Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin

Matthew K. Waldor* and John J. Mekalanos

_Vibrio cholerae_, the causative agent of cholera, requires two coordinately regulated factors for full virulence: cholera (CT), a potent enterotoxin, and toxin-coregulated pili (TCP), surface organelles required for intestinal colonization. The structural genes for CT are shown here to be encoded by a filamentous bacteriophage (designated CTXφ), which is related to coliphage M13. The CTXφ genome chromosomally integrated or replicated as a plasmid. CTXφ used TCP as its receptor and infected _V. cholerae_ cells within the gastrointestinal tracts of mice more efficiently than under laboratory conditions. Thus, the emergence of toxigenic _V. cholerae_ involves horizontal gene transfer that may depend on in vivo gene expression.

Bacterial virulence factors such as toxins are often encoded by accessory genetic elements (bacteriophages, plasmids, chromosomal islands, and transposons) (1). These genetic elements are thought to move horizontally as well as vertically through bacterial populations, conferring increased evolutionary fitness to their pathogenic host cells and, thus, to their own nucleic acids. In some cases, these elements can be transmitted between strains under laboratory conditions, but little is known about the natural environments that stimulate genetic exchange between bacterial species and clones.

The genes encoding CT (including ctxAB) reside on the CTX genetic element, a 7- to 9.7-kilobase segment of DNA present on the chromosome of toxigenic strains (frequently in multiple tandemly arrayed copies) but absent in nontoxigenic strains (2). The CTX genetic element has the structure of a compound transposon, with a 4.5-kilobase central core region flanked by one or more copies of a 2.7-kilobase repetitive sequence (RS) (2–4) (Fig. 1). The core of the CTX element is known to carry at least six genes, including ctxAB (encoding the A and B subunits of CT), _zot_ (encoding _zonula occludens toxin_), _cep_ (encoding core-encoded pilin) (4), _ace_ (encoding accessory cholera enterotoxin), and _orfU_ (encoding a product of unknown function) (6). The RS sequences encode at least four open reading frames (rstABCR) (3), which together determine expression of a site-specific recombinations system that catalyzes the integration of plasmids carrying portions of the CTX element into the chromosomes of _V. cholerae_ strains at a particular 17-kilobase target sequence termed attRS1 (4). Kaper and co-workers have examined the transmission of the CTX element into nontoxigenic, live attenuated _V. cholerae_ vaccine candidates and concluded that the element was not self-transmissible (7). We have reexamined transmission of the CTX element and found that under appropriate conditions this element is not only self-transmissible, but indeed replicates as a plasmid and gives rise to extracellular particles (CTXφ) containing single-stranded DNA composed entirely of the CTX element.

**Identification of CTXφ.** Two key experimental details allowed the isolation of the CTXφ. First, the CTX element had to be marked with an antibiotic resistance in a way that did not disrupt any genes other than the ctxAB operon. Second, the transducing particles now carrying the antibiotic resistance determinant had to be plated on recipient cells that had been grown under laboratory conditions that were permissive for expression of TCP pili (8). The expression of TCP pili is dependent on the ToxR regulatory system of _V. cholerae_, which also coordinately regulates the transcription of the CT genes (8, 9). Certain _V. cholerae_ strains of the classical biotype express TCP pili and CT under defined laboratory con-
Strain RV508 is a derivative of classical biotype strain SM69B, which is known to constitutively express CT, TCP pilus, and other ToxR-regulated gene products (10). Initially, we used plate matings to detect the transfer of the CTX element from the V. cholerae El Tor strain SM44 (11) to strain RV508. SM44 carries a single copy of the CTX element, marked by the insertion of a DNA segment encoding resistance to kanamycin (KmR) in place of ctxAB on its chromosome, but lacks plasmids, bacteriophages, and other known transmissible elements (Fig. 1). A low but detectable frequency of KmR recombinants occurred in these plate mating experiments. When we investigated the mechanism of this horizontal gene transfer, we found that cell-free culture supernatant fluids (sterilized by filtration through 0.22-μm filters) derived from KmR RV508 recipients transmitted KmR to a new classical recipient strain (V. cholerae O395) at high frequency. These results indicated that the CTX element was apparently capable of being transduced by a viral-like particle in the supernatant fluids. The transducing particle was heat- and chloroform-labile but resistant to deoxyribonuclease (DNase), ribonuclease, and pronase, which suggests that it was probably a bacteriophage.

Preparation of plasmid DNA from initial RV508 KmR recipients, as well as from O395 KmR transductants, provided evidence that a replicative form of the CTX genetic element was responsible for the generation of high titers of KmR-transducing particles. O395 KmR recipients carry a plasmid that has been shown by restriction mapping, Southern hybridization, and DNA sequencing to correspond exactly to a circularized portion of the chromosomal CTX element of donor strain SM44 (Fig. 1). This plasmid, pCTX-Km, is apparently the replicative form (RF) of the CTX-Km DNA. Its structure appears to have arisen by excision from the chromosome of SM44 by either homologous recombination between RS2b and RS1c direct repeats or, more likely, by a site-specific recombination reaction between the two attRSl sites indicated in Fig. 1. DNA sequencing using primers corresponding to the cep and ctxB coding sequence demonstrated that pCTX-Km is composed of a circularized core region separated by an RS2 sequence with a single attRSl site located at its novel junction and downstream from the ctxB gene (12).

The RF form of the CTXΦ was easily transformable into several other strains of V. cholerae by electroporation and selection for KmR. These included strains of V. cholerae that did not act as recipients when exposed to KmR-transducing particles (for example, TCP mutants). In some recipient strains such as VO7 (13), pCTX-Km integrated into the chromosome at a resident attRSl site, whereas in others (for example, strains of the classical biotype or El Tor strains carrying deletions of all attRSl sites), pCTX-Km replicated as a plasmid (Fig. 1). Whenever this occurred, the corresponding strain produced large amounts of KmR-transducing particles in supernatant fluids.

To investigate the morphology of these phage particles, we purified CTX-KmΦ virions from the culture supernatants of strain Peru-15 containing pCTX-Km. This El Tor strain does not produce flagella and does not harbor any known bacteriophages (14). Peru-15 (pCTX-Km) and control Peru-15 cultures were grown in parallel under conditions where no TCP pilus are expressed. Transduction was used to monitor the concentration and purification of CTX-KmΦ particles from culture supernatant fluids of Peru-15 (pCTX-Km) by steps that included precipitation with hexametaphosphate, ultracentrifugation, and banding on isopycnic sucrose gradients. In parallel, the corresponding fractions derived from the Peru-15 control culture were also prepared. Concentrated preparations containing 10⁶ KmR-transducing particles per milliliter were examined by electron microscopy and shown to contain numerous curved filaments with diameters of approximately 7 nm, which frequently "bundled" together (Fig. 2A). Immunoanalysis confirmed that these preparations lacked detectable TCP pilus, which are known to have similar morphology (8). Furthermore, corresponding preparations from the Peru-15 control culture contained similar background levels of membrane "blebs" but lacked detectable filaments of comparable length, concentration, or substructure (Fig. 2B).

Nucleic acid extracted from the partially purified particles obtained from Peru-15 (pCTX-Km) culture supernatant fluids was found to be composed of single-stranded DNA that corresponds to the coding sequence—that is, the (+) strand—of the CTX element. This was demonstrated by finding that DNA extracted from the purified particles served as a sequence template only with a primer derived from the (−) strand sequence and not from the (+) strand sequence of the CTX element (12). Other known filamentous bacteriophages also contain single (+) strand DNA (15). The particle-derived nucleic acid was resistant to restriction endonuclease digestion but sensitive to DNase I digestion (16). These data strongly suggest that the filamentous structures that copurified with KmR-transducing activity correspond to filamentous CTXΦ particles.

Strains carrying a chromosomally integrated copy of the CTX element typically produced no KmR-transducing particles in supernatant fluids (for example, SM44 in Table 1). However, like many temperate bacteriophages, CTXΦ was able to be induced by DNA-damaging agents. For exam-

![Fig. 2. (A) Electron microscopy of partially purified CTXΦ particles purified from Peru-15 (pCTX-Km) cultures. (B) Mock-purified preparations from control cultures of Peru-15. Both preparations, ×100,000.](image-url)
ple, growth of SM44 in the presence of mitomycin C led to the production of more than 10^5 CTX-KmΦ particles per milliliter in supernatant fluids (Table 1). Transduction of strain O395 with such mitomycin C–induced particles gave rise to Km^R recipient strains that harbored plasmids identical to pCTX-Km.

**Requirement of zot and orfU gene products for CTX phage morphogenesis.** These data suggest that pCTX-Km encodes gene products that probably participate in replication and morphogenesis of CTX-KmΦ–transducing phages in *V. cholerae*. In order to identify one or more gene products required for particle formation, we introduced mutations into pCTX-Km at two unique restriction enzyme sites (Fig. 1). Plasmid pCTX-Km was digested separately with the enzymes Mlu I and Sph I, which each cut once at internal sites within the zot and orfU genes, respectively. The overhanging 5' and 3' termini exposed by this digestion were modified by T4 polymerase, then were ligated to produce 4–base pair insertion or deletion mutations in zot and orfU, respectively.

Plasmids carrying these two mutations were recovered by electroporation into the El Tor strain Bahl-2 (4) and designated pMW101 and pMW102 (Fig. 1). When pCTX-Km was introduced into Bahl-2, this strain produced 2.5 × 10^4 transducing particles per milliliter of culture fluid (Table 1). In contrast, repeated attempts failed to detect any Km^R–transducing particles in supernatant fluids of Bahl-2 carrying either pMW101 or pMW102, although these plasmids replicated to at least the same copy number as pCTX-Km. All genes located in the core of the CTX element are oriented in the same direction as ctxAB, although little more is known about their transcriptional organization. Thus, although it is possible that the mutation in orfU could be polar on expression of genes downstream—for example, expression of ace or zot—it seems unlikely that the phenotype of the pMW101 zot mutation could be explained by a polar effect. The only genes that are immediately downstream of zot are the ctxAB genes, and these have been deleted and replaced by a Km^R cassette in pCTX-Km. We therefore conclude that both zot and orfU correspond to genes involved in CTXΦ morphogenesis.

**Amino acid sequences of CTX element gene products.** The roles of zot and orfU in CTXΦ morphogenesis prompted us to examine more closely the deduced amino acid sequences of these and other gene products encoded by the core of the CTX element. As noted by Koonin (17), the zot gene product is homologous to a family of proteins that include plasmid and filamentous phage–encoded homologs displaying a nucleoside triphosphate–binding sequence motif. This family of proteins includes the gene I product of male (F^+)–specific coliphage M13 (15) and the corresponding gene I products of several other filamentous bacteriophages of *Escherichia coli*, *Pseudomonas*, and *Xanthomonas* (17). The gene I product is an inner membrane protein required for assembly of filamentous phage (18). Given the role that the zot gene product has in CTXΦ phage morphogenesis and its homology to Gpl (16), it is apparent that the biological activity previously designated "zonula occuldens toxin" (5) is probably not directly associated with the zot gene product unless its protein has dual functions.

The first gene in the core, cep, is predicted to encode a 47-amino acid polypeptide after cleavage of its NH_2-terminal hydrophobic signal sequence (4). This short polypeptide very closely matches the major M13 virion capsid protein (the gene VIII product) in size and distribution of charged and nonpolar amino acids (Fig. 3). Thus, we propose that the cep gene product corresponds to the virion capsid protein of CTXΦ. The cep gene product, core-encoded pilin (4), was so named because of its homology to the mini-pilin subunit of the flexible pilus of *Aeromonas hydrophila* (encoded by fp) (19). Clearly, fp and cep belong to the gene VIII family (Fig. 3), which suggests that the plasmid-encoded, *A. hydrophila* flexible pilus may also be a filamentous phage particle rather than a pilus as originally proposed (19).

It has been observed that there is conservation in the genomic organization of morphogenesis genes of filamentous phages from different bacterial species (20). For example, in the case of coliphage M13, gene VIII, encoding the major capsid protein, is followed by a long open reading frame (ORF) corresponding to gene III, then a short ORF (gene VI) followed by another long ORF (gene I) (18). This genomic organization appears to have been preserved for the morphogenesis genes of CTXΦ as well (Fig. 3). Thus, the ORF following cep, orfU, closely matches the size of gene III of M13; ace, the next ORF, is approximately the same size as gene VI; and zot is comparable in size to gene I (Fig. 3). As noted earlier, the zot gene product is homologous to the gene I product of M13 (17). The orfU gene product does not display significant sequence similarity to the corresponding gene III products of other filamentous phages. However, there is little homology between the known gene III products of most filamentous bacteriophages (except for the related coliphages M13, fd, and ike) (16, 18). Perhaps gene III products are so different because they participate in receptor binding and the pilus receptors for different filamentous phages vary widely between bacterial species. In contrast, alignment of the ace gene product with the corresponding gene VI homolog of *Pseudomonas* filamentous phage P1 (ORF141) revealed approximately 61% similarity and 27% identity (21). Given that the gene VI product of coliphage M13 and probably other filamentous phages is a small hydrophobic protein that assembles into the virion particle (16), these observations may cast doubt on the proposed role of the ace gene product as an accessory cholera enterotoxin (6).
when grown at pH 6.5 (a ToxR-activated condition that is permissive for TCP expression) but were not transduced when grown at pH 8.5 (Table 2). An isogenic mutant of classical strain O395 [which is defective in the production of TCP pili (SC262) by virtue of a nonpolar point mutation in the tcpA gene (22)] was completely resistant to transduction by KmR CTX\(\Phi\) particles (Table 2). Other TCP-defective mutants of O395, including the toxR mutant JMJ43 (23) and tcpA mutant TCP2 (23), were similarly defective as recipient strains, whereas other strains carrying point mutations in tcpA that do not affect expression of TCP pili (SC253 and SC254) were active as phage recipients. When either SC262 or JJM43 was transformed with pCTX-Km by electroporation, both strains readily produced high titers of KmR-transducing particles, which indicated that tcpA mutations do not interfere with replication or morphogenesis of CTX\(\Phi\). Finally, CTX\(\Phi\) adsorbs efficiently to TCP pilated cells of V. cholerae but not to nonpiliated cells; antisera directed against TCP blocks phage infection, whereas control antiserum directed against CT does not. Thus, the ToxR-regulated TCP pili of V. cholerae apparently are the functional receptors for CTX\(\Phi\).

Enhancement of transmission of CTX\(\Phi\) by in vivo gene expression. Because an intestinal colonization factor (TCP) is the receptor for CTX\(\Phi\), we investigated whether transduction of CTX\(\Phi\) could be demonstrated within the gastrointestinal tract. Accordingly, bacterial cells of donor strain RV508 (pCTX-Km) and recipient strain Bah-2 or O395 were grown under ToxR-repressed conditions, washed extensively, diluted to a final cell density of \(2 \times 10^6\) cells/ml, and then mixed together. Approximately 50 \(\mu\)l of the mixed inocula was immediately administered by gastrointestinal inoculation to a group of CD-1 suckling mice (8). The same mixed inocula were spotted onto Luria agar plates or added to Luria broth and then incubated for 24 hours under various laboratory conditions, including those known to be permissive for expression of TCP pili. After 24 hours, the small intestines of infected mice were removed and plated on selective media to establish the percentage of the recipient strain that had been transduced to Km\(^R\) in vivo. The various in vitro cultures were analyzed in the same way.

As shown in Fig. 4, for the El Tor recipient strain Bah-2, approximately 0.5% of the cells grown in vivo were transduced to Km\(^R\) after intra-intestinal co-cultivation with the CTX\(\Phi\) donor strain. This is at least six orders of magnitude greater than the fraction of Bah-2 cells that were transduced in vitro regardless of the growth conditions. When the classical strain O395 was used as the recipient of the CTX\(\Phi\) under the same in vivo conditions, we observed that a large percentage of recipient cells (approximately 50%) had acquired the CTX\(\Phi\) (Fig. 4).

These data indicate that CTX\(\Phi\) has evolved to be a highly efficient transmissible agent within the gastrointestinal environment and provide strong genetic evidence for expression of TCP pili in vivo by both El Tor and classical strains. The classical strain O395 was also highly transduced under all in vitro conditions tested. This result is consistent with previous observations, which suggests that classical strains produce TCP pili more readily in vitro than do El Tor strains (10, 13, 24). These data further suggest that classical strains of V. cholerae may be less suitable as live attenuated cholera vaccines than El Tor strains, given their elevated potential for re-acquisition of functional CT genes by means of CTX\(\Phi\) transduction (25).

Conclusions. The results presented here provide a new mechanism by which horizontal gene transfer functions in the emergence of pathogenic microbes. Specifically, our results suggest that filamentous bacteriophages may represent a previously unrecognized family of viruses participating in lysogenic conversion of bacterial pathogens. filamentous viruses appear well suited for the transfer of genes between bacterial clones and for lysogenic conversion. filamentous phage morphogenesis is not severely hampered by the size of heterologous DNA inserted into their genomes, and the production of filamentous phage particles usually does not have a highly deleterious effect on bacterial host cells. The pili that filamentous phages use as their receptors are ubiquitous and conserved structures that are often encoded by transmissible elements.

Table 2. CTX-Km\(^R\) transduction of V. cholerae strains, which vary in expression of TCP pili. All strains are derivatives of the classical V. cholerae strain O395 and were grown overnight under in vitro growth conditions (Luria broth, pH 6.5, with aeration (10)) to maximize expression of TcpA unless otherwise indicated. The CTX-Km\(^R\) transduction efficiency (transductants/ml) was assayed on the indicated strains by determination of the number of Km\(^R\) colony-forming units after a 40-min incubation of the cultures with serially diluted CTX-Km\(^R\).

<table>
<thead>
<tr>
<th>Strain (reference)</th>
<th>Relevant characteristic or growth condition</th>
<th>CTX-Km(^R) transduction efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>O395 (10)</td>
<td>Wild-type classical strain grown at pH 6.5 (ToxR activated)</td>
<td>(3.6 \times 10^7)</td>
</tr>
<tr>
<td>O395</td>
<td>Wild-type classical strain grown at pH 8.5 (ToxR repressed)</td>
<td>0</td>
</tr>
<tr>
<td>JMJ43 (8)</td>
<td>Deletion of toxR</td>
<td>0</td>
</tr>
<tr>
<td>TCP2 (23)</td>
<td>Deletion of tcpA</td>
<td>0</td>
</tr>
<tr>
<td>SC253 (22)</td>
<td>V8M (Val(^{20})→Met) substitution in TcpA (TCP pili (^+))</td>
<td>(4.0 \times 10^7)</td>
</tr>
<tr>
<td>SC254 (22)</td>
<td>V20T (Val(^{20})→Thr) substitution in TcpA (TCP pili (^+))</td>
<td>(6.0 \times 10^6)</td>
</tr>
<tr>
<td>SC262 (22)</td>
<td>G(-1)S substitution in TcpA (TCP pili (^-))</td>
<td>0</td>
</tr>
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</table>
themselves. The fact that pili often are virulence and colonization factors in Gram-negative organisms supports our conclusion that many DNA transfer events mediated by filamentous phages may occur on host mucosal surfaces.

Our results also emphasize the co-evolution of genetic elements mediating the transfer of virulence genes with the pathogenic bacterial species they infect. Thus, a virulence factor (TCP) is the receptor for a bacteriophage encoding another virulence factor (CT), both of which are coordinately regulated by the same virulence regulatory gene (toxR). In this case, the natural habitat of both phage and pathogen is the gastrointestinal tract. It is apparent that this host compartment provides the necessary environmental signals required for the expression of essential gene products mediating interactions between all three participants, namely, bacterium, phage, and mammalian host.

REFERENCES AND NOTES


12. The primers used to determine the sequence of the (+) DNA strand upstream of cap were 5'-GGGAAA-AGACAAAGGAAAAC-3' and 5'-AGAACAAGACGAG-AAAGG-3'. The primers used to determine the DNA sequence of the (−) DNA strand downstream of ctxB were 5'-GATGATCCTC-3' and 5'-CGATGATGAAAAACG-3'. Dye terminator cycle sequencing with a kit using AmpliTaq DNA polymerase FS (Perkin-Elmer) was carried out according to the manufacturer's recommendations. DNA sequences were determined with an ABI 373A DNA sequencer.


21. Homology between the ace and orf141 gene products was detected with the BLASTP program [S. Altschul et al., J. Mol. Biol. 215, 403 (1990)] run on the National Center for Biotechnology Information server with the nonredundant protein databases as of 18 April 1996. The percent similarity and identity between the ace gene product and the orf141 product was determined with BESTFIT (Program Manual for Wisconsin Package, version 6.0, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI 53711, USA).


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Minimal Energy Requirements in Communication
Rolf Landauer

The literature describing the energy needs for a communications channel has been dominated by analyses of linear electromagnetic transmission, often without awareness that this is a special case. This case leads to the conclusion that an amount of energy equal to KT ln 2, where KT is the thermal noise per unit bandwidth, is needed to transmit a bit, and more if quantized channels are used with photon energies hv > KT. Alternative communication methods are proposed to show that there is no unavoidable minimal energy requirement per transmitted bit. These methods are invoked as part of an analysis of ultimate limits and not as practical procedures.

Information is inevitably tied to a physical representation, such as a mark on a paper, a hole in a punched card, an electron spin pointing up or down, or a charge present or absent on a capacitor. This representation leads us to ask whether the laws of physics restrict the handling of information and in particular whether there are minimal energy dissipation requirements associated with information handling. The subject has three distinct but interrelated branches dealing, respectively, with the measurement process, the communications channel, and computation. Concern with the measurement process can be dated back to Maxwell's demon (1). In the development of that subject, the notion that information is physical was introduced by Szilard (2), although it was not widely accepted for many decades. Concern with the communications channel became a subject of intense concern after Shannon's work (3). It is the newest of the three branches, computation, that has caused us to reexamine the perceived wisdom in the two earlier areas (1, 4–8). It was pointed out long ago (9) that the steps in the computational process that inevitably demand an energy consumption with a known and specifiable lower bound are those that discard information. It was also understood long ago (9) that operations that do throw away information, such as the logical AND and the logical OR, can be imbedded in larger operations that perform a logical 1:1 mapping and do not discard information. Nevertheless, a real understanding of what is now called reversible computation came from the work of Bennett (10, 11), who showed that computation can always be conducted through a series of logical 1:1 mappings. Bennett furthermore showed that physical implementations exist that allow this mapping to be utilized to perform computation with arbitrarily little dissipation per step, if done sufficiently slowly. Bennett's discussion envisioned classical machinery with viscous frictional forces proportional to the velocity of motion. It is these forces that can be made as small as desired, through slow computation.

The notion of logically reversible operations, which do not discard information, provides the unifying thread between the three fields of measurement, communications, and computation. In the measurement process, transfer of information from the system to be measured to the meter does not require any minimal and unavoidable diss-