

Lysogenic Conversion by a Filamentous Phage Encoding Cholera Toxin

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Vibrio cholerae, the causative agent of cholera, requires two coordinately regulated factors for full virulence: cholera toxin (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 CTX Φ), which is related to coliphage M13. The CTX Φ genome chromosomally integrated or replicated as a plasmid. CTX Φ 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.

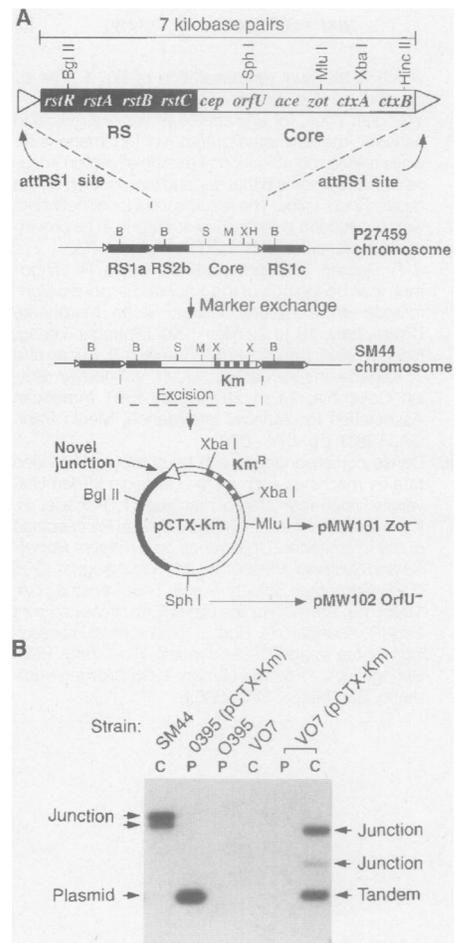
The genes encoding CT (including *ctxAB*) reside on the CTX genetic element, a 7- to 9.7-kb segment of DNA present on the chromosome of toxigenic strains (frequently in multiple tandemly arrayed copies) but absent in nontoxigenic strains (2). The CTX genetic element has the structure of a compound transposon, with a 4.5-kb central core region flanked by one or more copies of a 2.7-kb 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) (5), *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 recom-

bination system that catalyzes the integration of plasmids carrying portions of the CTX element into the chromosomes of nontoxigenic *V. cholerae* strains at a particular 17-bp target sequence termed *attRS1* (4). Kaper and co-workers have examined

Fig. 1. Structure of CTX element DNA and analysis of CTX Φ transduction and transformation events. **(A)** The CTX element is composed of three distinct subrepeats: the 4.5-kb core (open boxes), 2.5- to 2.7-kb RS sequences (shaded), and the 17- to 18-bp *attRS1* sites (open triangles) (4). The 10 known genes residing within the core and RS repeats are indicated within the corresponding boxes. Selected restriction enzyme sites (designated by the full name or first letter) are indicated over the genes in which they are located. The chromosomal CTX element of *V. cholerae* strain P27459 was modified by marker exchange, deleting *ctxAB* and substituting a *Km^R* cassette (11). Plasmid pCTX-Km (the replicative form of CTX-Km DNA) corresponds to the expected product of a chromosomal excision event mediated by recombination (dashed lines) between the two *attRS1* sites indicated in strain SM44. The unique *Mlu* I and *Sph* I sites of pCTX-Km were modified to give *Zot⁻* and *OrfU⁻* derivatives pMW101 and pMW102, respectively. **(B)** Southern blot analysis of CTX-Km Φ donor and recipient strains. Chromosomal (C) or plasmid (P) DNA prepared from donor strain SM44 or the indicated recipient strains was digested with *Mlu* I and analyzed after electrophoresis with a CTX element-specific gene probe (26). Plasmid DNA prepared from recipient strain O395 lacks all CTX element hybridizing sequences. In contrast, a typical CTX-Km Φ transductant, O395(pCTX-Km), displays a prominent plasmid band corresponding to the replicative form of the CTX-Km Φ DNA. Nontoxigenic strain VO7 lacks chromosomal CTX element hybridizing sequences before transformation with pCTX-Km plasmid DNA. However, a typical *Km^R* transformant, VO7(pCTX-Km), carries two tandemly inserted copies of pCTX-Km on its chromosome and no detectable plasmid DNA.

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 (CTX Φ) containing single-stranded DNA composed entirely of the CTX element.

Identification of CTX Φ . Two key experimental details allowed the isolation of the CTX Φ . 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-



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ditions (8, 10). In contrast, it is more difficult to define laboratory conditions that lead to detectable TCP expression by strains of the El Tor biotype, even though these strains still require TCP to colonize the intestine (10).

Strain RV508 is a derivative of classical biotype strain 569B, which is known to constitutively express CT, TCP pili, 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 (Km^R) in place of *ctxAB* on its chromosome, but lacks plasmids, bacteriophages, and other known transmissible elements (Fig. 1). A low but detectable frequency of Km^R 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- μ m filters) derived from Km^R RV508 recipients transmitted Km^R 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 viruslike 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 Km^R recipients, as well as from O395 Km^R transductants, provided evidence that a replicative form of the CTX genetic element was responsible for the generation of high titers of Km^R -transducing particles. O395 Km^R 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 Φ 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 attRS1 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 attRS1 site located at its novel junction and downstream from the *ctxB* gene (12).

The RF form of the CTX Φ was easily

transformable into several other strains of *V. cholerae* by electroporation and selection for Km^R . These included strains of *V. cholerae* that did not act as recipients when exposed to Km^R -transducing particles (for example, TCP mutants). In some recipient strains such as VO7 (13), pCTX-Km integrated into the chromosome at a resident attRS1 site, whereas in others (for example, strains of the classical biotype or El Tor strains carrying deletions of all attRS1 sites), pCTX-Km replicated as a plasmid (Fig. 1). Whenever this occurred, the corresponding strain produced large amounts of Km^R -transducing particles in supernatant fluids.

To investigate the morphology of these phage particles, we purified CTX-Km Φ 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 pili are expressed. Transduction was used to monitor the concentration and purification of CTX-Km Φ 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 10^9 Km^R -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). Immunoblot analysis confirmed that these preparations lacked detectable TCP pili, 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 Km^R -transducing activity correspond to filamentous CTX Φ particles.

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

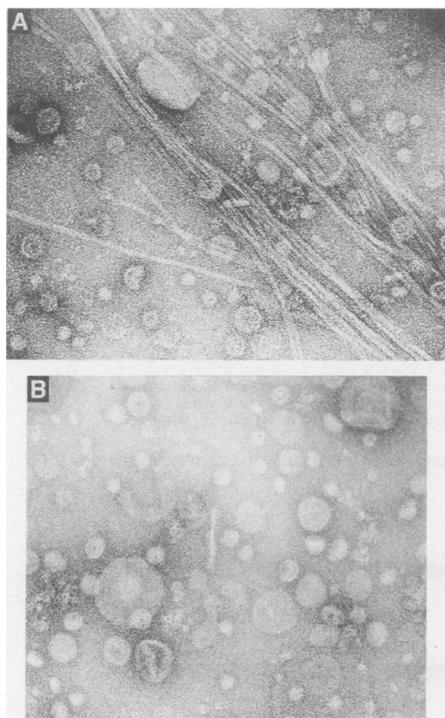


Fig. 2. (A) Electron microscopy of partially purified CTX Φ particles purified from Peru-15 (pCTX-Km) cultures. (B) Mock-purified preparations from control cultures of Peru-15. Both preparations, $\times 100,000$.

Table 1. Production of CTX-Km Φ by El Tor lysogens or by strains carrying wild-type or mutant pCTX-Km RF forms. The indicated strains were grown overnight at 30°C with aeration in Luria broth containing Km (50 μ g/ml) for all cultures except for Bah-2. Mitomycin C was added at a concentration of 20 ng/ml. We determined the titer of the CTX-Km-transducing particles in the supernatants by incubating serially diluted filtered supernatants with strain O395 (TCP pili⁺) and then determining the number of Km^R transductants after 30 min.

Strain	Titer of CTX-Km Φ in supernatant (particles/ml)
SM44 (P27459 <i>ctx::km</i>)	0
SM44 + mitomycin C	4.2×10^5
Bah-2 (E7946 $\Delta attRS1$)	0
Bah-2 pCTX-Km	2.5×10^7
Bah-2 pMW101	0
Bah-2 pMW102	0

ple, growth of SM44 in the presence of mitomycin C led to the production of more than 10^5 CTX-Km Φ 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-Km Φ -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 Bah-2 (4) and designated pMW101 and pMW102 (Fig. 1). When pCTX-Km was introduced into Bah-2, this strain produced 2.5×10^7 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 Bah-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 CTX Φ morphogenesis.

Amino acid sequences of CTX element gene products. The roles of *zot* and *orfU* in CTX Φ 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 CTX Φ phage morphogenesis and its homology to GpI (16), it is apparent that the biological activity previously designated “zonula occludens toxin” (5) is probably not directly associated with the *zot* gene product unless its product has dual functions.

The first gene in the core, *cep*, is predicted to encode a 47–amino acid polypeptide after cleavage of its NH₂-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 CTX Φ . 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 *fxp*) (19). Clearly, *fxp* 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 CTX Φ 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 lke) (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 Pf1 (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).

TCP pili as receptors for CTX Φ . The fact that several filamentous bacteriophages use pili as receptors for infection of host bacterial cells prompted us to evaluate the possibility that TCP pili play an analogous role for CTX Φ . O395 recipient cells were readily transduced by CTX Φ

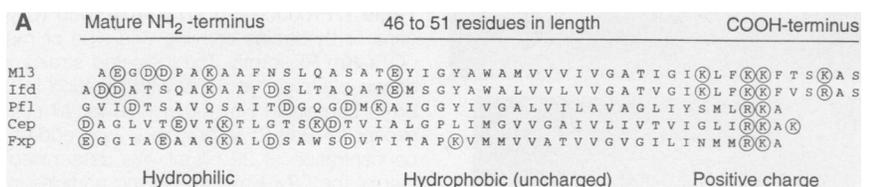
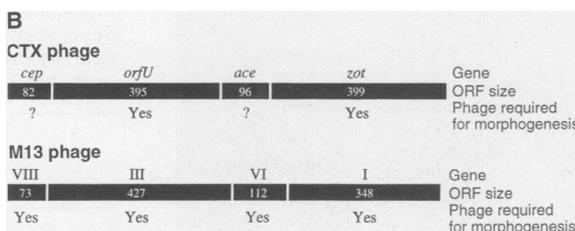


Fig. 3. Gene products of filamentous bacteriophages. **(A)** Amino acid sequence alignment of the predicted mature Cep gene product from *V. cholerae* (4) and Fxp gene product of *Aeromonas hydrophila* (19) with the processed capsid proteins (gene VIII products) of *Escherichia coli* phages M13 and Ifd (15) and of *Pseudo-*

monas aeruginosa phage Pf1 (20). All charged amino acid residues are circled. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. **(B)** Relative position and size of genes involved in the morphogenesis of CTX phage and coliphage M13. Gene size is indicated schematically by the filled bars and indicated number of codons.



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 Km^R CTX Φ particles (Table 2). Other TCP-defective mutants of O395, including the *toxR* mutant JJM43 (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 Km^R-transducing particles, which indicated that *tcpA* mutations do not interfere with replication or morphogenesis of CTX Φ . Finally, CTX Φ adsorbs efficiently to TCP piliated 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 Φ .

Enhancement of transmission of CTX Φ by in vivo gene expression. Because an intestinal colonization factor (TCP) is the receptor for CTX Φ , we investigated whether transduction of CTX Φ could be demonstrated within the gastrointestinal tract. Accordingly, bacterial cells of donor strain RV508 (pCTX-Km) and recipient strains Bah-2 or O395 were grown under ToxR-repressed conditions, washed extensively, diluted to a final cell density of 2×10^6 cells/ml, and then mixed together. Approximately 50 μ 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 Φ 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 Φ under the same in vivo conditions, we observed that a large percentage of recipient cells (approximately 50%) had acquired the CTX Φ (Fig. 4).

These data indicate that CTX Φ 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 Φ 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 filamen-

tous 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

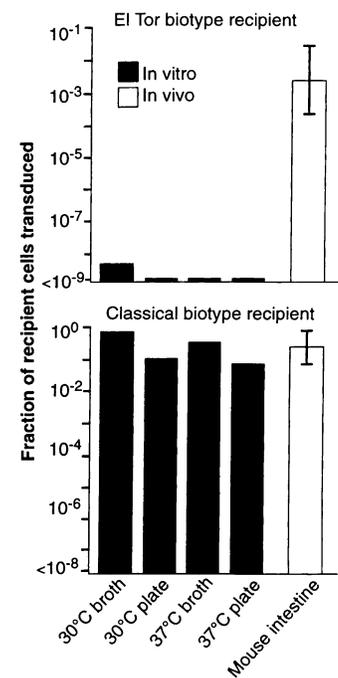


Fig. 4. Transduction efficiency of recipient strains in vitro and in vivo. Washed cells of donor strain RV508 (pCTX-Km) were mixed with either of two recipient strains (Bah-2 or O395) that had been grown in Luria broth [pH 8.5; nonpermissive conditions for expression of TCP pili (10)]. Approximately 10^5 total cells of each mixture was inoculated into 5 ml of Luria broth or onto the surface of a Luria agar plate and then incubated at the indicated temperature for 24 hours (the in vitro conditions). The same mixtures were also used to gastrointestinally inoculate groups of 5-day-old CD-1 suckling mice. There were at least five mice per group. After 24 hours, the mice were killed and intestinal homogenates prepared (the in vivo condition). All in vitro growth cultures and intestinal homogenates were then titered for Km^R (transduced) and sensitive (nontransduced) recipient cells; streptomycin was used to counterselect the RV508 donor cells. The ratio of the total number of transduced cells divided by the total number of recipient cells recovered equals the fraction of the recipient cells transduced. The median and range are shown on the bar graphs depicting the in vivo transduction experiments.

Table 2. CTX-Km Φ 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 TcxA unless otherwise indicated. The CTX-Km Φ 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 Φ .

Strain (reference)	Relevant characteristic or growth condition	CTX-Km Φ transduction efficiency
O395 (10)	Wild-type classical strain grown at pH 6.5 (ToxR activated)	3.6×10^7
O395	Wild-type classical strain grown at pH 8.5 (ToxR repressed)	0
JJM43 (8)	Deletion of <i>toxR</i>	0
TCP2 (23)	Deletion of <i>tcpA</i>	0
SC253 (22)	V9M (Val ⁹ →Met) substitution in TcxA (TCP pili ⁺)	4.0×10^7
SC254 (22)	V20T (Val ²⁰ →Thr) substitution in TcxA (TCP pili ⁺)	6.0×10^6
SC262 (22)	G(-1)S substitution in TcxA (TCP pili ⁻)	0

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.

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12. The primers used to determine the sequence of the (+) DNA strand upstream of *cep* were 5'-CGGAAA-ACAGCAAGAAAAC-3' and 5'-AGAACAAGACGAG-AACGG-3'. The primers used to determine the DNA sequence of the (-) DNA strand downstream of *ctxB* were 5'-AATAATAAAACGCCTCAT-3' and 5'-CGAT-GATGAGAAGCAACC-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.
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Minimal Energy Requirements in Communication

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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 $h\nu > kT$. 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 dissi-

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