MICROBIOLOGICAL REVIEWS, June 1987, p. 285–298
0146-0749/87/020285-14$02.00/0

Vol. 51, No. 2

Genetics of *Vibrio cholerae* and Its Bacteriophages

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INTRODUCTION

Vibrio cholerae strains of the O1 serotype are subdivided into two biotypes, classical and El Tor (24, 40, 113), both of which are capable of causing human cholera. These two biotypes differ in several characteristics. El Tor strains were originally identified as being hemolytic, whereas classical strains were not. However, further differences have subsequently been identified. El Tor strains produce a cell-associated hemagglutinin for chicken erythrocytes and are resistant to polymyxin B and to Mukerjee's group IV bacteriophages (83).

There have been seven recorded cholera pandemics produced by these two biotypes. Since 1960, epidemics of cholera have been caused predominantly by El Tor strains, whereas previous epidemics were due mainly to classical strains (25, 32). Cholera is still one of the most prevalent diseases of humans.

Until the last few years, *V. cholerae* has been relatively neglected in terms of genetic analysis. Most studies have related to the production of cholera toxin. However, there is now interest in other determinants of pathogenicity, particularly because *V. cholerae* is unusual in that it is capable of excreting a broad range of proteins.

GENETICS OF *V. CHOLERAE*

Plasmids in *V. cholerae*

A conjugal system in *V. cholerae* was discovered by Bhaskaran in 1958 (11). Fertile host cells contain a plasmid sex factor, P (9), and are referred to as P^+ cells. Such donor cells are capable of derepressed self-transfer of P to P^- recipients (96). Thus, the P sex factor is similar to the fertility factor F of *Escherichia coli* K-12. However, unlike the F factor, P cannot integrate stably into the host chromosome to form strains capable of high frequency of recombination (Hfr). This has been suggested to be due to the significant difference in the overall guanine-plus-cytosine composition of P deoxyribonucleic acid (DNA) relative to *V. cholerae* chromosomal DNA (42 and 48%, respectively) (21). This is in contrast to F, which has an average base composition close to that of its *E. coli* host. However, it would seem more likely that the difference is due to a lack of common insertion sequences in P and the *V. cholerae* chromosome.

A striking feature of the cholera conjugal system is the formation of lacunae (11, 124). These plaquelike clearings form when P^+ cells are plated on a lawn of P^- cells. They are probably sites of active mating between P^+ and P^- cells and represent zones of inhibited growth caused by lethal zygosis (96) rather than plaques caused by bacteriophages or bacteriocins as was first thought (10, 11).

The size of P has been reported by several workers (21, 54) and varies between 47×10^6 and 80×10^6 daltons. Precise restriction analysis, as well as electron microscope contour measurements, has recently demonstrated a length of 68 kilobases (kb) or 45×10^6 daltons (Fig. 1; E. J. Bartowsky, G. Morelli, M. Kamke, and P. A. Manning, Plasmid, in press).

Analysis of the P factors from a number of strains has shown that their structures are similar, but evidence of DNA inversions can be detected (E. J. Bartowsky and P. A. Manning, unpublished data).

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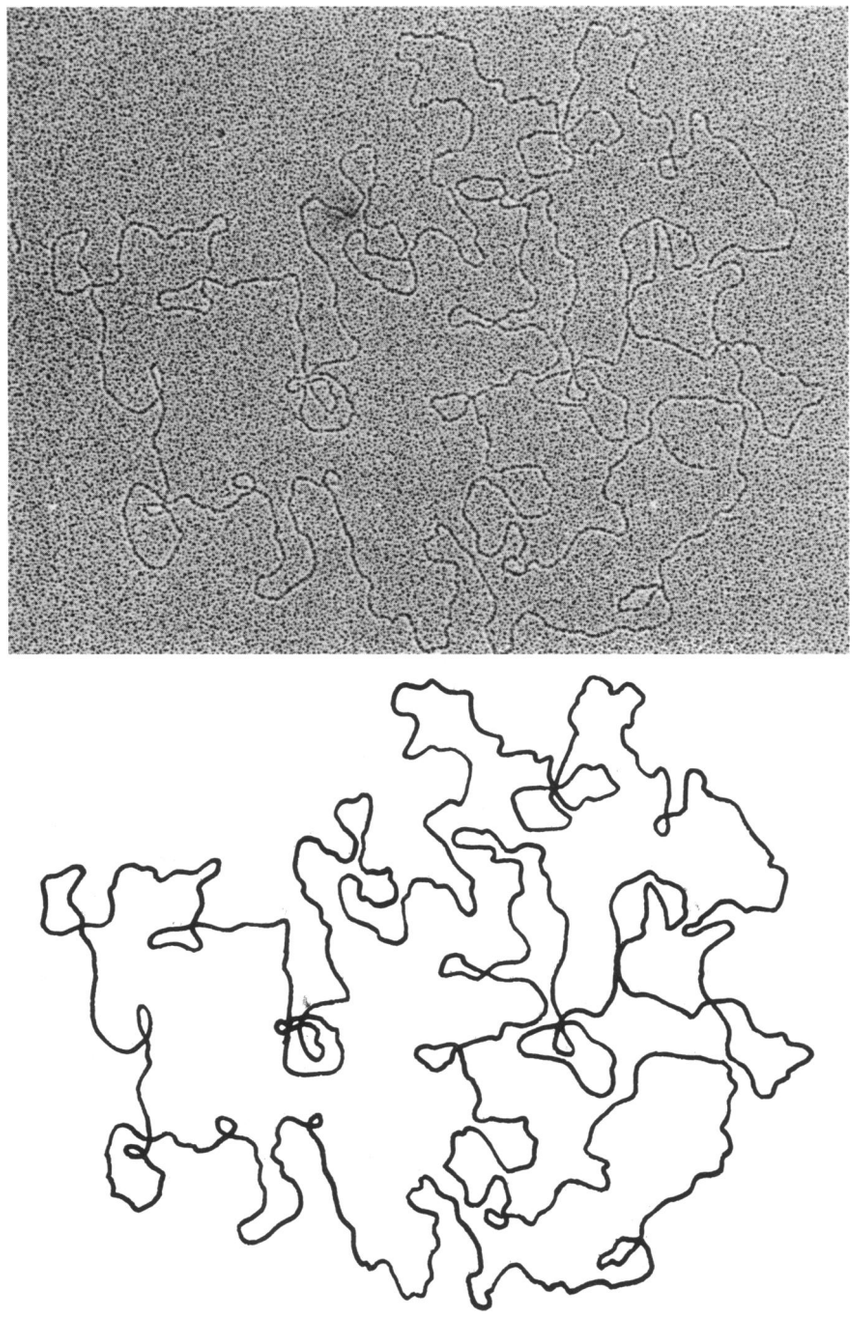


FIG. 1. Electron microscope analysis of the P sex factor of *V. cholerae* derived from strain O162 (courtesy of E. J. Bartowsky and G Morelli).

No simple phenotypic markers have been identified on P. However, P has been implicated in the suppression of the pathogenicity of *V. cholerae* (115). This is thought to be due to a decreased production of cholera toxin. The original observations have been confirmed by using temperature-sensitive derivatives of P, which, when present, result in about a 10-fold reduction in cholera toxin production (63).

The Bhaskaran strain 162 has been shown to contain two plasmids besides P (21, 54; Bartowsky et al., in press). These plasmids are 4.7 and 34 kb in size, and neither has any known function. Both have been cloned, and their presence in *V. cholerae* strains is being examined.

Few plasmids have been reported to be present in *V. cholerae*. However, a most notable exception is plasmids of the IncC incompatibility group (46, 100). The increase in incidence of R-factor-mediated drug resistance in *V. cholerae* has been due almost exclusively to plasmids of this group.

Genetic Mapping

Parker et al. (95) used P-factor crosses to obtain a linear linkage map of the *V. cholerae* classical strain 162, containing both selected and unselected markers. Seventeen markers were ordered, and five others were shown to be linked but were not ordered. This mapping procedure, however, is very inefficient, because transfer frequencies of chromosomal markers are low (10^{-5} to 10^{-6}) and nonselected makers are poorly linked to selected markers.

A system that significantly enhanced the mobilization of *V. cholerae* chromosomal genes by P was developed by Johnson and Romig (55) and has been termed transposon-facilitated recombination (Tfr). This system comprises a multistep procedure to construct *V. cholerae* donor strains that carry the ampicillin transposon Tn*1* within the chromosome. A P derivative, P::Tn*1*, is introduced into such Tfr donor strains, and the homology between the chromosome and P, provided by the transposon, allows recombination and subsequent transfer of genes at high frequency from origins specified by the chromosomally located Tn*1* (54). It is also possible to achieve transfer of chromosomal markers in the opposite direction from the Tn*1* site of insertion by using a Tn*1* insertion derivative of P in which the transposon is inserted in the opposite orientation with respect to the P-factor origin of transfer. Thus one can obtain transfer from a single site and in both directions around the chromosome (Fig. 2).

Tfr donors are obtained by the steps indicated in Fig. 3. The hybrid P plasmid, pSJ26 (P::Tn*9*Tn*1* *hts-25*) is chloramphenicol resistant (Cm^r) and ampicillin resistant (Ap^r) and contains an *hts* mutation (lethality to host cells at elevated temperatures). This plasmid is transferred to a recipient *V. cholerae* strain. Transconjugants from this mating are incubated at 42°C to promote the formation of deletion derivatives of pSJ26 that lack both *hts* and Tn*9* (Cm^r) but retain Tn*1* (Ap^r). Bacteria containing chromosomally located Tn*1* insertions are isolated from *hts* revertant strains by superinfecting with the incompatible plasmid pSJ25 (P::Tn*9* *hts*) and selecting for Cm^r. Cells that retain the Ap^r phenotype are cured of pSJ26 by selecting for growth at 42°C. Survivors that lose Cm^r but retain Ap^r represent cells with chromosomally inserted Tn*1*. These strains are converted into donors by having plasmid pSJ5 (P::Tn*1*) introduced into them. Such donor strains are able to transfer genes at frequencies 100- to 1,000-fold higher than conventional P donors.

This system has been used to obtain circular genetic maps of the El Tor strain RJ1 (S. R. Johnson, Ph.D. thesis, University of California, Los Angeles, 1978) and, more recently, the classical strain 162 (120). The latter study confirmed the gene order obtained in $P^+ \times P^-$ crosses (95) (Fig. 4A).

The genetic relatedness of the two *V. cholerae* biotypes was also demonstrated by transfer of functionally equivalent genes from El Tor Tfr donors to classical recipients (90). This occurred at frequencies equal to those of the homologous biotype cross. However, transfer frequencies from classical donors to El Tor recipients were generally at least 10-fold lower than to classical recipients. The reason for this unequal transfer is not known, but could be related to differences in DNA restriction and modification (51).

The Tfr system of Johnson and Romig has a number of disadvantages. A large number of manipulations are required to construct each Tfr donor, and Tn*1* chromosomal insertions are rare and therefore require enrichment techniques.

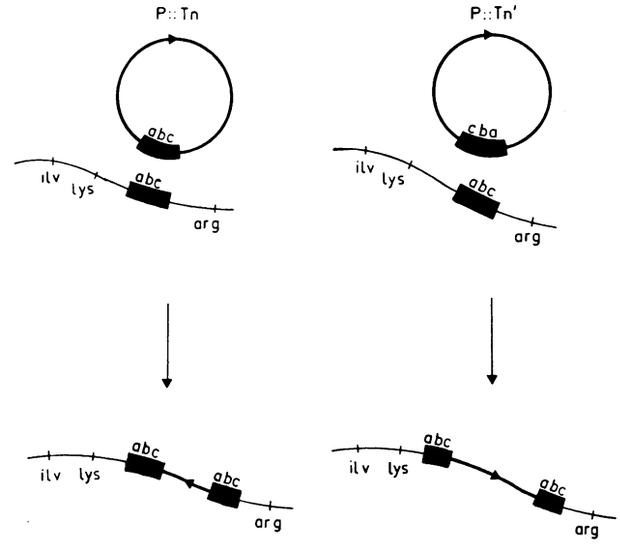


FIG. 2. Transfer of chromosomal markers in the opposite direction from a single transposon insertion. ■, Transposon (Tn*1*). The closed circle is a P sex factor derivative in which the arrowhead represents the origin and direction of transfer. Integration of P::Tn in the left panel leads to high-frequency transfer of *arg* but not *lys* and *ilv*. High-frequency transfer of *lys* and *ilv* occurs by integration of P::Tn'. The letters indicate orientation of the transposon. Adapted from reference 54.

This latter problem is compounded for classical strains, which contain cryptic plasmids, because Tn*1* transposes preferentially to plasmids (64). Also, attempts to extend this system to recent clinical isolates of *V. cholerae* have failed (90).

Newland and co-workers (90) therefore devised a single-step procedure for introducing transposons into the *V. cholerae* chromosome to produce Tfr donors. This was achieved by using the temperature-sensitive F' factors F'(Ts) *lac*$^+$ *trp*$^+$ Tn*5* (kanamycin resistance [Km^r]) or F'(Ts) *lac*$^+$ Tn*10* (tetracycline resistance [Tc^r]) as suicide vectors. Transposon-induced auxotrophs were generated with either of these vectors by incubation at elevated temperatures. Also, P::Tn*1* was mutagenized with each of these transposon vectors to produce two P::Tn*1*Tn*5* (pJN2 and pJN8) and two P::Tn*1*Tn*10* (pJN5 and pJN8) derivatives capable of marker transfer in opposite directions. Tfr donors were formed by introducing the appropriate mobilizing plasmid by conjugation into transposon-induced auxotrophs. These workers also obtained *V. cholerae* El Tor genetic map (Fig. 4B) in which 20 markers are located on a circular chromosome.

Comparison of the genetic maps of *V. cholerae* classical strain 162 and the El Tor strain GN6300 (Fig. 4A and B) reveals a high degree of similarity. However, the El Tor and classical genetic maps are inverted in the region containing the loci *pro-201–pyrA201–leu-1–ura-201*.

As mentioned, previously, the El Tor and classical biotypes differ in several characteristics (25). El Tor strains usually produce a soluble hemolysin, have a cell-associated hemagglutinin for chicken erythrocytes, and are resistant to polymyxin B and Mukerjee's group IV bacteriophages; classical strains lack these properties.

Using the procedure of Newland et al. (90) Green et al. (41) performed conjugal matings between El Tor donor

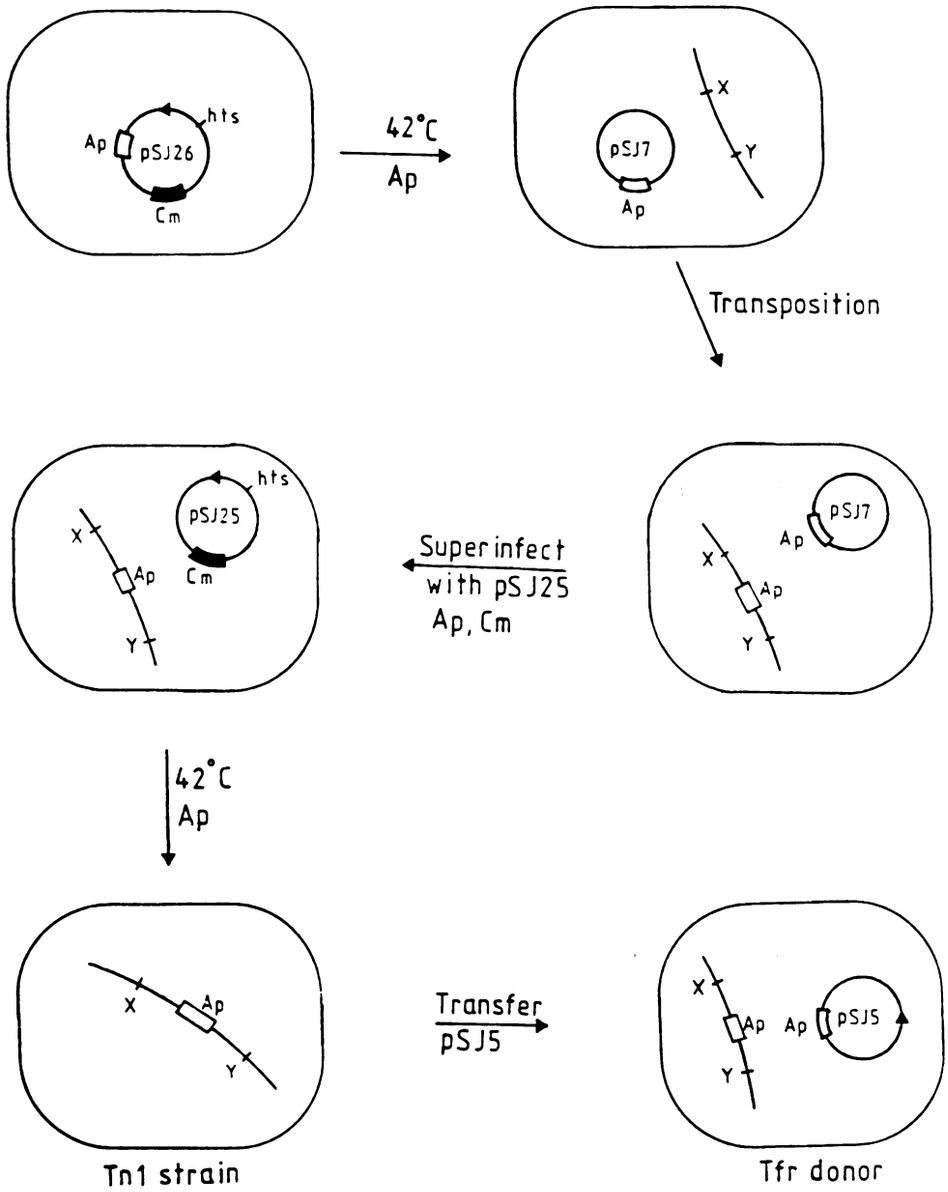


FIG. 3. Schematic representation of the steps involved in the construction of Tfr donor strains. Closed circles represent P sex factor derivatives, with the arrowhead depicting the origin and direction of P transfer. ——, bacterial chromosome with markers X and Y. ▬, Tn*9* (Cm^r); ▭, indicate Tn*1* (Ap^r). See text for details. Adapted from reference 54.

strains and classical recipient strains of *V. cholerae* to locate the loci responsible for hemolysin production (*hly*), chicken erythrocyte agglutination (*cha*), and polymyxin B resistance (*pmx*). These three genes are closely linked to each other and to the *leu* locus (Fig. 4B).

Recently, Goldberg and Mekalanos (35) cloned the *V. cholerae recA* gene responsible for homologous recombination by complementation of a *recA* mutant of *E. coli* K-12. By constructing a small insertion (4 base pairs) in the gene, they constructed a mutation which has been recombined into the *V. cholerae* chromosome. This typical *recA* mutant has been used to analyze cholera toxin gene amplification (36; see below). It should also be useful for recombination analysis.

ctxAB Locus and *toxR* (Toxin Production)

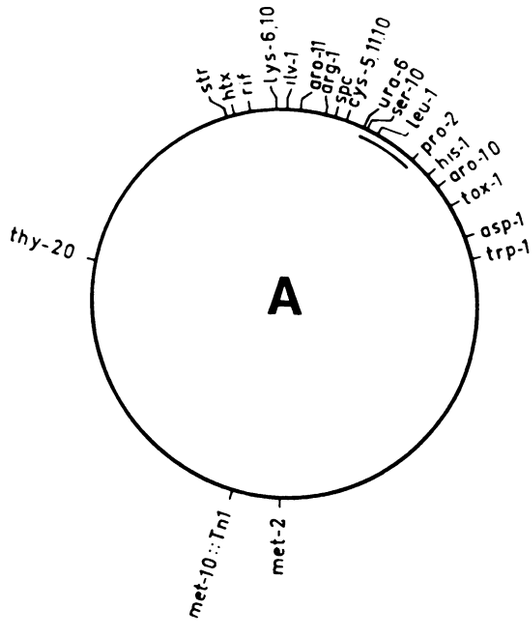
At the molecular level, studies have been confined mainly to determining the genetic organization and regulation of the genes involved in biosynthesis of cholera toxin. Mutants altered in the production of cholera toxin have been isolated in several laboratories. The *tox* class of mutations result in hypoproduction (1,000-fold decrease) of cholera toxin (29). Conjugal mapping of *tox* markers in classical biotype strain 569B shows that these mutations are closely linked to the *his-1* locus (6).

A second class of mutations, *htx*, leads to the hyperproduction of cholera toxin in *V. cholerae* (76, 77). This locus is closely linked to *rif* on the classical *V. cholerae* genetic map

(79). Hypertoxinogenic mutations, as well as all the *tox* mutations, appear to be regulatory mutations that alter the levels of toxin production.

The structural genes that constitute the cholera toxin operon, *ctxAB*, encoding the A and B subunits of cholera toxin, have been located on the chromosome of both classical and El Tor *V. cholerae* strains (58, 84).

By using the DNA sequence homology between cholera toxin and the heat-labile enterotoxin of *Escherichia coli* (84, 97), the *ctxAB* operon from classical strain 569B has been cloned into plasmid pBR322. The *ctx* genes are organized in a single transcriptional unit, with the A cistron (*ctxA*) preceding the B cistron (*ctxB*). The nucleotide sequence of the *ctxAB* operon has been determined (80).



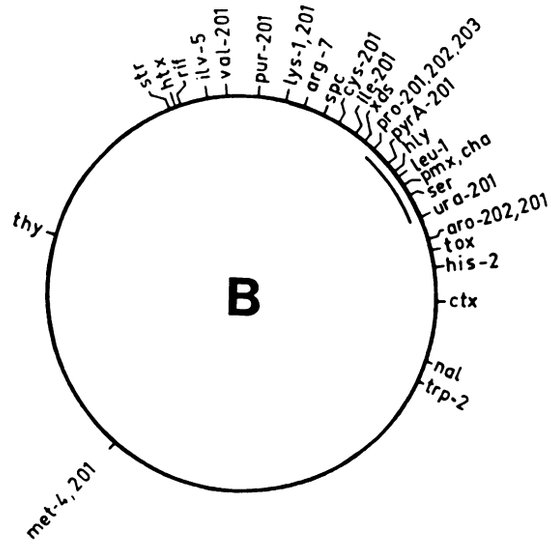


FIG. 4. Genetic map of the *V. cholerae* chromosome from (A) classical strain 162 and (B) El Tor strain GN6300, based on published linkage data (90, 120). Relative distances are proportional to genetic linkage. The double line corresponds to an inverted order of markers between the classical and El Tor genetic maps.

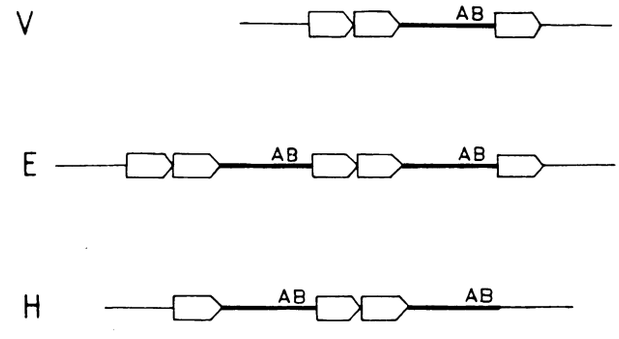


FIG. 5. Molecular organization of the *ctxAB* operon in three *V. cholerae* strains, VRL1795 (V), E7946 (E), and HK1 (H). Thick lines containing AB represent the *ctx*AB operon, and open boxes indicate the location and directionality of the RS1 repetitive sequence. Adapted from reference 75.

Honda and Finkelstein (50) were the first to describe a mutant strain, Texas star, that produces the B subunit but not the A subunit of cholera toxin. Although this nitrosoguanidine-generated strain has been extremely useful in analysis of the potential of nontoxigenic mutants of *V. cholerae* as oral vaccine candidates (66), the difficulty encountered in isolating this mutant is probably due to the presence of multiple copies of the *ctxAB* operon in the parent strain.

Classical strains all contain two widely separated copies of the *ctxAB* operon (97). El Tor strains have been reported to contain only a single copy of the operon (58, 84). However, several El Tor strains have now been identified that carry *ctxAB* duplications (75). Numerous copies of this operon have been detected in some strains. Such strains also display variation in the DNA structure surrounding the *ctx* operon. The multiple copies of *ctx* in El Tor strains are arranged on large tandem repeats which are either 7 or 9.7 kb in length. This variation in size reflects the different number of copies of the 2.7-kb sequence (RS1) that is located at the joint of the duplication, as well as upstream and downstream from *ctxAB* (Fig. 5). This RS1 element also appears to be responsible for the *ctx* amplification phenomenon which occurs during intestinal passage in animals (48, 75). Evidence that RS1 may be involved in illegitimate recombination, coupled with the observed duplication and amplification properties of this sequence, has led to the suggestion that *ctxAB* is contained within a genetically mobile element, resembling a transposon (75).

With *recA* mutants (35), it has been possible to analyze the importance of homologous recombination in *ctxAB* amplification (36); amplification did not occur in the absence of *V. cholerae* RecA protein. These results support the model that amplification (or deletion) occurs via intramolecular recombination involving unequal crossing over between the RS1 sequences flanking the *ctxAB* operon.

The inability to isolate structural gene mutations in *ctxAB* may be a consequence of the operon duplication observed in classical and some El Tor strains. The cholera toxin operon has been located in the El Tor strain RV79 (equivalent to MAK757) (116) between *nal* and *his* (Fig. 4B). At least one of the two *ctx* copies in the classical strain 569B appears to be located in the same relative chromosomal position as it is in

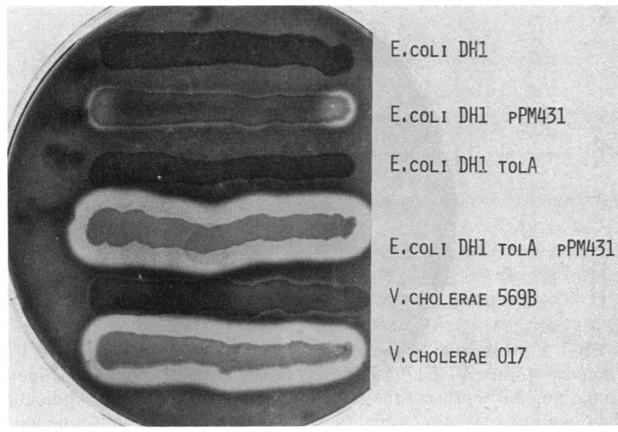


FIG. 6. Hemolysin production by *V. cholerae* El Tor strain O17 and classical strain 569B and *E. coli* K-12 strain DH1 and its *tolA* derivative harboring either the cloned hemolysin gene(s) encoded in plasmid pPM431 or the cloning vector pBR322 (69, 70).

strain RV79. The precise location of the other copy of *ctx* and the physical structure of the *ctxAB* duplication in strain 569B are not known.

As mentioned above, mutations affecting the level of cholera toxin have also been mapped. The *htx* locus maps in the *str* region, which is quite distant from *ctxAB* (6, 109, 126). Mutations in the *toxR* locus (see below) are, like *ctxAB*, linked to the *his* locus but are on the opposite side of *his* from *ctxAB*.

Recently, the positive regulatory gene (*toxR*) from strain 569B that activates cholera toxin transcription was cloned (82). Suppression of the hypotoxinogenic *tox* mutation by the cloned *toxR* gene indicates that *tox* mutants may have a defective *toxR* gene. Southern blot analysis with the cloned *toxR* gene as probe indicates that all examined classical strains contain *toxR* sequences. This is also true for El Tor strains and nontoxigenic strains, even though nontoxigenic strains lack the *ctxAB* operon. This observation poses interesting questions about the origin of the regulatory genes and the toxin genes they control. In fact, it is now known that *toxR* is a transmembrane DNA-binding protein which can function to control the expression of other virulence genes (82a). In particular, the *toxR* gene product is required for the synthesis of a pilus colonization factor encoded by the *tcpA* locus and an outer membrane protein encoded by the *ompU* gene (cited in reference 82a). Activation of the *ctxAB* operon occurs as a result of binding ToxR to the sequence TTTTGAT, which is tandemly repeated before the start of the operon (82a).

hly Locus (Hemolysin Production)

Although in the past the emphasis has centered on the structure and production of toxin in *V. cholerae*, other genetic loci have recently been characterized. One determines hemolysin production. Classical strains are invariably nonhemolytic (Hly^-, whereas El Tor strains generally synthesize and excrete hemolysin (Hly^+) (49). However, weakly hemolytic El Tor strains have been isolated (106), as well as strains that produce no hemolysin but have other biochemical properties characteristic of the El Tor biotype (23).

The hemolysin gene(s) from several El Tor strains have been cloned in *E. coli* K-12 (38, 69, 70; R. A. Alm and P. A. Manning, manuscript in preparation). Hemolytic strains of *V. cholerae* produce large zones of hemolysis on sheep erythrocyte plates; in contrast, *E. coli* cells harboring the cloned *hly* genes produce very small zones (Fig. 6). Hemolysin is produced in *E. coli*, but is not actively excreted into the extracellular medium (81). Similar phenomena have been observed when cloned genes for haemolysin and other extracellular proteins from a number of bacterial species were introduced into *E. coli* (20, 30a, 59, 97).

Both Manning et al. (69, 70) and Goldberg and Murphy (39) reported that the hemolysin structural gene (*hlyA*) encodes a protein of M_r ca. 80,000. However, excreted hemolysin has M_r 61,000 and appears to be identical to the hemolysin of non-O1 vibrios, as is predicted on the basis of Southern hybridization analysis (15, 130, 131). This disagrees with the M_r 20,000 value for the purified hemolysin reported by Honda and Finkelstein (49). However, this discrepancy could be accounted for if these authors purified the second hemolysin present in some strains.

In *E. coli* K-12, hemolysin is translocated across the cytoplasmic membrane but not the outer membrane, remaining predominantly in the periplasmic space (84). This has been demonstrated by cell fractionation and by the use of *tolA* and *tolB* mutants of *E. coli* K-12 which leak periplasmic proteins (2, 8). Such mutants release hemolysin into the medium. Nucleotide sequence analysis of *hlyA* also shows the presence of a typical signal sequence; however, an unusual cleavage must occur when this protein is excreted (R. A. Alm and P. A. Manning, manuscript in preparation).

Goldberg and Murphy (38) used the cloned *hlyA* gene as a probe to verify insertions in the hemolysin locus of *V. cholerae* to produce Tfr donors. These workers located and mapped the *hly* locus between *ilv* and *arg* on the El Tor chromosome. This contradicts linkage data by Green et al. (41), who did not observe significant linkage between *hly* and *arg*.

When El Tor strains, regardless of their hemolytic phenotype, and classical strains are analyzed by Southern blot hybridizations with an *hly*-specific probe, all can be shown to contain *hly*-homologous DNA (15, 38, 70). Furthermore, it has been shown that the DNA surrounding the *hly* gene cluster is highly conserved in both hemolytic and nonhemolytic strains as judged by a large number of restriction enzyme cleavage sites (15, 70). However, the observed pattern of *Bam*HI sites is an exception. Two patterns are observed, one which correlates with Hly production and one which correlates with the nonhemolytic phenotype. The source of this difference is situated about 10 kb upstream of the *hly* locus. It could represent the regulatory region.

Comparison of the cloned *hly* genes from nonhemolytic El Tor (RV79 Hly^-), classical (569B), and hemolytic El Tor (RV79 Hly^+) strains revealed more subtle DNA alterations in the vicinity of the structural gene (39). Restriction analysis indicated a difference, involving an apparent insertion of 10 to 15 base pairs, between an otherwise isogenic pair of Hly^+ and Hly^- El Tor strains and a 20-base-pair deletion in the 569B *hly* locus as compared with RV79. Location of this deletion in *hly* regulatory sequences would explain the nonreverting Hly^- phenotype in classical strains. The precise manner by which hemolysin production is regulated in either cholera biotype and in non-O1 vibrios is not known.

It has been proposed that a locus, designated *hlyR*, is responsible for the regulation of hemolysin production (127). This conclusion rests on the study of a single recombinant colony from a conjugation experiment; *hlyR* is very tightly linked to *toxR*. However, *toxR* would not appear to be involved in the regulation of *hlyA*, since the repeat se-

quences seen before *ctxAB* (see above) are not found within 500 bp of the start of *hlyA* (Alm and Manning, in preparation).

xds Locus (Extracellular DNase[s])

A third protein excreted by *V. cholerae*, an extracellular deoxyribonuclease (DNase), has recently come under investigation (30a, 89). The limited ability of *V. cholerae* strains to accept and maintain foreign plasmids may be related to the production of this DNase (30a). When compared with other enteric organisms, *V. cholerae* has a low incidence of R plasmids (46, 100).

A structural gene encoding extracellular DNase production (*xds*) has been cloned (30a, 89). Newland et al. (89) have narrowed the limits of the DNA region encoding nuclease activity to within 3.5 kb. In minicells, an M_r 100,000 polypeptide is produced. This contrasts with observations by Focareta and Manning (30a), who found the gene encoding DNase activity to be confined to a 1.5-kb fragment. A corresponding protein product of M_r 24,000 showed DNase activity. The relatively large zone of hydrolysis produced by the DNase even in *E. coli* K-12 strains carrying the cloned gene is consistent with the conclusion that the DNase protein is small and consequently readily diffusible (30a). Cell fractionations demonstrated that this protein is also localized to the periplasm of *E. coli* K-12 but to the extracellular environment of *V. cholerae*. Nucleotide sequence analysis of the DNase confirms that the protein has a size of 24.3 kilodaltons and is produced with an additional 18-amino-acid NH_2-terminal signal sequence. The DNase of Newland et al. (89) requires much longer incubation times for detection than that of Focareta and Manning (30a), suggesting a further difference. These discrepancies appear to have been resolved by Southern hybridization studies, which suggest that the two DNases are different (T. Focareta, unpublished data). The *xds* locus maps between the markers *pro-20* and *ile-201* on the genetic map (89) (Fig. 4). The locus of the other DNase gene (30a) has not been mapped.

Protein Excretion

Mutants of *V. cholerae* have been isolated which are defective in the excretion of one or more extracellular proteins. Holmes and co-workers (48, 86) isolated a class of mutant in which cholera toxin was localized intracellularly. This is not a general defect in export, because the mutant is indistinguishable from the parent in releasing other extracellular proteins (T. Focareta, unpublished results). Schneider and Parker (111) also isolated specific export mutants. Recently, Focareta and Manning (30) described the cloning of a potential excretion protein system. Further analysis suggests that this system may facilitate the excretion by *E. coli* K-12 of the extracellular DNase but not of the hemolysin. Taken together, these results suggest that *V. cholerae* has more than one excretion system and that these systems have a high degree of selectivity in the proteins which they release.

OmpV and Other Outer Membrane Proteins

The outer membrane of *V. cholerae* contains a group of major proteins with M_r 44,000 to 47,000, a heat-modifiable protein of M_r 35,000, and another of $M_r \sim 25{,}000$ (57, 60, 73). The profile of membrane proteins varied with media and culture conditions (57; R. A. Alm, A. Barker, and P. A. Manning, unpublished data). Similar results have been observed with other gram-negative organisms, including *E. coli* (67,110). Variations in protein composition among different *Vibrio* species have also been demonstrated (73). The role of these major outer membrane proteins is unknown; however, the M_r 44,000 to 47,000 proteins are most probably porins and the M_r 35,000 protein has been shown to behave like the OmpA protein of members of the family *Enterobacteriaceae* (1).

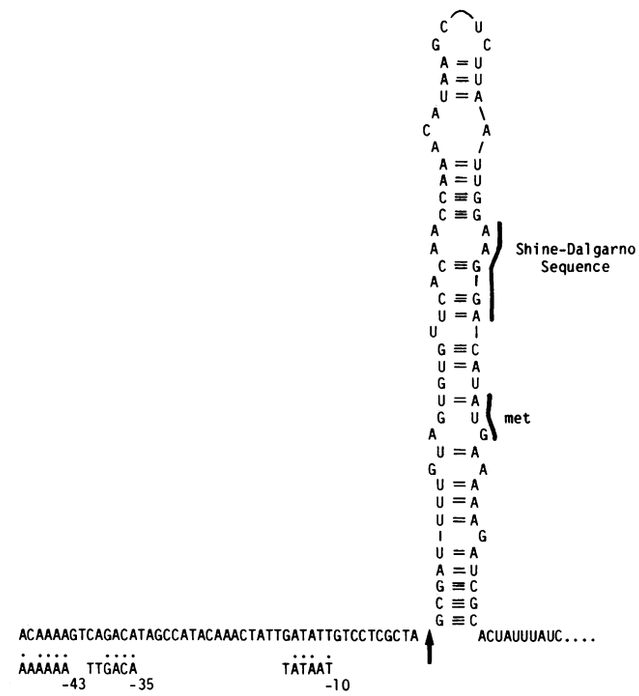


FIG. 7. Regulatory region of the DNA sequence prior to the start of the *ompV* gene. The promoter consensus sequences, the Shine-Dalgarno sequence (ribosome-binding site), and the initiation codon (met) are shown for comparison. The start of the 5′ end of the messenger ribonucleic acid is indicated with an arrow (A. Barker and P. A. Manning, unpublished data).

Immunochemical studies demonstrate that strains of *V. cholerae* belonging to both the major serotypes (Inaba and Ogawa) and that both biotypes have cross-reacting protein antigens located in their outer membrane (71). In fact, antibodies to certain cell envelope proteins have been demonstrated to be protective (4, 87, 88).

Recombinant DNA clones encoding several potentially immunologically important proteins have been obtained by use of antisera prepared against either live cells or purified protein. One such clone encodes an M_r 22,000 minor outer membrane protein, which, when expressed in *E. coli* K-12, was exposed on the cell surface of *E. coli* K-12 (68). The DNA encoding the gene was shown to be conserved among the biotypes. The immunological importance of this protein is currently being established. It would appear to be similar to a protein that is readily detected with convalescent-phase human antisera.

The gene for a very immunogenic outer membrane protein has been cloned and termed *ompV* (74, 119). The OmpV protein is the M_r 26,200 major outer membrane protein of *V. cholerae*; its gene is poorly expressed in *E. coli*. A possible explanation for this poor expression comes from analysis of the DNA sequence (98). Prior to the initiation codon is an

excellent Shine-Dalgarno sequence. However, this sequence falls within a region capable of forming a stem-loop structure in the messenger ribonucleic acid (Fig. 7) that could reduce translation by inhibiting the binding of the messenger ribonucleic acid the 16S ribosomal subunit. Operon fusion studies have confirmed the notion of translational control (A. Barker, unpublished data). It has been proposed that a positive regulatory element is involved which is present in *V. cholerae* but absent from *E. coli* K-12. Further analysis of the DNA sequence and the amino acid sequence of the mature protein demonstrated that OmpV is synthesized in a precursor form with a 19-amino-acid NH_2-terminal extension or signal sequence (98). The derived amino acid sequence has marked hydrophilic regions, several of which correspond to the antigenic determinants on the native and denatured forms of the protein (98, 99).

The immunogenicity of OmpV has been investigated. OmpV is present on all *V. cholerae* strains, irrespective of their biotype or serotype (71), but is not present in other vibrios such as *V. mimicus* and *V. fluvialis* (P. A. Manning, unpublished data). It is not known whether OmpV is a protective antigen, but antibodies to OmpV have been detected in some convalescent-phase human sera (Manning, unpublished data).

Hemagglutinins

V. cholerae produce at least four distinct hemagglutinins, all of which differ with respect to their spectrum of erythrocyte activity, sugar sensitivity pattern, Ca^{2+} requirement, and phase of expression (45). A cell-associated hemagglutinin is present in both biotypes. The El Tor hemagglutinin, the chicken hemagglutinin used in biotyping *V. cholerae*, is inhibited by D-mannose and D-fructose. The classical cell-associated hemagglutinin is inhibited by L-fucose. A soluble hemagglutinin, which is not inhibited by any of these sugars, has been found in culture supernatants of strains of both biotypes.

The soluble hemagglutinin has been purified and shown to possess several biological activities (28, 121). In addition to its ability to agglutinate erythrocytes, this soluble hemagglutinin has protease activity and is able to hydrolyze fibronectin, ovomucin, and lactoferrin (27) and to nick and activate the A subunit of cholera toxin (12). The protein may also be involved in the adherence of cholera cells to the intestinal epithelium (26) and therefore represents an important factor in the pathogenesis of *V. cholerae*.

Using antiserum provided by R. A. Finkelstein, Franzon and Manning (31) cloned the gene for a cell-associated hemagglutinin. A number of strains from both biotypes express this hemagglutinin, and Southern hybridization analysis suggests that the gene encoding this protein has been conserved between both biotypes. However, van Dongen and de Graaf (125a), who subsequently cloned this gene, have detected some strains in which it is absent. The effects of mutations in this gene on the pathogensis of cholera are not known. This hemagglutinin differs antigenically from the soluble hemagglutinin, but, as for the soluble hemagglutinin, it is resistant to all sugars tested (28, 31).

Srivastava and Srivastava (117) have described the isolation of a mutant defective in the production of a mannose-sensitive hemagglutinin. The encoding gene, *ams* (mannose-sensitive adherence), is closely linked (75%) to the *pur* locus and less closely linked (60%) to *ilv*. This location clearly differentiates this hemagglutinin from the chicken erythrocyte hemagglutinin (*cha*) of El Tor strains (Fig. 4B).

VARIABILITY IN THE O ANTIGEN OF THE LPS

The lipopolysaccharide (LPS) from *V. cholerae*, like LPS from other gram-negative bacteria, contains lipid A, a core region, and O-antigenic side chains. Two major subclasses, Ogawa and Inaba, are recognized in *V. cholerae*. Strains of the Ogawa serotype possess antigens A and B and a small amount of antigen C, whereas those of the Inaba serotype are said to have only antigens A and C (105, 108). A third, rare, serotype subclass, Hikojima, contains the three antigenic factors A, B, and C (17). Bhaskaran (9) has mapped a locus, *oag*, associated with the serotype specificity of the O antigen. He suggested that the Hikojima serotype may be an artifact and that it represented some form of merodiploid. Perhaps Hikojima serotypes represent strains undergoing conversion (see below).

Evidence for serotype conversion has come from a number of studies. Gangarosa et al. (33) detected changes in the serotype of *V. cholerae* being excreted by a confined patient. Sack and Miller (107), using germfree mice, also demonstrated progressive changes in the serotypes during a *V. cholerae* infection. It has also been possible to isolate serotype convertants by treating cultures with antiserum (108). More recently, Ogg et al. (92, 93) have implicated bacteriophage CP-T1 in a lysogenic conversion which leads to a change in the serotype of the host (see below).

The composition and structure of the lipid A component of cholera LPS have been determined (3, 14). The fatty acid content of cholera LPS is complex; the Inaba and Ogawa serotypes have similar compositions.

The chemical composition of the polysaccharide portion of cholera LPS has received widespread attention (47, 52, 61, 102, 104, 105, 112). Cholera LPS contains several unusual sugars, including 2-amino-2,6-dideoxy-D-glucose (quinovosamine), 4-amino-4-deoxy-L-arabinose, and 4-amino-4,6-dideoxy-D-mannose (perosamine). LPS from both the Ogawa and the Inaba serotypes contain quinovosamine and perosamine; however, Ogawa LPS contains the additional sugar 4-amino-4-deoxy-L-arabinose.

Unlike *E. coli* and *Salmonella* spp. *V. cholerae* possesses only a single 2-keto-3-deoxyoctulosonic acid unit in its core (13). It seems likely that in *V. cholerae*, fructose replaces the second 2-keto-3-deoxyoctulosonic acid unit.

The difference in sugar composition has led to investigations into the role of particular sugars in the serological specificity of LPS. Structural studies on LPS reveal that the side chain is a linear homopolymer of D-perosamine, of approximately 60 repeating units (61, 103), in which the terminal amino group is acylated with quinovosamine (62). This structure appears to be common to both the Ogawa and Inaba serotypes. It has therefore been suggested that this backbone structure of the LPS represents the antigenic determinant A, which is common to both Inaba and Ogawa strains. In addition, Redmond et al. (105) demonstrated a loss of serological activity concomitant with the release of fructose, suggesting the involvement of a fructo-furanoside linkage either associated with or anchoring the A specificity. The presence of 4-amino-4-deoxy-L-arabinose in the Ogawa serotype may implicate this sugar in determining antigenic factor B specificity. The isolation of monoclonal antibodies directed against cholera LPS (44) should provide valuable information about the chemical nature of O-antigenic specificity.

Confusion about the precise nature of the antigenic factors present on the three cholera serotypes remains. It is often assumed that Ogawa strains carry only antigens A and B,

Inaba strains carry only antigens A and C, and Hikojima strains carry all three antigens. However, Sakazaki and Tamura (108) suggested that Ogawa strains produce the three somatic antigens, with high levels of determinants A and B relative to C. They also suggested that the Inaba serotype possesses no B antigen and that the Hikojima serotype contains decreased levels of B and increased levels of C antigens relative to the Ogawa serotype. These observations are supported by similar results obtained by Redmond et al. (105) and Hisatune and Kondo (47).

However, if the Ogawa-to-Inaba conversion occurs at a higher frequency than the reverse, as suggested by Sakazaki and Tamura (108), then the level of convertants in a culture could provide the C determinant. Studies must be done with single cells to resolve these possibilities. This could perhaps be done by using A-, B-, and C-specific monoclonal antibodies and a cell sorter. The composition and structure of each serotype are not affected by biotype.

The genes determining the biosynthesis of the Inaba and Ogawa serotypes have been cloned and expressed in *E. coli* K-12 (72); the *E. coli* K-12 LPS core is substituted with the *V. cholerae* O antigen (R. Morona, M. W. Heuzenroeder, and P. A. Manning, unpublished data). Restriction analysis of these clones and determination of the chromosomal organization in both Inaba and Ogawa strains by Southern hybridization suggest that only minor changes, which must lie in this region, are associated with serotype conversion (M. Kamke, G. Morelli, H. M. Ward, R. Morona, J. A. Hackett, J. Yeadon, and P. A. Manning, Gene, in press). These genes are present in a cluster of about 16 to 19 kb in length.

Expression in *E. coli* K-12 of the LPS O antigen of the Ogawa and Inaba serotypes from cloned *V. cholerae* genes may allow the investigation of phage conversion caused by phage CP-T1 at the molecular level.

V. CHOLERAE BACTERIOPHAGES

One approach that has contributed greatly to our present understanding is the use of vibriophages as tools for studying the *V. cholerae* chromosome.

Contributions of Vibriophages to Genetic Analysis

Genetic analysis of *V. cholerae* has progressed slowly, owing mainly to the inability to introduce exogenous DNA into this organism. Although transfection of *V. cholerae* with phage DNA has been reported (7), it has not proven to be reproducible.

The inability to make *V. cholerae* cells competent for transformation or transfection may be twofold. Production of an active extracellular DNase by *V. cholerae* may significantly inhibit the entry of intact DNA molecules into the cell. A DNase-negative strain has been constructed by transposon insertion into the cloned DNase gene, followed by reintroduction of this insertion mutation into *V. cholerae* by mobilization and then into the chromosome by recombination (92). This procedure is much more laborious and less efficient than that used to transform *E. coli* K-12. The possible utility of this DNase-negative strain has not yet been demonstrated.

Restriction is a second barrier that exogenous DNA must overcome upon entering the cell. Imbesi and Manning (51) have reported the presence of biotype-specific restriction and modification systems in the two cholera biotypes. Restriction-negative strains have not been identified.

Generalized transducing phage particles have only recently been isolated from *V. cholerae* (94; D. A. Relman, M.D. thesis, Harvard-Massachusetts Institute of Technology, Cambridge, 1982). A cholera phage capable of specialized transduction has yet to be isolated.

Despite these limitations, vibriophages have been useful tools in extending our knowledge of their host. This is particularly true for a small group of phages that are serologically related to the common kappa-type phage (18, 122). VcA-1, VcA-2 (34, 129), and VcA-3 (37) are temperate phages that are capable of lysogeny in vibrios irrespective of the host biotype. This finding is surprising, because kappa-type phages were thought to be confined mainly to El Tor strains (122). However, induction of classical strains can release either complete or defective bacteriophages that are serologically and morphologically related to kappa-type phages (34). VcA-1 and VcA-3 are very similar morphologically and are homoimmune (37). Even though these two phages share DNA homology as determined by Southern hybridization analysis, the restriction endonuclease patterns of their purified DNA are markedly different, suggesting considerable evolutionary divergence between VcA-1 and VcA-3.

An important feature shared by the three phages VcA-1, VcA-2, and VcA-3, which makes them useful instruments in performing genetic experiments with *V. cholerae*, is their ability to integrate randomly into many different sites in the host chromosome (37, 56). Thus lysogeny of host bacteria may result in the inactivation of the particular gene into which the prophage has integrated.

The mutator property of these three phages closely resembles that of phage Mu (16, 125) and has been used to generate a large number of auxotrophic mutations (37, 53). Mekalanos et al. (78), using VcA-1 and a temperature-sensitive derivative of VcA-2, VcA-2cts1, have generated deletion strains lacking the cholera toxin gene, making them suitable for analysis as potential vaccine strains.

Mutagenic vibriophage VcA-1 can be used to form Tfr donors by virtue of its homology with a hybrid P factor, P::Tn*1* (pSJ15), containing a defective VcA-1 genome. The cholera toxin operon was mapped by using such a system (116). In a similar fashion, the *hly* locus of *V. cholerae* was mapped (38). In this instance, VcA-3 was used to generate a Tfr donor strain.

The contribution of phages to *V. cholerae* genetics has increased with the isolation of the transducing phage CP-T1 from an apparently lysogenic El Tor strain (93, 94). Several properties of CP-T1 endow this bacteriophage with great potential for further study of its host (see below). Besides its ability to transduce widely separated markers, it is capable of propagating on host cells irrespective of biotype (51, 93) (see below for a more detailed discussion of transduction).

Lysogeny in *V. cholerae*

Upon adsorption of a temperate phage onto a susceptible host cell and penetration of its nucleic acid into the host cytoplasm, one of two mutually exclusive pathways is followed (118); the lytic pathway leading to the assembly of large numbers of new phage particles, or entrance into the prophage state. In the prophage state, the viral gene may exist as a plasmid or an insertion into the chromosome of the host. In either case, few phage genes are expressed and the

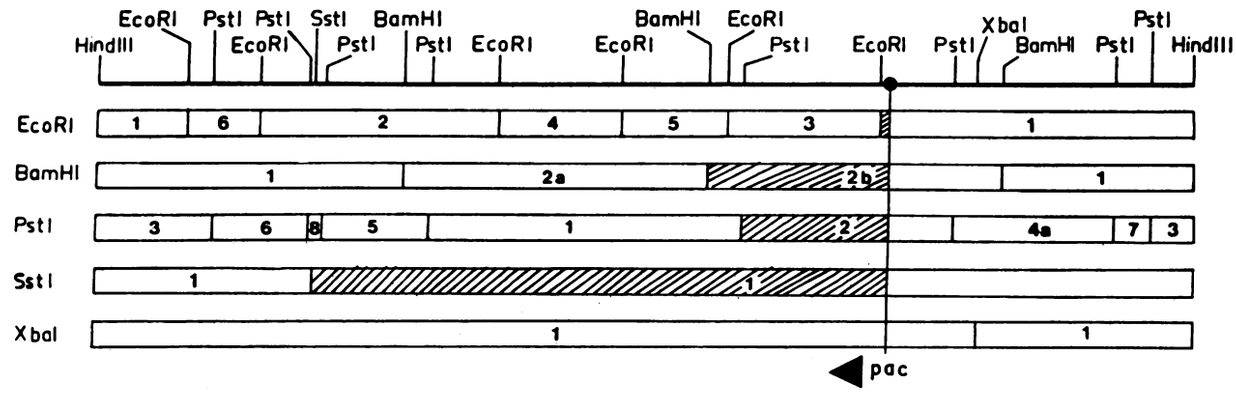


FIG. 8. Restriction map of the CP-T1 genome, indicating the position of *pac* and the direction of packaging from this site. ▨, Fragments obtained after digestion with the respective restriction enzyme and present in a nonmolar ratio to the other fragments.

viral genome is replicated in synchrony with the host chromosome.

Lysogens that produce infectious phages appear to be rare in the *V. cholerae* classical biotype but extremely common in El Tor vibrios (91). Temperature phages released by lysogenic El Tor strains have a characteristically narrow host range and were termed kappa-type phages by Takeya and Shimodori (123). These workers have also demonstrated a possible relationship between lysogeny and virulence of El Tor strains. El Tor strains could be prophage typed into two groups, those that release temperate phage and those that do not. Upon examination of El Tor strains isolated during epidemics between 1930 and 1960, the former of these two groups appeared to be responsible for severe disease, while the latter was associated with mild or relatively asymptomatic cholera.

Phage Conversion

The association of lysogeny with virulence of *V. cholerae* appears to be an example of a phage conversion. Lysogeny of the El Tor strains appears to change characteristic properties of the bacterial population.

Phage conversion leading to changes in the serotype of *V. cholerae* has been reported (93). The production of antigenic factor C has been suggested to be associated with the infection of the classical Ogawa strain O29 with phage CP-T1. That is, strains undergo a conversion from the Ogawa serotype (AB) to the Hikojima serotype (ABC). Similar experiments revealed antigenic changes associated with CP-T1 infection in El Tor Ogawa strains (92). The isolates surviving phage infection produced altered agglutination reactions and, unlike those of the classical biotype, were variable and highly unstable, with a large proportion agglutinating in anti-rough antisera. Strains maintaining their converted characteristics were resistant to infection by CP-T1 but could not be induced to release phage. Thus it is not known whether the stable seroconverted strains were lysogens.

Phage conversion by CP-T1 of the two cholera biotypes is markedly different (92, 93). The variation in surface antigens on phage-treated El Tor strains may be due to selection of phage-resistant derivatives, which have various degrees of roughness and therefore different reactivity with the antisera. In contrast, classical strains appear to undergo a more defined conversion, which may be related directly to lysogeny by CP-T1. These phenomena are complicated by the fact that CP-T1 uses the O antigen as its receptor (42). Thus, phage treatment may simply be selecting resistant mutants and not convertants. Furthermore, the temperate nature of CP-T1 is in doubt. Relman (M.D. thesis) suggested that CP-T1, like the related phages VP-M, VP-2, and VP-T, probably forms only pseudolysogens. Guidolin and Manning (42) also were unable to demonstrate lysogeny by CP-T1. By Southern hybridizations with cloned CP-T1 DNA fragments, they were unable to detect CP-T1-related sequences in strain 1633, a supposed CP-T1 lysogen (93), even though this strain had all the other characteristics of strain 1633. Unraveling this dilemma could provide valuable information on the structure and synthesis of the *V. cholerae* LPS molecule.

TRANSDUCTION AND BACTERIOPHAGES

Transduction

Bacteriophages capable of specialized transduction of *V. cholerae* have not been found; generalized transduction of this organism has only recently been demonstrated. The first such phage, CP-T1, was later joined by the three isolates VP-M, VP-2, and VP-T (94; Relman, M.D. thesis).

Ogg et al. (93) showed that classical strains infected by a phage released from an apparent El Tor lysogen underwent a biotype conversion. That is, they became resistant to polymyxin B, a property of the El Tor biotype. Later, transduction of a number of characters was acquired at frequencies ranging between 10^{-5} and 10^{-8} (94). These workers, however, were not able to demonstrate cotransduction of genetic markers by CP-T1, presumably because the amount of DNA between selectable markers exceeded the encapsulating capacity of CP-T1. Transduction by CP-T1 is biotype independent.

Bacteriophages VP-M and VP-2 are very similar to CP-T1; they mediate generalized transduction and are genetically homologous. Indeed, the restriction patterns of CP-T1 and VP-M are identical; those of VP-2 differ only slightly (Relman, M.D. thesis). Thus CP-T1 and VP-M may be independent isolates of the same phage, which is closely related to VP-2. A third generalized transducer obtained by Relman (M.D. thesis), VP-T, appears to be totally unrelated to either of these phages. Its DNA is not cleaved by many of the common restriction enzymes.

Two other bacteriophages have been studied in some detail. ϕ149 is a Mukerjee group IV phage (85, 101). A physical map of this phage has been described (114). Another phage, ϕ138, has also been examined and appears to be similar in size and in its replication to CP-T1 (19).

Molecular Analysis of Bacteriophage CP-T1

Much of the genome of phage CP-T1 has been cloned, and the entire genome has been analyzed by restriction enzyme mapping (43). These studies establish that the phage is terminally redundant and circularly permuted. The site, *pac*, at which DNA packaging into mature phage particles commences was mapped (Fig. 8). The DNA sequence around the *pac* site has been determined; within it lies a gene encoding a 12,900-dalton DNA-binding protein, which, by analogy with studies of *Salmonella* bacteriophage P22 (5, 22, 65, 128), could be the enzyme that recognizes and cleaves at *pac* (A. Guidolin, Ph.D. Thesis, University of Adelaide, Adelaide, Australia, 1985; A. Guidolin and P. A. Manning, submitted for publication). A transposon containing *pac* has been constructed, and attempts are being made to use it to obtain high-frequency generalized transducing lysates of *V. cholerae*. The rationale is that if the packaging system has a high affinity for *pac*, then the introduction of this signal into the chromosome would result, upon CP-T1 infection, in packaging commencing at this chromosomal site. This could produce phage particles containing chromosomal DNA, and the frequency at which markers were present would be dependent upon the distance from the *pac* site and the orientation of *pac*.

Plasmid vectors which would be transduced between *V. cholerae* O1 strains are also feasible, with the observation that the presence of *pac* leads to an elevated frequency of CP-T1-mediated plasmid transduction (U. Stroeher and P. A. Manning, manuscript in preparation).

SUMMARY

Within the last few years, there has been a major increase in the genetic analysis of *V. cholerae* and its phages. Much of this effort has been associated with the acceptance of the need for a live oral vaccine against cholera. Thus one approach has been to eliminate the ability of *V. cholerae* strains to produce cholera toxin, since it is well documented that survivors of a cholera infection are immune. Another approach has been to identify protective somatic antigens and to introduce these into suitable carrier strains (72). In addition, the need to expand the genetic tools available to manipulate *V. cholerae* and the possibility of understanding the phenomena of protein excretion and gene conversions have prompted new areas of research.

ACKNOWLEDGMENTS

We thank the National Health and Medical Research Council of Australia, the Australian Research Grants Scheme, the Clive and Vera Ramaciotti Foundations, and the World Health Organization for support both continued and in the past.

Thanks also to colleagues who have generously supplied strains, materials, and information which have aided us in our studies of *V. cholerae*.

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