA fragment in conferring phage ϕ149 resistance to the el tor biotype, attempts were made to delete amino acids from the N-terminal end of either the 14,000- or 22,000-Da protein, keeping the other intact. Surprisingly, neither of the two proteins could be detected in maxicell experiments with the deleted 1.2-kb DNA segment, in spite of the coding region of at least one of the proteins being kept intact. It is possible that the expression of these two genes is coordinately regulated. This might also explain why the transcripts for both these proteins are missing in the classical vibrios (Fig. 5, lane b).

The genes coding for the 14,000- and 22,000-Da proteins are not transcribed in the classical vibrios (Fig. 5, lane b) although sequences homologous to the 1.2-kb *V. cholerae* biotype el tor DNA segment are present in the classical cells (Fig. 4, lane c). In Southern blot hybridization with the 1.2-kb *V. cholerae* biotype el tor DNA fragment as a probe, the intensities of bands obtained with the DNA of both the biotypes were similar (Fig. 4), indicating that there is no difference in the copy number of the genes coding for the 14,000- and 22,000-Da proteins in the two biotypes. Hence, the failure to detect transcripts in the classical biotype (Fig. 5, lane b) is not due to the fact that the genes are present in lower copy numbers in this biotype than in the biotype el tor.

The hydropathic character of the 14,000-Da protein suggests that it is possible for some parts of this protein to intercalate into the hydrophobic interior of the cell membrane while the rest of it can protrude out into the cytoplasm. The presence of this protein in the inner membrane might hinder the binding of the phage ϕ149 concatemeric DNA replicative intermediate at the functional sites essential for packaging of mature phage DNA into phage heads. This predicted mechanism of inhibition of phage ϕ149 replication in *V. cholerae* biotype el tor cells is unique for group IV choleraphages because replication of circularly permuted, double-stranded DNA phages belonging to other groups can occur normally in both the biotypes (10, 24). The present study shows that the presence of the 14,000- and 22,000-Da proteins in the classical biotype has no effect on phage ϕ138 replication, a phage belonging to group II choleraphages.

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