JOURNAL OF BACTERIOLOGY, Mar. 1986, p. 723–731
0021-9193/86/030723-09$02.00/0

Vol. 165, No. 3

Effect of a *recA* Mutation on Cholera Toxin Gene Amplification and Deletion Events

INA GOLDBERG AND JOHN J. MEKALANOS*

Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

Received 19 June 1985/Accepted 12 November 1985

The cholera toxin operon (*ctxAB*) is located on a 7-kilobase pair variable genetic element which undergoes genetic duplication and amplification events in *Vibrio cholerae*. Amplification of the *ctx* genetic element was investigated by substituting the resident *ctx* loci of two *V. cholerae* strains with a DNA fragment encoding resistance to kanamycin. Although these strains were not normally resistant to >150 μg of kanamycin per ml, spontaneous derivatives could be obtained that grew well on 3 mg of kanamycin per ml. Southern blot analysis of these highly resistant isolates demonstrated that the *ctx* element was amplified approximately 20-fold. This amplification process was completely inhibited in the absence of a functional *recA* gene. The *V. cholerae* RecA protein, therefore, is essential for cholera toxin gene amplification. Spontaneous deletions of the *ctx* structural genes were observed in both *recA*$^+$ and *recA*$^-$ *V. cholerae* strains, although such deletions occurred at a 21-fold-lower frequency in the latter case. Structural analysis of these *ctx* amplification and deletion events supports a model for their formation that involves unequal crossing over between repetitive sequences located upsteam and downstream of the *ctx* operon.

One of the classical mechanisms of bacterial pathogenicity is the elaboration of potent exotoxins. Where the genetic basis for toxinogenicity has been studied in detail, it has often been the case that these proteins are encoded by accessory genetic elements (bacteriophages, plasmids, and transposons). For example, the genes specifying diphtheria toxin (8), streptococcal erythrogenic toxin (40), botulinum toxins C and D (5), *Escherichia coli* shiga-like toxin (27), and staphylococcal enterotoxin A (1a) are all carried by temperate bacteriophages. The *E. coli* heat-labile and heat-stable toxins (13), anthrax toxin (24), and tetanus neurotoxin (15) are plasmid encoded. In addition, some toxin genes are located on transposable elements. The gene for *E. coli* heat-stable toxin is flanked by inverted repeats of IS*1* and has been shown to transpose (31). In contrast, the genes for *E. coli* heat-labile toxin (38) and hemolysin (14, 17) are flanked by repeated sequences and thus have structures similar to transposons.

Over the last few years, we have been involved in the characterization of a genetic element which encodes the enterotoxin of *Vibrio cholerae* (Fig. 1). This element is composed of a central core region containing the cholera toxin operon (*ctxAB*), flanked by copies of a 2.7-kilobase pair (kb) repetitive sequence called RS1 (21). The *ctx* element (or rearranged forms of it) is present in toxinogenic strains of *V. cholerae* but absent in nontoxinogenic strains (26). The element can be found in multiple chromosomal locations (34) and is also capable of increasing its copy number within a strain. These gene amplification events are of particular interest, since they are selected in *V. cholerae* animal infections and thus have been implicated in the enhancement of this organism's pathogenicity (21).

Investigators have shown that amplification of certain bacterial genes coding for resistance to a given antibiotic can be selected by increasing the concentration of the antibiotic in the growth medium (6, 29, 37, 39). The results presented here show that *ctx* gene amplification can also be selected in this manner after modifying the *ctx* element with a gene encoding resistance to kanamycin. This amplification was completely dependent on the *recA* gene product of *V. cholerae* and appears to rely on recombination occurring within copies of RS1 than flank the *ctx* genes.

* Corresponding author.

MATERIALS AND METHODS

Bacterial strains and plasmids. *V. cholerae* strains, *E. coli* strains, and plasmids are listed in Table 1. The plasmid content of a strain is given in parentheses after its name in the text.

Media. LB, TYCC, trimethoprim medium, brain heart infusion, and M63 minimal medium have been described previously (9, 10, 25). Unless otherwise stated, antibiotics were present in the following concentrations: 100 μg of streptomycin, 75 μg of spectinomycin, 45 μg of kanamycin, 15 μg of tetracycline, 30 μg of gentamicin, 15 μg of chloramphenicol, and 50 μg of ampicillin per ml.

Construction of strain SM44. *V. cholerae* SM44, derived from strain P27459 (21), has its resident *ctx* copy replaced by the *ctx* construction (Δ*ctxABN4* Km^r) present on plasmid pJM290.3 (Fig. 2). This plasmid is a derivative of pGP6 which contains the cloned *ctx* locus from *V. cholerae* strain E7946 (21, 22). pGP6 DNA was digested by *Sal*I and the cohesive ends were filled in, using the Klenow fragment of DNA polymerase I. *Eco*RI DNA linkers (New England Biolabs, Beverly, Mass.) were ligated to the blunt ends with T4 DNA ligase. Any remaining circular molecules with *Sal*I sites were converted to poorly transformable linear molecules by digestion with *Sal*I, and *E. coli* SY327 was transformed with the DNA mixture. Amp^r colonies were screened for plasmids with the desired restriction pattern, and one of these was named pGP6E. The two *Nde*I sites within the *ctx* locus of this plasmid were replaced by a single *Xba*I site as follows: pGP6E DNA was cut with *Nde*I, thereby removing a 776-base pair DNA fragment containing essentially all of the *ctxA* and part of the *ctxB* sequences (22). The *Nde*I overhangs on the plasmid were converted to blunt ends by filling in these cohesive ends with T4 DNA polymerase. *Xba*I linkers were ligated to the blunt ends with T4 DNA ligase, and the linear DNA molecules were circularized with

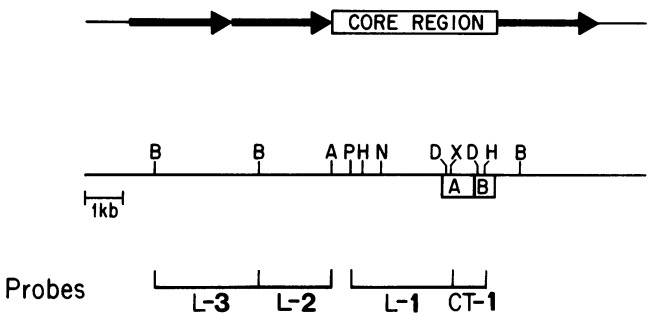

FIG. 1. Restriction map of the *ctx* genetic element. At the top is shown a schematic diagram of the element with its core region (open box), a 4.3-kb DNA region containing the *ctx* operon, flanked by copies of the 2.7-kb repeated sequence RS1 (horizontal arrows). Under this is shown the position of selected restriction endonuclease sites which are located within this element and the position of the *ctx* operon (AB). The origin of three DNA probes used in this study, L-3, L-1, and CT-1, is indicated below the restriction map. Abbreviations used for restriciton endonuclease sites are as follows: A, *Ava*I; B, *Bgl*II; H, *Hin*cII; D, *Nde*I; N, *Nru*I; P, *Pst*I; X, *Xba*I.

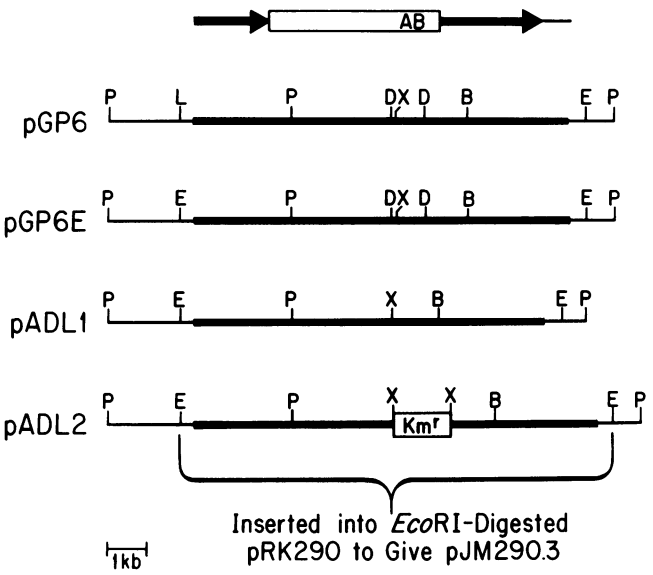

FIG. 2. Construction of plasmid pJM290.3. Shown are the restriction maps of plasmids involved in the construction (see Materials and Methods for details). The schematic diagram at the top shows what portions of the RS1 copies (arrows) and the core region (open box) of the *ctx* genetic element correspond to the cloned *V. cholerae* fragments (heavy lines). The abbreviations for restriction endonuclease sites are as noted in the legend to Fig. 1 with the addition of *Sal*I (L).

T4 DNA ligase to give plasmid pADL1. A 1,400-base pair *Xba*I-*Xba*I DNA fragment coding for kanamycin resistance isolated from plasmid pJM23.21 (22) was ligated into the single *Xba*I site of pADL1. SY327 was transformed with the DNA preparation and the Kan^r Amp^r Tet^s phenotype was selected. Such isolates were surveyed for plasmids with the desired construction (pADL2), which resulted in a net increase of about 600 base pairs in the cloned DNA fragment. Finally, the entire reconstructed *ctx* region (Δ*ctxABN4* Kan^r) was excised from pADL2 by *Eco*RI digestion and ligated into *Eco*RI-cut pRK290, yielding plasmid pJM290.3. Strain SM44 was created by replacing the *ctx* sequences on the bacterial chromosome of SM43 with the Δ*ctxABN4* Km^r allele on pJM290.3, using an in vivo marker exchange procedure adapted from Ruvkun and Ausubel (30). This procedure involved superinfection with plasmid pPH1JI and was previously described (22, 34).

Cholera toxin gene amplification. *V. cholerae* strains car-

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Other information
V. cholerae strains		
P27459	Prototrophic	El Tor biotype (21)
P27459 derivatives		
SM43	*str-43*	Spontaneous Str^r mutant of P27459
SM44	Δ*ctxABN4* Kan^r *str-43*	From SM43 by marker exchange with pJM290.3
SM105	Δ*ctxABN4* Kan^r *str-43 thy-105*	From SM44 by selection with trimethoprim and thymine
SM106	Δ*ctxABN4* Kan^r *str-43 thy-105 recA24*	From SM105 by marker exchange with pIS24 (10)
SM107	Δ*ctxABN3* Kan^r-3[b] *str-43*	From SM44 after selection for resistance to 3 mg of kanamycin per ml
SM108	Δ*ctxABN4* Kan^r-3L[c] *str-43 thy-108*	From SM107 by selection with trimethoprim and thymine
SM109	Δ*ctxABN4* Kan^r-3L *str-43 thy-108 recA-24*	From SM108 by marker with pIS24
SM110	*spc-110 nal-110*	From P27459 by spontaneous resistance
SM117	Δ*ctx-117 str-43 thy-105*	Spontaneous Kan^s derivative of SM105
SM118	Δ*ctx-118 str-43 thy-105 recA-24*	Spontaneous Kan^s derivative of SM106
GP100	Δ*ctx-100 str-43*	Spontaneous Kan^s derivative of SM44 (G. Pearson)
E7946	Prototrophic	El Tor biotype (21)
E7946 derivatives		
SM114	*str-114*	Spontaneous mutation
SM115	Δ*ctxABN4* Kan^r *str-114*	From SM114 by marker exchange with pJM290.3
SM116	Δ*ctxABN4* Kan^r *str-114*	From SM114 by marker exchange with pJM290.3
Plasmids		
pRK290	IncP Tc^r	30
pJM290.3	Tc^r Δ*ctxABN4* Km^r	This work

[a] Designations correspond to previously established conventions for naming markers and alleles (10).
[b] The Kan^r-3 designation indicates the presence of approximately 20 tandem copies of the Δ*ctxABN4* Kan^r allele.
[c] The Kan^r-3L designation indicates the reduction of the copy number of the Δ*ctxABN4* Kan^r-3 allele from 20 copies to approximately 2 to 3 copies per genome.

rying the Δ*ctxABN4* Kan^r allele were grown overnight in LB medium containing 45 μg of kanamycin per ml. The cultures were diluted 10-fold into 10 ml of LB and grown at 37°C for several hours. After concentrating the cell suspension 10-fold, dilutions were plated onto LB plates containing 3 mg of kanamycin per ml. Plates were incubated at 37°C overnight.

Isolation of Kan^s derivatives of strains containing the ΔctxABN4 Kan^r allele. Cultures were diluted 1:2,000 into fresh LB broth daily and grown with agitation at 37°C. After about 100 generations, the cultures were plated onto LB medium so as to obtain approximately 300 colonies per plate and replica plated onto LB-kanamycin plates. After overnight incubation at 30°C, Kan^s colonies were picked and repurified on LB plates. Chromosomal DNA was prepared from all Kan^s colonies, and the structure of the *ctx* region was analyzed by Southern blot hybridization.

Nucleic acid preparation and analysis. Chromosomal DNA was prepared as described by Mekalanos (21). Restriction enzymes were purchased from New England Biolabs. Genomic digests were fractionated on 0.7% agarose gels in TEA buffer and transferred to nitrocellulose sheets as described by Southern (32).

Hybridization of radioactive probes to the nitrocellulose was performed as previously described (21). The L-1 probe is a 2.5-kb *Pst*I-*Xba*I fragment from plasmid pGP6, and the L-3 probe is an RS1-specific 2.7-kb *Bgl*II fragment from the same plasmid (21). The CT-1 probe is a 918-base pair *Xba*I-*Hin*cII fragment from plasmid pJM17 (28). The location of these probes on the *ctx* genetic element's restriction endonuclease cleavage map is shown in Fig. 1. CsCl-purified DNA of each plasmid was simultaneously digested with the two specified restriction endonucleases overnight. The DNA was phenol extracted, ethanol precipitated, and nick translated with [^{32}P]dATP (800 Ci/mmol; Amersham Corp. Arlington Heights, Ill.). The desired fragment was subsequently isolated from a 1% low-gelling-temperature agarose gel (type BII; Sigma Chemical Co., St. Louis, Mo.). The DNA was extracted from the agarose with phenol and chloroform according to Maniatis et al. (19) and ethanol precipitated prior to its use as a radioactive probe in Southern blot hybridizations.

RESULTS

Amplification of the cholera toxin genetic element in derivatives of El Tor strains P27459 and E7946. Amplification of the *ctx* region has been previously observed after intestinal growth of *V. cholerae* in laboratory animals (21). We investigated the possibility that high-level resistance to kanamycin might be used to select for cholera toxin gene amplification in derivatives that have had the *ctx* genetic element modified with a DNA fragment encoding resistance to kanamycin. Plasmid pJM290.3 (Fig. 2) was used to recombine the Δ*ctxABN4* Kan^r allele onto the chromosome of strain SM43, a Str^r derivative of strain P27459. The Δ*ctxABN4* Kan^r allele is an internal deletion of the *ctxA* and *ctxB* genes substituted with a fragment encoding resistance to kanamycin (see Materials and Methods and Fig. 2). The resultant strain, SM44, is therefore kanamycin resistant but produces neither the A nor the B subunit of cholera toxin.

Strain SM44 showed nearly the same efficiency of plating on media containing 150 μg of kanamycin per ml as on media containing no antibiotic. Media containing higher levels of kanamycin produced a marked drop in the plating efficiency of this strain. This result suggsts that a single copy of the Δ*ctxABN4* Kan^r allele encodes resistance to approximately 150 μg of kanamycin per ml. We then isolated derivatives of SM44 which were able to grow on LB plates containing 3 mg of kanamycin per ml. Such derivatives were obtained at a frequency of 9.4×10^{-8}.

Colonies resistant to this concentration of kanamycin were grown in the presence of 3 mg of kanamycin per ml and analyzed by Southern blot hybridization (Fig. 3). The lower bands (6.2 and 8.9 kb) in Fig. 3 represent the DNA sequences spanning the novel joints formed during duplication and amplification of the *ctx* genetic element. This was demonstrated by showing that these amplified bands also hybridized to the CT-1 probe (Fig. 1), while the 20-kb bands did not (data not shown). The CT-1 probe detects DNA sequences which remain downstream of the *Xba*I sites flanking the Km^r fragment of the Δ*ctxABN4* Kan^r allele (that is, the DNA between the *Nde*I site located early in the *ctxB* gene and the *Hin*cII site located in the middle of the *ctxB* gene). As discussed in detail in a earlier report (21), chromosomal *Xba*I fragments that hybridize to both the CT-1 probe and the L-1 probe must represent tandem fragments (Fig. 3). Moreover, the hybridization intensity of such a tandem fragment is proportional to the degree of *ctx* amplification (21). Densitometry analysis indicates that the strains able to grow on 3 mg of kanamycin per ml contain about 20 tandemly amplified *ctx* genetic elements (data not shown).

Strain SM44 is able to amplify its *ctx* element with either one or two RS1 copies in between (Fig. 3, lanes 3 and 4). The 6.2-kb fragment occurs when one RS1 is present at the novel joint, and the 8.9-kb fragment, 2.7 kb larger, is seen when two RS1 copies are located at this position. The discrepancy between these molecular masses and the usual sizes of 7.0 and 9.7 kb observed for tandem signals in other *V. cholerae* strains (21) is due to the *Nde*I-*Nde*I deletion present in the Δ*ctxABN4* Kan^r allele in these strains. The Km^r insert does not contribute to the size of these fragments because it is precisely excised by restriction enzyme *Xba*I used in this analysis. The broad bands of hybridization seen in Fig. 3 are probably due to degradation or random shearing (or both) of the chromosomal DNA of these amplified derivatives. This smearing occurs when other restriction enzymes are used in the analysis and is not as prominent when less DNA is loaded on gels (see Fig. 4 and 5). Because these DNA preparations are each derived from single colonies resistant to 3 mg of kanamycin per ml and this smearing is never resolved into any other distinct bands of single-copy intensity, we conclude that the amplification observed is occurring in the tandemly repeated structures shown in the lower part of Fig. 3. We cannot rule out the possibility that in a minority of colonies amplification has also occurred by nontandem duplication (transposition) of the *ctx* genetic element.

In addition to SM44, we constructed similar Δ*ctxABN4* Kan^r allele-containing derivatives of E7946, a clinical isolate with two tandem *ctx* elements (21). The two resident toxin operons of the E7946 derivative strain SM114 were exchanged for the Δ*ctxABN4* Kan^r allele present on plasmid pJM290.3 by the marker exchange procedure used in the construction of SM44.

Several Km^r SM114 isolates were analyzed for the structure of their *ctx* region by Southern blot hybridization with the L-1 probe. Two types of Km^r recombinants were identified. Type 1 recombinants (SM115) have a Δ*ctxABN4* Kan^r allele recombined into each of the two *ctx* elements originally present in E7946 (Fig. 4, lane 1). Type 2 recombinants (SM116) have both of their resident *ctx* elements replaced by a single copy of the ΔctxABN4 Kan^r allele and appear to be identical to strain SM44 on Southern blots (Fig. 4, lane 5).

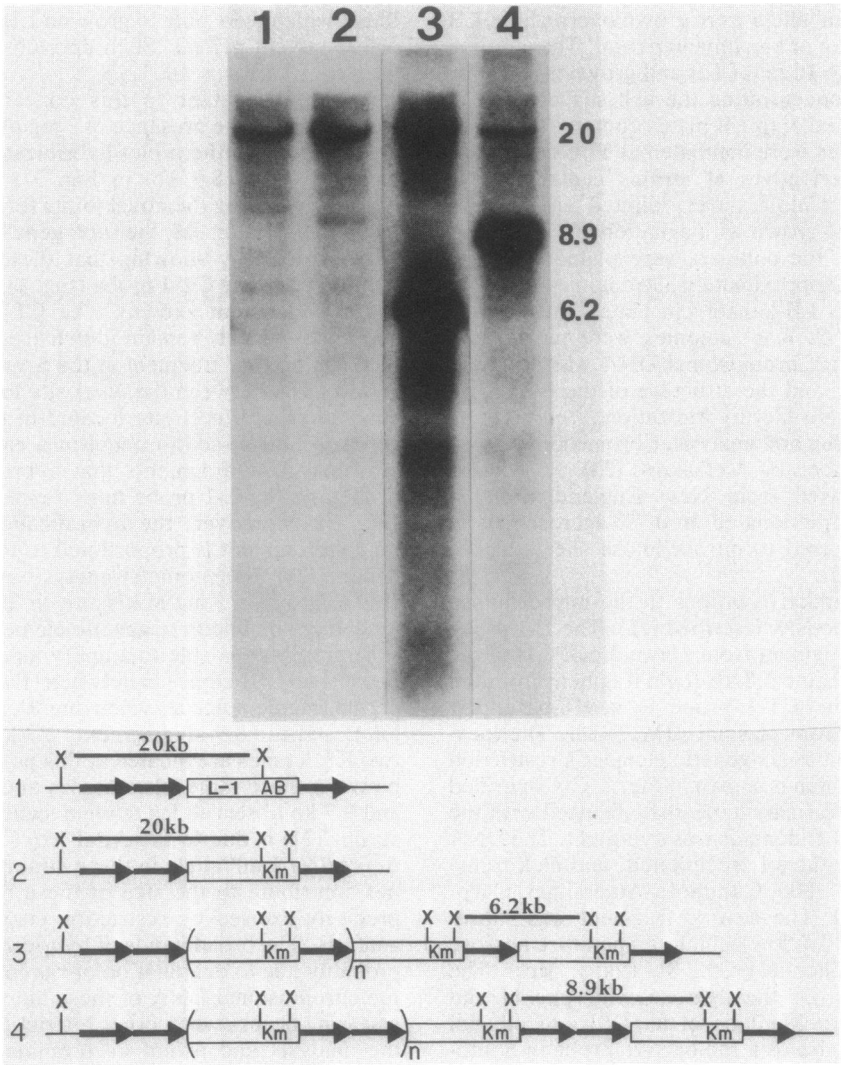

FIG. 3. Southern blot analysis of *ctx* region amplification in *V. cholerae* strain SM44. Chromosomal DNAs of SM43, SM44, and derivatives of SM44 resistant to 3 mg of kanamycin per ml were digested with *Xba*I, electrophoresed in a 0.7% agarose gel, and transferred to nitrocellulose paper. Southern blot hybridizations were performed with the radioactive L-1 probe (see Fig. 1). Numbers on the right refer to the size of bands in kilobases. Lane 1, SM43; lane 2, SM44; lanes 3 and 4, two different derivatives of SM44 resistant to 3 mg of kanamycin per ml. A schematic diagram of the corresponding amplification events is given at the bottom. Arrows and boxes indicate RS1 and core sequences, respectively. L-1 gives the location of sequences in the core region that hybridize to the L-1 probe. The bracketed regions show the two types of amplified tandem repeats observed. Digestion of DNA carrying these repeats with the enzyme *Xba*I (X) gives tandem fragments that are either 6.2 or 8.9 kb in size.

The former recombinant class is characterized by the appearance of two bands in Southern blots of 20 and 8.9 kb, while the latter does not contain the lower-molecular-weight band, which is characteristic for the tandemly duplicated *ctx* genetic element.

Derivatives of SM115 and SM116 resistant to 3 mg of kanamycin per ml were obtained at frequencies of 3.2×10^{-8} and 5.6×10^{-9}, respectively. This result suggests that a derivative of a given strain which has two copies of the *ctx* genetic element amplifies it more efficiently than a derivative that has a single copy. Figure 4 shows that amplification of the *ctx* region in these derivatives can be detected by staining of the agarose gel with ethidium bromide as well as by hybridization.

SM115 always amplified its *ctx* elements with two RS1 copies in between (Fig. 4, lanes 1 through 4). SM116, however, which contains only one Δ*ctxABN4* Kan^r allele, can amplify with either one (lanes 6 and 7) or two (lane 8) RS1 copies between its *ctx* elements. Thus, the number of RS1 copies present at the novel joint of a *ctx* duplication tends to be maintained after subsequent amplification.

Effect of a *recA* mutation on cholera toxin gene amplification. The ability to select amplification events in the laboratory allowed us to test whether this process was dependent on homologous recombination. Therefore, the *recA24* mutant strain SM106 and its parent (SM105), which both carry the Δ*ctxABN4* Kan^r allele (11), were subjected to the amplification protocol. While derivatives of SM105 which grew normally on 3 mg of kanamycin per ml could be easily isolated, no derivatives of SM106 could be obtained which grew well on this concentration of kanamycin. Southern blot hybridization was performed on the largest colonies obtained for both strains (Fig. 5). SM105 had amplified its *ctx* copy, as expected, with either one or two RS1 copies at the

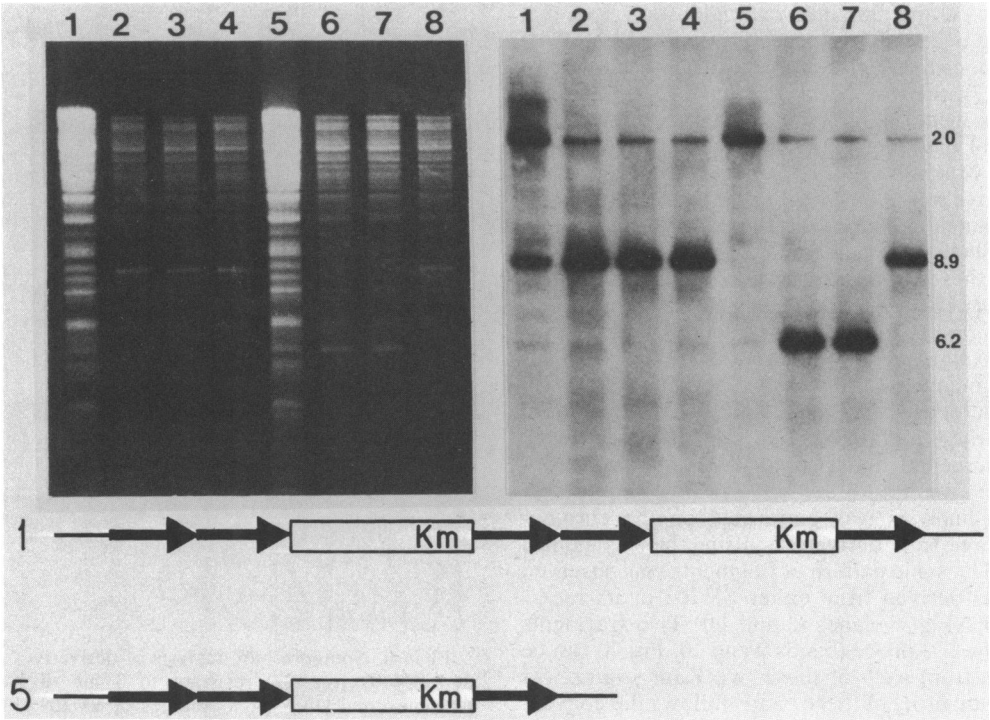

FIG. 4. Southern blot analysis of *ctx* region amplification in *V. cholerae* strains SM115 and SM116. Chromsomal DNAs from SM115, SM116, and isolates of these two strains resistant to 3 mg of kanamycin per ml were prepared and digested with restriction endonuclease *Xba*I. After agarose gel electrophoresis, the gel was stained with ethidium bromide (0.5 μg/ml) and photographed with Polaroid 667 film under UV light with a yellow filter (left panel). Lane 1, SM115; lanes 2 through 4, SM115 derivatives resistant to 3 mg of kanamycin per ml; lane 5, SM116; lanes 6 through 8, SM116 derivatives resistant to 3 mg of kanamycin per ml. Approximately 10 times as much DNA was loaded in lanes 1 and 5 as in the other lanes. The organization of the *ctx* region of SM115 and SM116 is given at the bottom. The organization of the amplified regions in lanes 2 to 4 and 6 to 8 are shown in the lower half of Fig. 3 depending upon whether the strain displays an 8.9- or 6.2-kb amplified band.

novel joint (lanes 2 and 3). However, no derivatives of strain SM106 with more than one copy of the *ctx* element were observed (lanes 5 through 8).

Although the chromosomal DNAs derived from only four colonies are represented in Fig. 5, a total of 10 colonies at least partially resistant to 3 mg (or 600 μg) of kanamycin per ml were analyzed and all demonstrated the same hybridization pattern. Attempts to obtain amplified derivatives of the *recA* mutant strain SM106 have failed even when batch culture of this strain were grown serially in media containing increasing amounts of kanamycin up to a final concentration of 3 mg/ml. Thus, resistance to high levels of kanamycin in the *recA* strain SM106 occurs by a mechanism other than duplication or amplification of the Δ*ctxABN4* Kan^r allele. These results indicate that amplification of the *ctx* element is dependent on the *recA* gene product.

The amplification of a single *ctx* genetic element can be formally divided into two steps: the initial duplication which generates two directly repeated copies of the *ctx* core region, and the subsequent amplification of this structure (21). We therefore considered the possibility that amplification of the *ctx* region might be *recA* independent for strains that already carry a duplication of the core region of the *ctx* element.

To address this possibility, a procedure identical to the one described for the construction of strain SM106 (10) was used to introduce the *recA24* mutation into an SM44 derivative (SM107) which had previously amplified its resident *ctx* genes (Fig. 3) but had subsequently lost its amplified state. During the course of these studies we observed that constant selection pressure was necessary to maintain the amplified *ctx* regions of derivatives of SM44 resistant to 3 mg of kanamycin per ml. Within 25 generations of growth in TYCC medium, only 50% of the colonies obtained from strain SM107 were still able to grow on 3 mg of kanamycin per ml. We used this observation to facilitate the construction of a *recA* strain carrying two tandem copies of the *ctx* element. During the introduction of the *recA24* mutation into strain SM107, this strain was not maintained on media containing high levels of kanamycin. As can be seen by Southern blot hybridization (Fig. 6, lane 3), the resultant strain, SM109 (*recA*), had lost the amplified *ctx* region of SM107 and had reduced its copy number of *ctx* elements to approximately two copies per genome. Concomitant with the loss of the amplified structure, high-level resistance to 3 mg of kanamycin per ml was lost.

We then attempted to increase the copy number of *ctx* elements in strain SM109 once again by growth on 3 mg of kanamycin per ml. However, no colonies of a size comparable to those of SM107 were observed on plates containing this high concentration of kanamycin. Twelve of the largest colonies present were picked and their chromosomal DNA was analyzed by Southern blot hybridization. As can be seen for four of these derivatives (Fig. 6, lanes 4 through 7), no amplification of *ctx* copies was observed in these *recA* mutants. Thus, the presence of an initial core duplication did not aid the amplification process in the *recA* strain SM109.

Isolation of Kan^s derivatives of strains SM105 and SM106. The results presented in the previous section indicate that amplification of the *ctx* region is dependent on homologous recombinational events. Moreover, the structure of the

tandemly duplicated and amplified *ctx* regions suggests that unequal crossing over between directly repeated copies of RS1 may be responsible for these events (21). According to this model (Fig. 7), the reciprocal products of such unequal crossover events would be deletion mutations that remove the central part of the *ctx* genetic element corresponding to the core region. Mutants of *V. cholerae* carrying these *ctx* core deletions have not been previously observed (21).

We have obtained deletions of the *ctx* core region in SM105 and SM106 by taking advantage of the fact that such deletions render these strains sensitive to kanamycin, due to the loss of the Δ*ctxABN4* Kan^r allele. Thus, Kan^s colonies of these two *V. cholerae* strains were identified by replica plating after approximately 100 generations of growth in LB broth without kanamycin. For strain SM105, about 1,500 colonies were screened and nine Kan^s derivatives were found. For the *recA* mutant SM106, approximately 7,000 colonies were screened and two Kan^s isolates were obtained.

Southern blot analysis was performed on the chromosomal DNA of three Kan^s derivatives, using the L-2 and L-3 probes (Fig. 8). The same pattern of fragments was observed for Kan^s variants derived from either SM105 or its *recA*$^-$ derivative SM106 (Fig. 8, lanes C and D). Two fragments homologous to the L-2 probe (bands 9 and 10, Fig. 8) can be seen when DNA from each of these two Kan^s strains was analyzed. The numbering of these bands follows the convention of a previously published analysis of the *ctx* region (21). When hybridized to the L-3 probe, DNA from these two Kan^s strains (SM117 and SM118) also had band 6 (data not shown). In another experiment, a Kan^s derivative of SM44 was obtained by a similar procedure, and DNA from this strain (GP100) gave in this analysis only bands 6 and 9 with the L-3 probe (Fig. 8, lane H) and only band 9 with the L-2 probe (data not shown; G. Pearson, and J. Mekalanos, unpublished results).

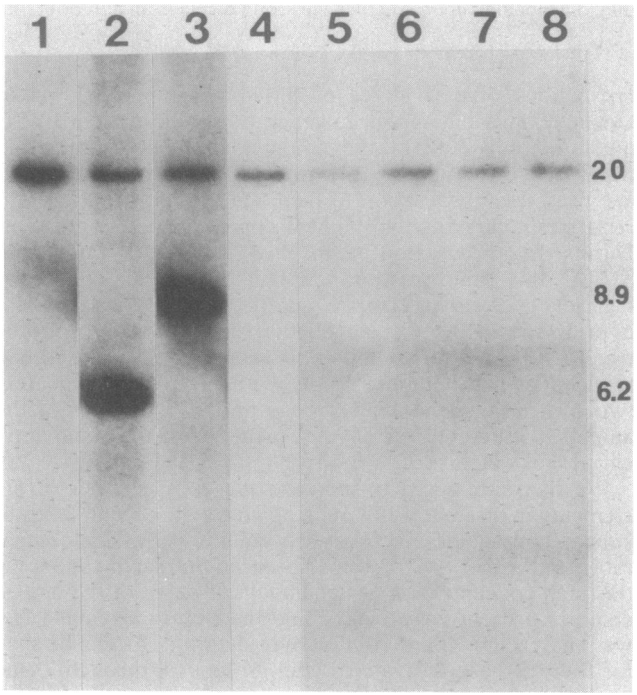

FIG. 5. Southern blot analysis of derivatives of SM105 (*recA*$^+$) and SM106 (*recA24*) resistant to 3 mg of kanamycin per ml. Chromosomal DNAs were digested with *Xba*I and processed for Southern blot hybridization with the L-1 probe. Lane 1, SM105; lanes 2 and 3, derivatives of SM105 able to grow on 3 mg of kanamycin per ml; lane 4, SM106; lanes 5 through 8, isolates of SM106 able to grow on 3 mg of kanamycin per ml.

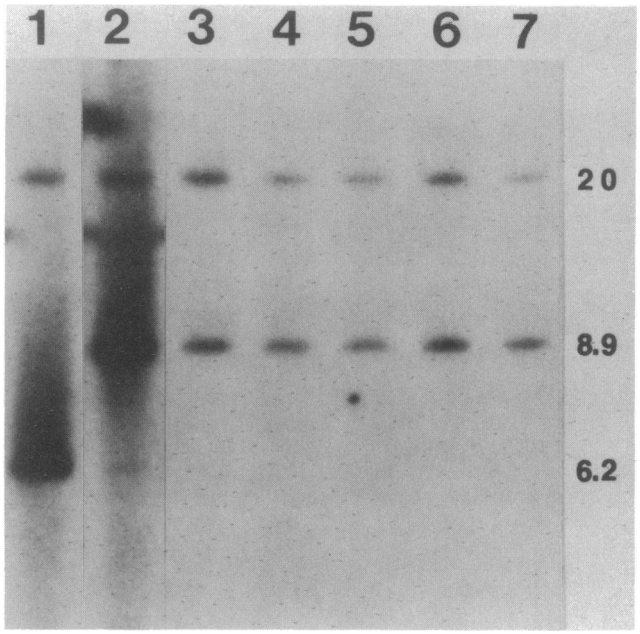

FIG. 6. Southern blot analysis of derivatives of SM105 (*recA*$^+$) and SM109 (*recA24*) resistant to 3 mg of kanamycin per ml. Chromosomal DNA from colonies of SM109 able to grow on this concentration of kanamycin was digested with *Xba*I and processed for Southern blot hybridization with the L-1 probe. Lanes 1 and 2, Amplified derivatives of SM105; lane 3, SM109; lanes 4 to 7, isolates of SM109 able to grow on 3 mg of kanamycin per ml.

The sizes of these fragments indicate that two RS1 copies, in tandem remain on the bacterial chromosome at the original site occupied by the *ctx* genetic element in the Kan^s derivatives SM117 and SM118. In contrast, the Kan^s strain GP100 has only a single RS1 copy left at the same site on the chromosome. Core deletions of both types are predicted by the unequal crossover model presented in Fig. 7.

These data demonstrate that recombination between RS1 copies (Fig. 7) is probably responsible for the *ctx* deletion events observed here. Furthermore, these recombination events still occur in the presence of the *recA24* mutation but at a 21-fold reduced frequency when compared with the *recA*$^+$ case.

DISCUSSION

The kanamycin resistance phenotype of the ΔctxABN4 Kan^r allele has allowed us to study amplification of the *ctx* genetic element and to demonstrate that *ctx* amplification can be selected in the laboratory with high levels of kanamycin. When amplified strains were grown in the absence of kanamycin, the copy number of the *ctx* element decreased, consistent with the reported instability of such tandemly repeated DNA in other systems (1, 20, 35–37).

The mechanism by which the *ctx* region is amplified appears to depend upon an initial duplication generated by unequal crossing over between RS1 sequences (Fig. 7). If unequal crossovers are repeated a few times, large tandem arrays can quickly be accumulated. In our experiments,

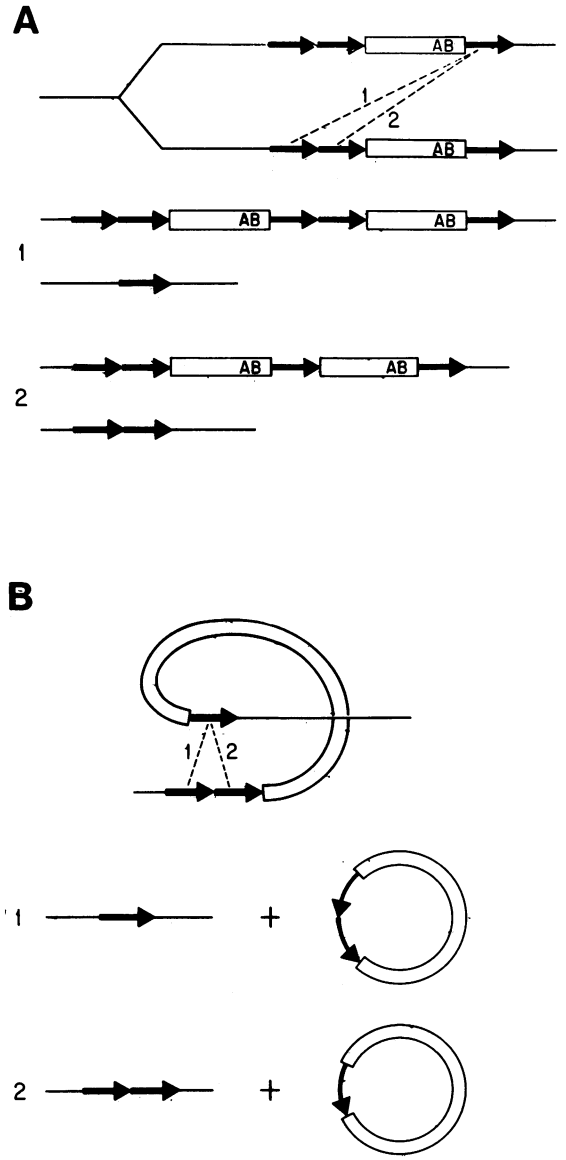

FIG. 7. Models for the generation of *ctx* deletion and amplification events. (A) Schematic representation of unequal crossover events between RS1 sequences. Shown is the *ctx* region of a typical El Tor strain such as P27459, which carries a total of three copies of RS1 (arrows) flanking a core region (open box) containing the *ctxAB* operon. Unequal homologous recombination (dashed lines) could occur at two different positions to produce a tandem duplication of the *ctx* region. For position 1, the products are a 9.7-kb tandem duplication and, if reciprocal, a deletion that retains one RS1 copy at the deletion junction. For position 2, the products are a 7-kb tandem duplication and a deletion that retains two tandem RS1 copies at the deletion junction. (B) Schematic representation of possible *ctx* operon deletion events via intramolecular recombination. The example presented here shows a *ctx* core region (open box) flanked by one RS1 (arrow) at one end and two RS1 copies at the opposite end. Recombination between the RS1 copies would result in one (event 1) or two (event 2) RS1 copies remaining on the chromosome and the formation of a circular segment of DNA consisting of a core region with either two or one copy of RS1, respectively.

strains SM44 and SM116 produced amplified structures with wither one or two RS1 copies between the *ctx* core regions. If unequal crossing over occurred randomly among RS1 copies subsequent to the initial core duplication event, it would be expected that most amplified derivatives would contain a variable number of RS1 copies between the repeated *ctx* core regions. Instead, the amplification process appears to be very specific in that strains tend to maintain either one or two RS1 copies located at the novel joints. Moreover, a strain that starts out with a give type of *ctx* tandem duplication always maintained the same number of RS1 between the core regions after amplification.

These results suggests that, unlike the initial core duplication event, the amplification mechanism may be largely independent of recombinational events occuring with RS1. Thus, the core region of the *ctx* element might be the preferred site for the unequal crossover events that ultimately lead to amplification. This may be due to the core's larger size (4.5 kb) relative to RS1 (2.7 kb) or other factors.

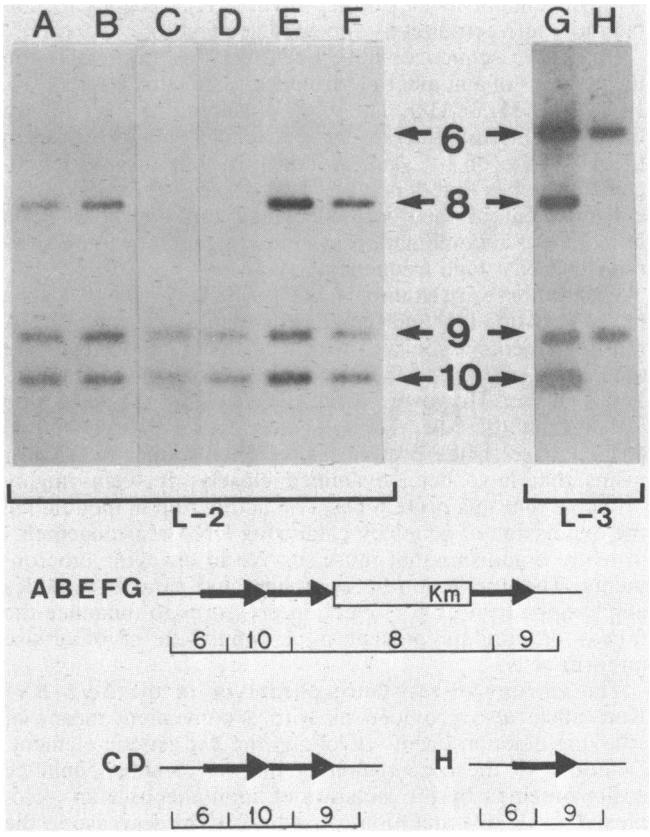

FIG. 8. Southern blot analysis of spontaneous Kan^s isolates of *recA*$^+$ and *recA V. cholerae*. (Top) Chromosomal DNAs from SM105 (lane A), SM106 (lane B), SM44 (lane G), and Kan^s derivatives of these strains (lane C, SM117; lane D, SM118; lane H, GP100) were digested with restriction endonuclease *Bgl*II. The DNA fragments were electrophoresed in a 0.7% agarose gel and processed for Southern blot hybridization with the L-2 and L-3 probes (see Fig. 1). Two other Kan^r derivatives of strain SM105 are shown in lanes E and F for comparison. (Bottom) A schematic representation of the analysis is shown. The numbering of the various fragments corresponds to the convention of Mekalanos (21). The approximate position of fragment 6 is indicated and was present in lanes A through F when hybridized with the L-3 probe (data not shown). Sizes are given in kilobase pairs: (6) 16.0, (8) 7.6, (9) 3.5, (10) 2.7.

Whatever the reason, the formation of a *ctx* core duplication is apparently the rate-limiting step in the amplification process. This conclusion is supported by the observation that a derivative which contained a duplication of the Δ*ctxABN4* Kan^r allele amplified its *ctx* regions six times more efficiently than one carrying only a single copy of this allele.

We also examined whether duplication of the *ctx* element and its subsequent amplification were dependent on homologous recombination. In the *recA* strain SM106, we have not observed the presence of *ctx* core duplications among derivatives able to grow on 3 mg of kanamycin per ml. Moreover, even a strain that started with a duplicated *ctx* region (SM109) was unable to amplify this region further in the presence of the *recA24* allele. Thus, within the resolution of the experiments reported here, *ctx* amplification is *recA* dependent regardless of whether the critical unequal crossover events occur within the core region of the *ctx* element or within RS1 sequences.

The above observations are largely consistent with the literature (1, 4–6, 12, 20, 35–37, 39). In most cases, gene duplication and amplification events have been shown to be *recA* dependent, because they involve homologous recombination between directly repeated sequences.

Repetitive sequences have been found to be involved in the genetics of a number of virulence-associated traits (2, 11, 14, 15, 17, 31, 33, 38). The work presented here has raised the possibility that the *recA* gene may play an indirect role in the virulence of *V. cholerae* and other organisms. Pathogenic microbes that depend on DNA rearrangements for the enhancement of their virulence may turn out to rely on homologous recombination systems to generate these rearrangements at high frequency.

Clearly there are examples, such as *Salmonella* sp. flagella phase variation (41) and type 1 pili variation (9), where DNA rearrangements associated with changes in gene expression are catalyzed by *recA*-independent, site-specific recombination systems. However, these may well be the exception rather than the rule. Homologous recombination, and in particular *recA*-like proteins, have been found in all organisms that have been examined closely. It seems highly probable that this protein plays an active role in modulating the expression of genes by catalyzing DNA rearrangements in many organisms that must survive in changing environments. The interaction between host and parasite provides ample oppourtunity for selection pressures to influence the fitness of variants present in a population of otherwise uniform cells.

The kanamycin resistance phenotype of the Δ*ctxABN4* Kan^r allele also provided us with a convenient means of studying deletion events involving the *ctx* genetic element. Deletions of the core region of the *ctx* element could be easily obtained by the isolation of spontaneous Kan^s colonies. The *recA24* mutation caused a 21-fold decrease in the frequency of Kan^s colonies recovered in strain SM106 compared with strain SM105. Southern blot analysis revealed that, in all cases, one or two RS1 copies remained on the bacterial chromosome after loss of the Δ*ctxABN4* Kan^r allele.

The simplest mechanisms for the formation of *ctx* deletions in a *recA*$^+$ background involve homologous recombination events between RS1 copies. Two formal possibilities exist. First, *ctx* deletions can occur during replication by an unequal crossover event between the RS1 posterior to the *ctx* operon on one daughter DNA strand and either one of the two RS1 copies anterior to the *ctx* operon on the other daughter strand (Fig. 7A). In this proposed mechanism, the deletion event is the reciprocal of the duplication event described earlier but occurs on the other daughter strand, producing either one or two tandem copies of RS1. Alternatively, the deletion events can take place by a loop-out mechanism in which the DNA in between two RS1 copies is excised as a circle (Fig. 7B).

The *ctx* deletion events observed in the *recA* background could occur by a similar recombinational mechanism since these events also left behind two RS1 copies on the chromosome. However, the source of the recombinational activity responsible for the deletions in the *recA* strain is not clear. The deletions might be promoted by residual *recA*-mediated recombinational activity associated with the *recA24* mutation or possibly an alternate *recA*-independent recombinational pathway (16). Deletions of the central region of Tn*9* mediated by flanking direct repeats of IS*1* have been shown to occur in *recA E. coli* and to be mediated by an IS*1*-specific product (3). Similar site-specific recombinational activity has been observed with the transposon Tn*3* (18). It is therefore possible that the *recA*-independent *ctx* deletion events might be mediated by site-specific recombinational activity associated with the RS1 sequence. The possibility that RS1 may be a type of *V. cholerae* insertion sequence is supported by its occasional transposition in *V. cholerae* and its involvement in several novel recombinational events (I. Goldberg, G. Pearson, and J. Mekalanos, manuscript in preparation).

ACKNOWLEDGMENTS

We thank Alison Delong for her help in plasmid constructions.

This work was supported by Public Health Service grant AI-18045 from the National Institutes of Allergy and Infectious Disease.

LITERATURE CITED

1. **Anderson, R. P., and J. R. Roth.** 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. **31**:473–504.

1a. **Betley, M. J., and J. J. Mekalanos.** 1985. Staphylococcal enterotoxin A is encoded by phage. Science **229**:185–187.

2. **Borst, P., and G. A. M. Cross.** 1982. Molecular basis for typanosome antigenic variation. Cell **29**:291–303.

3. **Braedt, G.** 1985. Recombination in *recA* cells between direct repeats of insertion element Is*1*. J. Bacteriol. **162**:529–534.

4. **Chandler, M., E. Boy de la Tour, D. Willems, and L. Caro.** 1979. Some properties of the chloramphenicol resistance transposon Tn*9*. Mol. Gen. Genet. **176**:221–231.

5. **Edlund, M. W., and F. T. Poysky.** 1974. Interconversion of type C and D strains of *Clostridium botulinum* by specific bacteriophages. Appl. Microbiol. **27**:251–258.

6. **Edlund, T., and S. Normark.** 1981. Recombination between short DNA homologies causes tandem duplication. Nature (London) **292**:269–271.

7. **Finn, C. W., R. P. Silver, W. H. Habig, M. C. Hardegree, G. Zon, and C. F. Garon.** 1984. The structural gene for tetanus neurotoxin is on a plasmid. Science **224**:881–884.

8. **Freeman, V. J.** 1951. Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae*. J. Bacteriol. **61**:675–688.

9. **Freitag, C. S., J. M. Abraham, J. R. Clements, and B. I. Eisenstein.** 1985. Genetic analysis of the phase variation control of expression of type 1 fimbriae in *Escherichia coli*. J. Bacteriol. **162**:668–675.

10. **Goldberg, I., and J. J. Mekalanos.** 1985. Cloning of the *Vibrio cholerae recA* gene and construction of a *Vibrio cholerae recA* mutant. J. Bacteriol. **165**:715–722.

11. **Goldson, G. N., J. Ellis, P. Svec, D. H. Schlesinger, and V. Nussenzweig.** 1983. Identification and chemical synthesis of a tandemly repeated immunologenic region of *Plasmodium*

knowlesi circumsporozoite protein. Nature **290**:29–32.
12. **Gutterson, N. I., and D. E. Koshland.** 1983. Replacement and amplification of bacterial genes with sequences altered *in vitro*. Proc. Natl. Acad. Sci. USA **80**:4894–4898.
13. **Gyles, C., M. So, and S. Falkow.** 1974. The enterotoxin plasmids of *Escherichia coli*. J. Infect. Dis. **130**:40–49.
14. **Hacker, J., S. Knapp, and W. Goebel.** 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an *Escherichia coli* O6 strain. J. Bacteriol. **154**:1145–1152.
15. **Hagblom, P., E. Segal, E. Billyard, and M. So.** 1985. Intragenic recombination leads to pilus antigenic variation in *Neisseria gonorrhoeae*. Nature (London) **315**:156–158.
16. **Jones, I. M., and S. B. Primrose, and S. D. Ehrlich.** 1982. Recombination between short direct repeats in a *recA* host. Mol. Gen. Genet. **188**:486–489.
17. **Knapp, S., J. Hacker, I. Then, D. Mueller, and W. Goebel.** 1984. Multiple copies of hemolysin genes and associated sequences in the chromosomes of uropathogenic *Escherichia coli* strains. J. Bacteriol. **159**:1027–1033.
18. **Krasnow, M. A., and N. R. Cozzarelli.** 1983. Site-specific relaxation and recombination by Tn*3* resolvase: recognition of the DNA path between oriented *res* sites. Cell **32**:1313–1324.
19. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
20. **Mattes, R., H. J. Burkhardt, and R. Schmitt.** 1979. Repetition of tetracycline resistance determinant genes on R plasmid pRSD1 in *Escherichia coli*. Mol. Gen. Genet. **168**:173–184.
21. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.
22. **Mekalanos, J. J., D. J. Swartz, G. D. N. Pearson, N. Harford, F. Groyne, and M. de Wilde.** 1983. Cholera toxin genes: nucleotide sequence, deletion analysis and vaccine development. Nature (London) **306**:551–557.
23. **Meyer, J., and S. Iida.** 1979. Amplification of chloramphenicol resistance transposons carried by phage P1Cm in *Escherchia coli*. Mol. Gen. Genet. **176**:209–219.
24. **Mikesell, P., B. E. Ivins, J. D. Ristroph, and T. D. Dreier.** 1983. Evidence for plasmid-mediated toxin production in *Bacillus anthracis*. Infect. Immun. **39**:371–376.
25. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Miller, V. L., and J. J. Mekalanos.** 1984. Synthesis of cholera toxin is positively regulated at the transcriptional level by *toxR*. Proc. Natl. Acad. Sci. USA **81**:3471–3475.
27. **O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal.** 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis on infantile diarrhea. Science **226**:694–696.
28. **Pearson, G. D. N., and J. J. Mekalanos.** 1982. Molecular cloning of the *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA **79**:2976–2980.
29. **Rownd, R. H., D. Perlman, and N. Goto.** 1975. Structure and replication of R factor DNA in *Proteus mirabilis*, p. 74. *In* D. Schlessinger (ed.), Microbiology—1974. American Society for Microbiology, Washington, D.C.
30. **Ruvkun, G. B., and F. M. Ausubel.** 1981. A general method for site-directed mutagenesis in procaryotes. Nature (London) **289**:85–88.
31. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat-stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature (London) **277**:453–456.
32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Spanier, J. G., S. J. C. Jones, and P. Cleary.** 1984. Small DNA deletions creating avirulence in *Streptococcus pyogenes*. Science **225**:935–938.
34. **Sporecke, I., D. Castro, and J. J. Mekalanos.** 1984. Genetic mapping of the *Vibrio cholerae* enterotoxin structural genes. J. Bacteriol. **157**:253–261.
35. **Tlsty, T. D., A. M. Albertini, and J. H. Miller.** 1984. Gene amplification in the *lac* region of *E. coli*. Cell **37**:217–224.
36. **Wiebauer, K., S. Schraml, S. W. Shales, and R. Schmitt.** 1981. Tetracycline resistance transposon Tn*1721*: *recA*-dependent gene amplification and expression of tetracycline resistance. J. Bacteriol. **147**:851–859.
37. **Yagi, Y., and D. B. Clewell.** 1976. Plasmid-determined tetracycline resistance in *Streptococcus faecalis*: tandemly repeated resistance determinants in amplified forms of pAM 1 DNA. J. Mol. Biol. **102**:583–600.
38. **Yamamoto, T., and T. Yokota.** 1981. *Escherichia coli* heat-labile enterotoxin genes are flanked by repeated deoxyribonucleic acid sequences. J. Bacteriol. **145**:850–860.
39. **Young, M.** 1984. Gene amplification in *Bacillus subtilis*. J. Gen. Microbiol. **130**:1613–1621.
40. **Zabriskie, J. B.** 1964. The role of temperate bacteriophage in the production of erythrogenic toxin by group A Streptococci. J. Exp. Med. **119**:761–779.
41. **Zieg, J., M. Hilmen, and M. Simon.** 1978. Regulation of gene expression by site-specific inversion. Cell **15**:237–244.