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Cloning of the *Vibrio cholerae recA* Gene and Construction of a *Vibrio cholerae recA* Mutant

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A recombinant plasmid carrying the *recA* gene of *Vibrio cholerae* was isolated from a *V. cholerae* genomic library, using complementation in *Escherichia coli*. The plasmid complements a *recA* mutation in *E. coli* for both resistance to the DNA-damaging agent methyl methanesulfonate and recombinational activity in bacteriophage P1 transductions. After determining the approximate location of the *recA* gene on the cloned DNA fragment, we constructed a defined *recA* mutation by filling in an *XbaI* site located within the gene. The 4-base pair insertion resulted in a truncated RecA protein as determined by minicell analysis. The mutation was spontaneously recombined onto the chromosome of a derivative of *V. cholerae* strain P27459 by screening for methyl methanesulfonate-sensitive variants. Southern blot analysis confirmed the presence of the inactivated *XbaI* site in the chromosome of DNA isolated from one of these methyl methanesulfonate-sensitive colonies. The *recA V. cholerae* strain was considerably more sensitive to UV light than its parent, was impaired in homologous recombination, and was deficient in induction of a temperate vibriophage upon exposure to UV light. We conclude that the *V. cholerae* RecA protein has activities which are analogous to those described for the RecA protein of *E. coli*.

The RecA protein of *Escherichia coli* plays a pivotal role in homologous recombination and DNA repair (4, 29). Purified RecA protein has been shown to catalyze a variety of reactions in vitro, including proteolysis of the phage lambda repressor protein and the *lexA* gene product (7, 12, 24). This activity leads to induction of prophage lambda and activation of the SOS responses. RecA protein also catalyzes DNA strand exchanges (D-loop formation) which may be required for homologous recombination (14, 26).

Evidence is accumulating that RecA-like proteins with similar functions are present in a variety of both gram-negative and gram-positive bacteria. Recently, the *recA* genes of several bacterial species have been cloned and were shown to encode proteins analogous to the *E. coli* RecA protein (3, 9).

Vibrio cholerae is the etiologic agent of cholera, a diarrheal disease induced by the action of a protein exotoxin secreted by this organism. Recently, genetic analysis of cholera toxin has shown that the genes for this toxin are located on a genetic element that is present in toxinogenic strains but absent in nontoxinogenic *V. cholerae* (15, 20). The toxin genetic element can exist at multiple sites on the cholera chromosome (15, 28) and can undergo tandem duplication and amplification (15).

The involvement of homologous recombination in rearrangements involving the *ctx* genetic element has not been evaluated previously because recombination-deficient mutants of *V. cholera* have not been available. In this paper, we report the cloning and characterization of the *recA* gene of *V. cholerae*. An insertion mutation was introduced into the cloned DNA fragment, thereby inactivating the *recA* gene, and this mutation was recombined back onto the *V. cholerae* chromosome. The *V. cholerae recA* strain behave similarly to an *E. coli recA* mutant with respect to its sensitivity to DNA-damaging reagents, recombinational activity, and lysogenic prophage induction. The RecA proteins of *E. coli* and *V. cholerae* apparently have analogous activities. In an accompanying paper (6), the *V. cholerae recA* mutant described here is further characterized for its ability to perform cholera toxin gene duplication, amplification, and deletion events.

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 and mating procedures. LB medium, brain heart infusion, and M63 minimal medium have been described previously (19, 28). Adenine and thiamine were always added to M63 minimal medium to final concentrations of 20 and 2.5 μg/ml, respectively. TYCC medium contained 10 g of tryptone, 1 g of yeast extract, 1 g of dextrose, 8 g of NaCl, and 0.5 g of $CaCl_2$ per liter; the pH was adjusted to 7.8 with 4 N NaOH. Trimethoprim medium consisted of M63 medium containing 50 μg of thymine, 10 μg of trimethoprim, and 20 μg of methionine per ml. Plates contained 15 g of Bacto-Agar (Difco Laboratories, Detroit, Mich.) per liter. Soft agar for overlays consisted of TYCC medium and 5 g of Bacto-Agar per liter. When Thy^- strains were used, all media were supplemented with 100 μg of thymine per ml. Amino acids and nucleotide bases (Sigma Chemical Co., St. Louis, Mo.) were added as needed at a concentration of 100 μg/ml. Methyl methanesulfonate (MMS) plates were prepared by suspending 2 μl of MMS stock (Sigma) in 0.3 ml of LB and spreading this solution onto a plate containing 25 ml of LB medium.

Unless otherwise stated, antibiotics (Sigma) were present at the following concentrations: 100 μg of streptomycin or spectinomycin per ml, 15 μg of chloramphenicol per ml, and 50 μg of ampicillin per ml. Nalidixic acid was used at a concentration of 2 μg/ml to select spontaneous single-step resistant mutants. This low concentration of nalidixic acid

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype[a]	Other Information
V. cholerae strains		
P27459	Prototrophic	El tor biotype (15)
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 (6)
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
E. coli strains		
SY203	F$^-$ *araD* Δ(*lac pro*) *argE rif gyrA*	R. Isberg
SY327	F$^-$ *araD* Δ(*lac pro*) *argE rif nal recA56*	R. Isberg
P678-54	F$^-$ *thr leu thy lac gal xyl mal mtl*	2
MC4100	F$^-$ *araD139* Δ*lac strA thiA rpsL relA rbsR*	J. Beckwith
Plasmids		
pSJ5	P::Tn*1* Ap^r	8
pRK290	IncP Tc^r	25
pJM290.3	Tc^r Δ*ctxABN4* Km^r	6
pJM2013.9	pRK2013::Tn*9* Cm^r Km^r	18
pIS1	Ap^r *recA*$^+$	This work
pIS2	Ap^r *recA*$^+$	From pIS1
pIS18	Ap^r *recA*$^+$	From pIS1
pIS24	Ap^r *recA24*	From pIS18
pIS26	Ap^r *recA24*	From pIS18
pIS27	Ap^r *recA24*	From pIS24

[a] Designations correspond to previous established conventions for naming markers and alleles (8, 24, 28).

was used to avoid possible linkage problems associated with two-step, high-level resistance (22). Mating procedures were performed as described previously (28).

Construction of vibriophage VcA2 lysogens. *V. cholerae* strains were infected with vibriophage VcA2 (5) stocks by the agar overlay method (1) so as to obtain 10^3 to 10^4 plaques per plate. Plates were incubated at 30°C overnight. A sample of the overlay was suspended in LB broth, grown at 37°C with gentle shaking for 2 h, and streaked out for single colonies on TYCC plates. The colonies were purified by restreaking on TYCC plates and were confirmed to be vibriophage lysogens by stab inoculation into a lawn of phage-sensitive RV79 indicator cells. A zone of clearing around an individual colony indicated phage release and therefore lysogeny.

Transfer of plasmid pIS24 from *E. coli* into *V. cholerae*. Plasmid pIS24 was mobilized with the *tra*$^+$ plasmid pJM2013.9. *E. coli* SY327 containing pIS24 was mated to *E. coli* MM294(pJM2013.9) as follows. A loopful of freshly agar-grown cells of each strain was suspended in 1 ml of LB broth. A 20-μl sample of each resuspension was then mixed on a TYCC plate and incubated at 37°C for 3 to 4 h. The area of growth which appeared was collected with a loop and streaked onto an LB plate selecting for the presence of both plasmids. After overnight incubation at 37°C, cells from the confluent region of the plate were suspended in 1 ml of LB broth and mated to an Str^r *V. cholerae* strain (SM105) by the same method as above. *E. coli* cells were counterselected with streptomycin. *V. cholerae* colonies containing pIS24 were purified two times and tested for loss of the mobilizing plasmid pJM2013.9 by scoring for sensitivity to chloramphenicol.

Bacteriophage P1 transductions. For P1 transductions into SY327 derivatives, the procedure of Miller (19) was followed.

Isolation of Thy$^-$ mutants of *V. cholerae*. *V. cholerae* strains were grown overnight in 2 ml of LB-thymine broth. A 0.1-ml sample of the cell culture was plated onto a trimethoprim plate and incubated for 48 h at 37°C. Colonies were repurified on trimethoprim plates and tested for their Thy$^-$ phenotype on M63 plates with or without thymine.

Construction of a *V. cholerae* plasmid-based library. A genomic library constructed by Mekalanos et al. (18) was used as a source of the cloned *V. cholerae recA* gene. This library was constructed by insertion of 7- to 15-kilobase (kb) *Sau*3A partially digested fragments of *V. cholerae* 569B DNA into the *Bam*HI site of pBR327. The library was originally constructed in *E. coli* MS371 (F$^-$ *gal thi endA sbcB hsdR4 hsdM*$^+$). The plasmids making up this library were prepared from a mixed chloramphenicol-amplified culture and transformed into *E. coli* SY327 to screen for suppression of the *recA56* mutation.

Construction of *recA V. cholerae* strains. Strain SM105(pIS24) was grown overnight in 2 ml of LB-ampicillin-thymine. The culture was washed two times in M63-ampicillin broth. Cells were then diluted 1:20 into 10 ml of M63-ampicillin and grown at 37°C for 4 h with agitation. A 0.5-ml sample was transferred into 10 ml of LB-ampicillin-thymine and grown overnight. The next morning the culture was diluted 1:50 into LB-ampicillin-thymine and grown to an optical density at 590 nm of 0.3, corresponding to approximately 10^8 cells per ml. The culture was spread onto LB-ampicillin-thymine plates to give approximately 300 colonies per plate. After incubation at 30°C overnight, the plates were replica plated onto LB-ampicillin-thymine plates containing 2 μl of MMS. Colonies which appeared to be MMS sensitive were picked and restreaked onto LB-thymine plates two times to cure them of plasmid pIS24. They were then retested for MMS sensitivity. MMS^s colonies were streaked in a straight line across an LB-thymine plate, and sections of the plate were exposed to a General Electric G15T8 germicidal lamp for 0, 10, and 20 s to test for

sensitivity to UV light. A complete UV killing curve was constructed by using the procedure of Miller (19).

UV induction of lysogenic vibriophage VcA2. Overnight cultures of *V. cholerae* SM105(VcA2) and SM106(VcA2) were diluted 1:50 into 10 ml of LB-thymine and incubated at 37°C to an optical density at 590 nm of 0.6. The cells were collected by centrifugation and suspended in 10 ml of M63 salts. The resuspended cells were transferred to sterile petri plates and irradiated under a General Electric germicidal lamp for various periods of time. Bacteria were collected by centrifugation, suspended in 10 ml of LB-thymine, and incubated at 37°C for 1 h to allow for phage propagation. The cultures were then centrifuged at 10,000 rpm, and the supernatant fluids were titrated for viable phage on a lawn of RV79 indicator cells.

Thymine starvation procedure. Strains SM105 and SM106 were grown overnight in M63-thymine broth at 37°C. The cultures were diluted 1:20 into 10 ml of M63-thymine and grown at 37°C to an optical density at 590 nm of 0.2. The cells were collected by centrifugation and, after being washed two times in M63 salts, were suspended in an equal volume of M63 broth without thymine. The cultures were then incubated with shaking at 37°C. Samples were taken at various time intervals, and the number of viable cells was determined on LB-thymine plates.

Minicell analysis. One loopful of cells of a plasmid-carrying derivative of *E. coli* minicell strain P678-54 (2) was suspended in 1 ml of LB and inoculated into 500 ml of LB. The culture was incubated at 37°C with agitation overnight. The culture was divided into three centrifuge bottles and centrifuged at 6,000 rpm for 5 min in a Sorvall GSA rotor. The pellets were discarded and the supernatant fluid was recentrifuged at 9,000 rpm for 15 min. The small bacterial pellets were suspended in a total of 1.5 ml of BSG buffer (8.5 g of NaCl, 0.3 g of KH_2PO_4, 0.6 g of Na_2HPO_4, and 0.1 g of gelatin per liter) and vortexed vigorously for 1 min to dissociate the minicells. The suspension was layered onto a 35-ml 5 to 20% sucrose gradient formed in BSG buffer. After centrifuging at 4,500 to 5,000 rpm in an HB4 swinging-bucket rotor for 15 min, the upper three-quarters of the upper minicell band were carefully removed with a syringe and needle. The minicell band was centrifuged as described above, washed once with BSG, and rebanded on a second gradient. The band of minicells was collected as before and suspended in 1 to 2 ml of M63 medium containing all amino acids except methionine. A 1-ml sample of the suspension was incubated with shaking at 37°C for 1 h. At that time, 80 μCi of [^{35}S]methionine (>800 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added and the incubation was continued for 45 min. The radioactive label was chased with 0.1 ml of a 1% methionine solution for 5 min, and the minicells were pelleted in a Microfuge (Beckman Instruments, Inc., Fullerton, Calif.). The minicells were washed once with 1 ml of M63 salts, repelleted, and resuspended in 0.05 ml of sodium dodecyl sulfate loading buffer (0.0625 M Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.005% bromophenol blue). The sample was boiled for 3 to 5 min in a water bath and centrifuged for 2.5 min in a Microfuge to pellet any bacterial debris. The supernatant was recovered and stored at −20°C until use.

Samples (5 to 20 μl) were electrophoresed on a 12% sodium dodecyl sulfate-polyacrylamide gel (11) at 40 V overnight. Molecular weight marker proteins were phosphorylase B (M_r 97,400), bovine serum albumin (M_r 69,000), ovalbumin (M_r 46,000), carbonic anhydrase (M_r 30,000), and lactoglobulin A (M_r 18,367). The gel was fixed in a 10% methanol–10% acetic acid solution for 1 h with agitation. After rinsing for 5 min in distilled water, the gel was washed two times for 15 min in 10% methanol. The gel was treated with 1 M salicylic acid (pH 6.5) in 10% methanol for 1 h before being dried at high vacuum for 1.5 h at 175°F. Protein bands were visible after exposure to X-ray film for 24 h.

Recombinant DNA techniques. Quick plasmid DNA preparations (1.5 ml of culture) were performed by using the alkaline lysis method (13). For large-scale preparations (500 ml of culture), the plasmid DNA was further purified with CsCl-ethidium bromide equilibrium gradients (13). Restriction endonuclease digests of plasmid DNA were performed according to the manufacturer's specifications and analyzed on 1% agarose minigels in TEA buffer (0.04 M Tris [pH 8.0], 0.02 M sodium acetate, 0.001 M disodium EDTA). The overhanging ends of DNA restriction fragments were filled in with the Klenow fragment of DNA polymerase I by the procedure of Maniatis et al. (13).

Southern blot analysis. Chromosomal DNA was prepared as described by Mekalanos (15). Restriction enzymes were purchased from New England Biolabs, Inc., Beverly, Mass. Genomic digests were fractionated on 0.7% agarose gels in TEA buffer and transferred to nitrocellulose sheets as described by Southern (27). Hybridization of radioactive probes to the nitrocellulose was performed as described by Mekalanos (15). The RecA probe was prepared by nick translation (13) of CsCl-purified plasmid pIS2, using [^{32}P]dATP (800 Ci/mmol; Amersham Corp.).

RESULTS

Isolation of the *V. cholerae recA* gene. To study recombinational events involving the *ctx* genetic element (e.g., deletion, amplification, and transposition), we required a mutant of *V. cholerae* deficient in homologous recombination. Our first step in the construction of such a mutant was the cloning and characterization of the *recA* gene of *V. cholerae*.

A recombinant plasmid containing the *V. cholerae recA* gene was isolated from a strain 569B library of DNA sequences constructed in pBR327, using an *E. coli recA* mutant strain (SY327) as the host. The library was screened for colonies resistant to the DNA-damaging agent MMS. The usefulness of this procedure has been previously demonstrated by Better and Helinsky (3), who isolated the *recA* gene of *Rhizobium meliloti* in this manner. In our experiments, the concentration of MMS was 2 μl of MMS stock solution per 25-ml agar plate (MMS concentration, approximately 1.3 mM). This concentration allowed growth of *recA*$^+$ *E. coli* cells. Thus, only colonies in which the *E. coli recA56* mutation was complemented by a *V. cholerae* gene were able to grow on these plates after 18 h of incubation at 37°C. Plasmid DNA was purified from 12 MMS^r colonies, and restriction analysis demonstrated that all 12 clones harbored the same recombinant plasmid. Thus, the 12 colonies were apparently sibs, and we did not determine whether any other recombinant plasmids in the library carried genes encoding resistance to MMS. The MMS^r phenotype was shown to be linked to the plasmid carried by these clones by transformation of the plasmid into new SY327 cells (50 of 50 ampicillin-resistant transformants were also MMS^r). We called the recombinant plasmid encoding MMS^r pIS1 and showed by restriction analysis that it contained a 6-kb insert of *V. cholerae* DNA (Fig. 1).

Deletion of specific restriction fragments of pIS1 was used to determine the approximate location of the gene encoding MMS^r. A plasmid containing a 3-kb deletion of the DNA

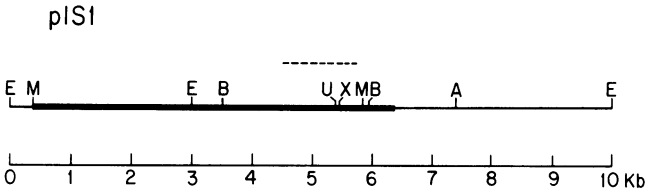

FIG. 1. Restriction map of plasmid pIS1. The heavy line represents the cloned insert of *V. cholerae* DNA; the light line represents pBR327 (5) sequences. The dashed line shows the approximate location of the *recA* gene as suggested by deletion and minicell analysis. Abbreviations for restriction endonuclease sites are as follows: A, *Ava*I; M, *Bam*HI; B, *Bgl*II; E, *Eco*RI; U, *Pvu*II; X, *Xba*I.

region between the two *Eco*RI sites (pIS2) was still able to confer MMS resistance onto SY327. This deletion eliminates the *tet* promoter. Since transcriptional activity in the opposite direction originating from vector promoters is minimal, the MMS^r phenotype was likely to be dependent on transcription originating within the cloned *V. cholerae* DNA. In contrast to pIS2, a pIS1 derivative containing a 5.5-kb deletion between the two *Bam*HI sites lost the MMS^r phenotype. Additional experiments demonstrated that inactivation of the *Xba*I site located at position 5.4 kb on the restriction map also abolished MMS resistance (see below). These data indicate that the gene encoding MMS^r is located in the vicinity of this *Xba*I site (Fig. 1).

Determination of P1 transduction frequencies in SY327 containing plasmid pIS2. To confirm that the gene encoding MMS^r was indeed the *V. cholerae recA* gene, we tested the ability of pIS2 to restore recombinational proficiency to *E. coli* SY327. Strains SY327(pBR327), SY327(pIS2), and SY203(pBR327) were tested as recipients for *arg*$^+$, and *str*^{r} markers in bacteriophage P1 transductions. Only SY327(pIS2) and SY203(pBR327) were capable of genetic recombination (Table 2). That the cloned DNA sequence was able to complement a *recA56* mutation in *E. coli* SY327 strongly suggests that the *recA* gene of *V. cholerae* is indeed carried by pIS1.

Construction of a defined *recA* mutation. We attempted to construct a deletion mutation within the cloned *recA* gene. The *Pvu*II site located at 5.5 kb on the restriction map (Fig. 1) was converted into a *Xba*I site by digestion with *Pvu*II and ligation of an *Xba*I linker at this site. This plasmid was cleaved with *Xba*I, religated, and transformed into SY327. The final construction contained one functional *Xba*I site and an approximately 50-base pair (bp) deletion encompassing the DNA between the original *Xba*I and *Pvu*II sites. Three different DNA fragments (encoding resistance to kanamycin, mercury, or chloramphenicol) were inserted in separate constructions into the remaining *Xba*I site to provide a detectable marker linked to the *recA* mutation. These constructions were subcloned into pRK290 by using the two *Bgl*II sites flanking the *Xba*I site of pIS1. We attempted, though unsuccessfully, to transfer these *recA* constructions onto the *V. cholerae* chromosome by the in vivo marker exchange procedure which had previously been used to construct various *ctx* mutant alleles (18, 25).

We then used an alternative method of creating a *V. cholerae recA* strain. It has been shown in *E. coli* that mutations involving only small alterations in a DNA sequence can spontaneously recombine from a plasmid onto the *E. coli* chromosome at a detectable frequency (10). The entire 6-kb *recA* fragment was subcloned from pIS1 into pBR322 (pIS18) since pBR327 does not contain a DNA sequence permitting mobilization and thus cannot be mobilized by a transmissable plasmid into *V. cholerae*. The cloned *recA* gene was then inactivated by digesting with *Xba*I, filling- in the cohesive ends with the Klenow fragment of DNA polymerase I, and blunt-end ligation (pIS24). In this manner, a 4-bp insertion mutation was introduced into the *recA* gene. As expected, SY327(pIS24) was no longer able to grow on MMS-containing medium. The 4-bp insertion mutation present on pIS24 is called the *recA24* allele and was successfully recombined onto the *V. cholerae* chromosome (see below).

Minicell analysis was used to determine the effect of the *recA24* insertion mutation on the RecA protein. Plasmids pIS18 and pIS24 were digested with *Eco*RI and religated to delete the 3-kb *Eco*RI fragment known to be outside the *recA* gene (Fig. 1), giving rise to plasmids pIS26 and pIS27, respectively. The smaller plasmids were preferred for minicell analysis to eliminate any possible proteins not involved with *recA* activity. Plasmids pIS26 and pIS27 were transformed into *E. coli* minicell strain P678-54. Two transformants were selected, minicells were prepared, and proteins were labeled with [^{35}S]methionine as described in Materials and Methods.

For pIS26 (*recA*$^+$), following polyacrylamide gel electrophoresis, two bands of M_r 44,000 and 41,000 are observed in addition to an M_r 29,000 band representing β-lactamase (Fig. 2, lane A). The larger, M_r 44,000 band has a molecular weight similar to that of the *E. coli* RecA protein (7) when determined by relative mobility in polyacrylamide gels (M_r 43,000) and therefore most likely represents the intact *V. cholerae* RecA protein. The smaller band probably represents a RecA degradation product given that the *E. coli* RecA protein is known to be unstable and sensitive to proteases (L. Gudas, personal communication). These conclusions are supported by the observation that both bands disappear and a new, smaller band of M_r 35,000 is visible in the lane containing labeled proteins from the RecA$^-$ plasmid pIS27 (Fig. 2, lane B). The loss of MMS resistance in pIS27 therefore apparently correlates with a significant alteration in the presumptive RecA protein and its degradation product.

The most likely explanation of these data is that the filling in of the *Xba*I site introduced a frameshift or nonsense mutation into the *recA* gene which leads to the production of a truncated RecA protein of 80% its original size. It is interesting to note that the *Xba*I site itself contains an amber codon and that filling in this site generates an additional amber codon in a different reading frame. The RecA protein may therefore be terminated directly at this site. With this assumption, 20% of the *recA* coding sequence would lie on one side of the *Xba*I site and 80% would lie on the other side.

TABLE 2. Effect of pIS2 on bacteriophage P1 transduction frequencies in *E. coli*[a]

Recipient	Phenotype	No. of recombinants[b]	
		Arg$^+$	Str^r
SY327(pBR327)	RecA$^-$	0	0
SY327(pIS2)	RecA$^+$	163	584
SY203(pBR327)	RecA$^+$	115	590

[a] Bacteriophage P1 lysates were grown on strain MC100.
[b] Arg$^+$ recombinants were scored after incubation at 37°C for 48 h and Str^r recombinants were scored after incubation for 24 h.

Thus the *recA* gene would be located on pIS1 approximately as indicated by the dashed line in Fig. 1. The reverse orientation would also be possible, but seems less likely because this orientation would leave barely enough room to encode the entire M_r 44,000 protein.

Transfer of the *recA24* mutation onto the *V. cholerae* chromosome. It was anticipated that the *recA24* allele would spontaneously recombine from pIS24 onto the homologous region of the *V. cholerae* chromosome, since the homology between the cloned *recA* fragment and the chromosome was only slightly disturbed.

Plasmid pIS24 was mobilized into the El Tor strain SM105, using pRK2013 as the mobilizing plasmid (19). Strain SM105 is a *thyA* derivative of strain SM44. The latter strain has been useful in the analysis of *ctx* amplification and deletion events. SM105(pIS24) was grown overnight in rich medium, subjected to thymine starvation in minimal medium for 4 h, and again grown to saturation in rich medium. This thymine starvation step was included as a possible enrichment for *recA* cells. Although strain SM105 does not contain any detectable lysogenic vibriophages, it is possible that the strain contains defective phage genomes which are common in *V. cholerae* strains (5). During thymine starvation of *E. coli*, bacterial cells can be killed by the induction of lysogenic phages, which is in turn due to the proteolytic activity of activated RecA protein (12). Lysogenic cells containing *recA* mutations are therefore likely to be more resistant to the effects of thymine starvation, while the survival of nonlysogenic *E. coli* cells is not affected by *recA* mutations (21).

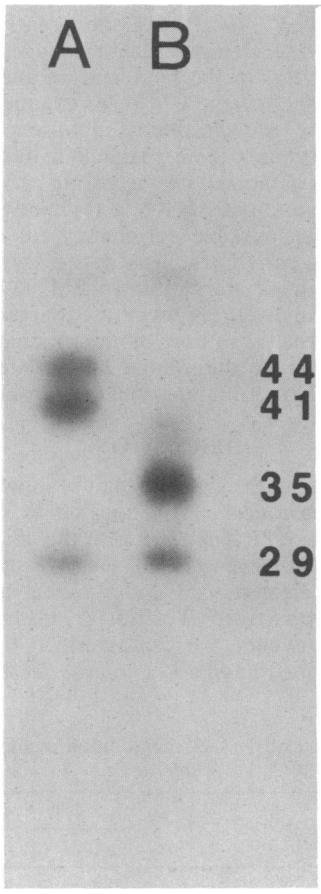

FIG. 2. Analysis of proteins encoded by plasmids pIS26 and pIS27 in minicells. Minicells were harvested as described in Materials and Methods, and proteins were labeled with [^{35}S]methionine for 1 h. The samples were electrophoresed in a 12.5% polyacrylamide gel. The autoradiogram shown was obtained after exposure of the dried gel to X-ray film. Lane A, pIS26; lane B, pIS27. Molecular weights of the protein bands seen are given $\times 10^3$.

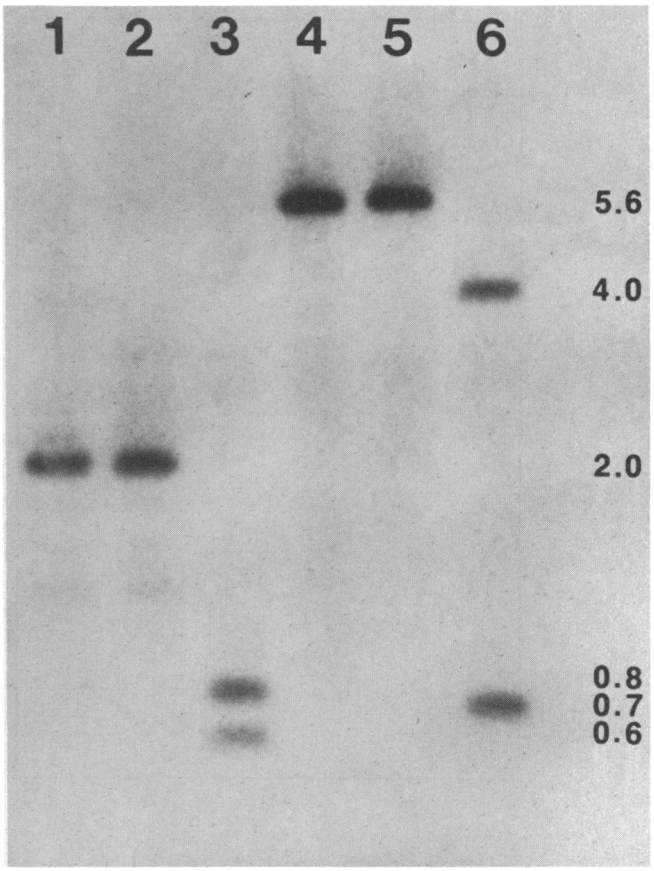

FIG. 3. Southern blot analysis of the *recA* region of *V. cholerae* SM105 and SM106. Chromosomal DNA from SM105 (*recA*$^+$) and SM106 (*recA*) was digested with restriction endonucleases and fractionated by electrophoresis in a 0.7% agarose gel. DNA fragments were transferred to a nitrocellulose filter and hybridized to ^{32}P-labeled, nick-translated pIS2 DNA. Lanes 1, 3, 4, and 6 represent SM105 DNA digested with *Pst*I, *Pst*I + *Xba*I, *Ava*I, and *Ava*I + *Xba*I, respectively. Lanes 2 and 5 contain SM106 DNA digested with *Pst*I + *Xba*I and *Ava*I + *Xba*I, respectively. The approximate molecular weight of each fragment is given in units $\times 10^3$.

Approximately 2,000 colonies of SM105(pIS24) surviving the thymine starvation step were replica plated onto MMS-containing plates. MMS^s colonies representing potential *recA* strains appeared at a frequency of 5×10^{-3}. Several of these were cured of pIS24 by restreaking onto LB-thymine plates and then were retested for MMS sensitivity. One of the MMS^s colonies so obtained, SM106, was selected for further analysis.

Chromosomal DNAs of SM105 and SM106 were prepared and digested with either of two restriction endonucleases, *Pst*I or *Ava*I, with or without *Xba*I. Southern blot hybridization was subsequently performed, using nick-translated pIS2 DNA as a radioactive probe (Fig. 3). Because the restriction enzymes *Pst*I and *Ava*I do not cleave within the cloned *V. cholerae* DNA carried by pIS2, there should be

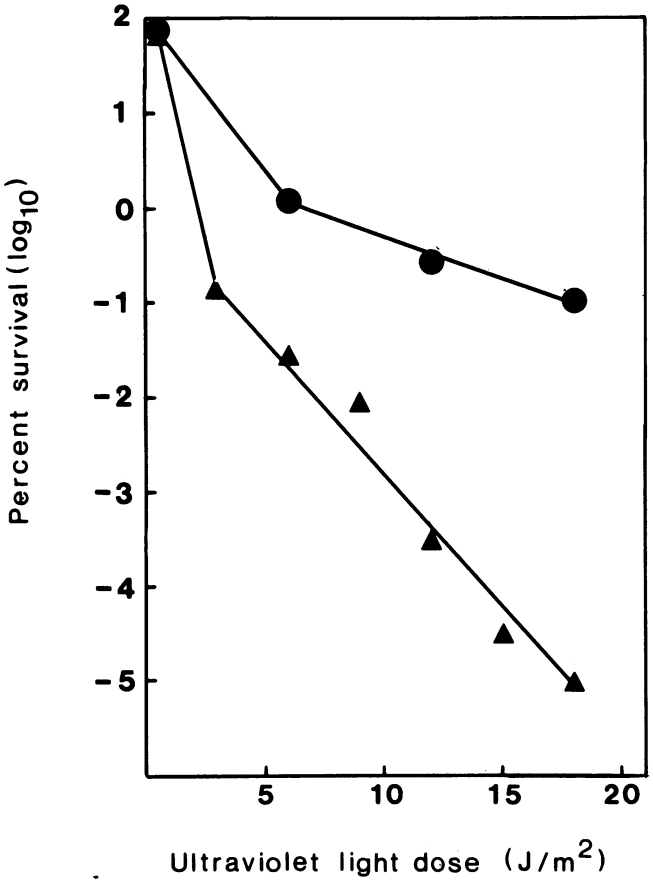

FIG. 4. Survival of SM105 and SM106 after exposure to UV light. Bacteria were irradiated with increasing amounts of UV light, and the percentage of surviving cells was determined by comparison with a nonirradiated cell culture. Symbols: ●, SM105; ▲, SM106.

only a single restriction fragment in each of these genomic digests that hybridize to pIS1. Each of these fragments should contain an *Xba*I site if the strain contains a wild-type *recA* gene but no *Xba*I site if it contains a *recA* gene inactivated by the mutation carried by pIS24. One band of 2.0 kb appears when SM105 DNA is digested with *Pst*I (Fig. 3, lanes 1 and 3), which is cleaved into two smaller bands of 0.8 and 0.6 kb when the DNA is simultaneously digested with *Pst*I and *Xba*I. When SM106 DNA is digested with both *Pst*I and *Xba*I (lane 2), only one band is visible, which is identical in size to the one obtained when SM105 DNA is digested with *Pst*I alone (lane 1). Similar results were observed with analyses using *Ava*I alone or *Xba*I plus *Ava*I double digests (lanes 4 through 6). The data demonstrate that SM106 contains no *Xba*I site within its chromosomal *recA* gene but that the structure of this region is otherwise unaltered. This analysis confirmed that the *recA24* mutation of pIS24 was successfully transferred onto the *V. cholerae* chromosome for strain SM106.

Analysis of the effects of the *recA24* mutation on *V. cholerae* SM106. Strain SM106 was highly sensitive to UV light as determined by a streak test. A UV survival curve was constructed for SM105 and SM106 (Fig. 4) which demonstrates the increased UV sensitivity of the *recA* SM106 strain. At a UV light dose of 15 J/m^2, the *recA* mutant is approximately 1,000-fold more UV sensitive than its parent strain.

To investigate the ability of SM106 to carry out genetic recombination, a mating experiment was performed. A donor strain, SM110(pSJ5), was constructed with the genotype *nal spc*. Each mutation occurred spontaneously and in a single step. Strains SM105 and SM106 were used as recipients. Recombinants were selected for spectinomycin or nalidixic acid resistance. For both markers tested, SM105 consistently showed at least a 30-fold-higher frequency of recombination than SM106 (Table 3). In other mating and transduction experiments, SM106 has shown <1% the recipient activity of SM105 (data not shown). Thus, the *recA-24* mutation greatly reduces homologous recombination in *V. cholerae*, analogous to *recA* alleles in *E. coli*.

We also determined the effect of the *recA24* mutation on UV light induction of a temperate vibriophage. VcA2 (5, 16) lysogens of SM105 and SM106 were isolated as described in Materials and Methods. The lysogenic cells were exposed to UV light for various periods of time. After incubation in LB-thymine broth for 1 h, 0.1 ml of chloroform was added, bacterial debris was pelleted, and the supernatant fluids were titrated for PFU. The $recA^+$ lysogen exhibited a significantly higher level of spontaneous induction than the *recA24* lysogen (Fig. 5). After exposure to 6 J of UV light per m^2, 10^5-fold more phages could be recovered from the supernatant of the $recA^+$ lysogen compared with the *recA24* lysogen. Thus, analogous to the RecA gene product of *E. coli*, the RecA protein of *V. cholerae* is apparently required for efficient induction of a temperate vibriophage. Curiously, the page induction ratios of the *recA* mutant and the wild-type strain are similar across the UV doses examined. This result suggests that the *recA24* allele might encode a protein product which retains some residual activity in SOS response reactions catalyzed by the native *recA* molecule.

During the isolation of SM106, a thymine starvation step was included as a possible enrichment for *recA* cells. To determine if this enrichment was necessary, we compared the effect of thymine starvation on SM106 and its parent strain SM105. The results demonstrate that over a 6-h period both strains decreased in titer by approximately 80% (data not shown). Thus, no differential killing effect of thymine starvation on the *recA* mutant could be observed.

DISCUSSION

Genetic mapping experiments have shown that the *V. cholerae ctx* sequences can be located at more than one chromosomal site (28). Together with the observed duplication and amplification of *ctx* (15), these results suggest that the *ctx* genetic element can undergo rearrangements and possibly transposition. To better understand these recombinational events, we characterized the *V. cholerae recA* gene and constructed a *V. cholerae recA* mutant strain.

TABLE 3. Effect of *recA-24* mutation on recombination in *V. cholerae*[a]

Recipient	No. of recombinants[b]	
	Spc^r	Nal^r
SM105 (*recA*$^+$)	28	32
SM106 (*recA24*)	1	0

[a] Strain SM110(pSJ5) was the donor and was counterselected with streptomycin.

[b] Recombination frequencies are relatively low ($<10^{-8}$) with derivatives of strain P27459 in conjugative crosses with pSJ5 for unknown reasons. Controls in these crosses indicated that the recombinants obtained were probably not spontaneous mutants.

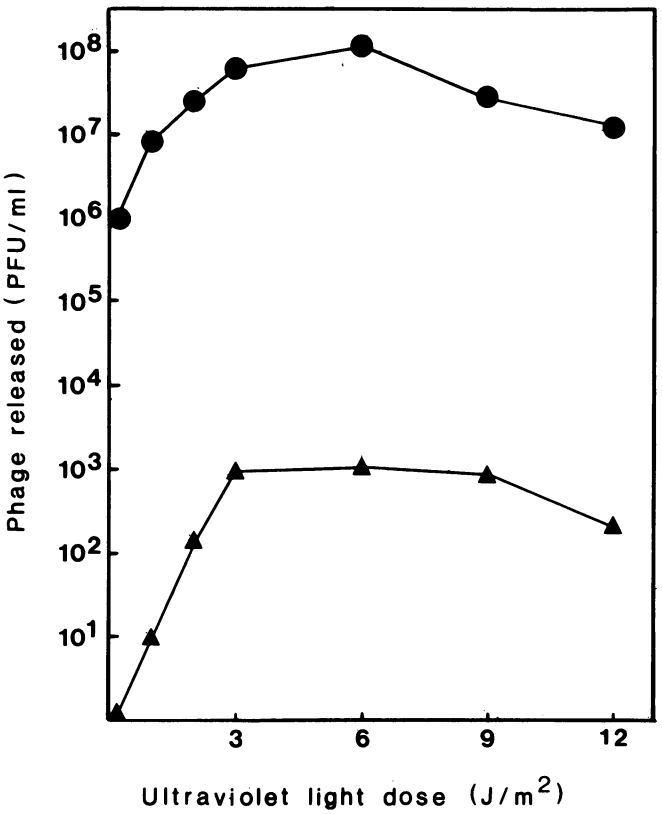

FIG. 5. Induction of vibriophage VcA2 following exposure to UV light. Cell cultures of SM105 and SM106 were irradiated with increasing doses of UV light. Supernatant fluids of the cultures were assayed for the presence of PFU on indicator strain RV79. Symbols: ●, SM105; ▲, SM106.

We have isolated a clone containing the *recA* gene from a strain 569B library by interspecific complementation. This technique was first described by Better and Helinsky (3), who used it to clone the *recA* gene of *R. meliloti*. The *R. meliloti recA* gene was shown to partially restore genetic recombination in an Hfr cross and suppressed the UV sensitivity phenotype of an *E. coli recA* strain. A later publication by Keener et al. (9) described the cloning and characterization of *recA* genes from *Proteus vulgaris*, *Erwinia carotovora*, *Shigella flexneri*, and *E. coli* B/r. All of these heterologous *recA* genes were able to reconstitute UV resistance and recombination proficiency in their *recA* host strains. Keener et al. (9) also conducted experiments investigating the structural similarity among the cloned RecA proteins. Western blot analysis demonstrated that monoclonal antibody prepared against the *E. coli* K-12 RecA protein cross-reacted with all of the cloned *recA* gene products, although it seemed to react less well with the *P. vulgaris* RecA protein. These results demonstrate that the RecA proteins of different bacterial species have been functionally and structurally conserved.

We have identified a recombinant plasmid containing the *recA* gene of *V. cholerae* by the following criteria. First, the cloned gene was able to complement a *recA56* mutation in *E. coli* SY327 for both MMS resistance and recombination proficiency during bacteriophage P1 transduction (Table 2). Second, when the cloned gene was inactivated and then exchanged in place of the wild-type copy, the resultant *V. cholerae* recombinant had many characteristics in common with *E. coli recA* mutants (see below).

Initially, several attempts were made to construct deletion and insertion mutations within the *recA* gene. A deletion was made in pIS2 between the *Xba*I and *Pvu*II restriction sites on this plasmid, and a DNA fragment coding for an antibiotic resistance was inserted at this position. This construction had the advantage of being a stable null mutation in the *recA* gene which could be easily transferred to other strains via its antibiotic resistance phenotype. All of our efforts to recombine this construction back on the *V. cholerae* chromosome by the marker exchange technique of Ruvkun and Ausubel (25) failed. The most likely explanation for this result is that the DNA sequences flanking the *recA* gene on one or both sides were too short to permit the required double-crossover event at a detectable frequency. Another explanation involves a possible lethal polarity effect of the resistance insert on an essential gene downstream of *recA*.

An alternative method of constructing a *V. cholerae recA* strain was successful. The *Xba*I site of pIS2 was inactivated by digestion with *Xba*I, followed by filling in of its cohesive ends and blunt-end ligation. Plasmids carrying this mutation were no longer able to confer MMS resistance onto *E. coli* SY327. The inactivation of the *Xba*I site led in theory to a 4-bp insertion mutation in the *recA* gene. Minicell analysis showed that the mutation caused a 9,000-dalton decrease in the size of the presumptive RecA protein (Fig. 2). One explanation of these data is that the 4-bp insertion adds a second amber codon (TAG) to the DNA sequence in a frame different from the TAG stop codon already present in an *Xba*I site (TCTAGA). Alternatively, the 4-bp insertion mutation could have caused a shift in reading frame to one which contains a stop codon at a site somewhere downstream in the sequence. In either case, our results show that the *recA* tanslational product has been significantly altered by the 4-bp insertion, which is consistent with the MMS^s phenotype resulting from inactivation of the *Xba*I site.

The 4-bp insertion was apparently a sufficiently minor alteration in the DNA sequence to enable the mutation to recombine spontaneously from a high-copy-number plasmid onto the *V. cholerae* chromosome. The *recA24* mutation is also less likely to produce transcriptional polarity effects on downstream genes in the same operon.

Several effects of the *recA* mutation were investigated. Compared to its parent SM105, strain SM106 was highly UV sensitive (Fig. 4), was unable to recombine markers onto the chromosome during P-factor-mediated matings (Table 3), and was deficient in UV induction of the lysogenic vibriophage VcA2 (Fig. 5). All of these data suggest that the *V. cholerae* RecA protein not only complements *recA* muations in *E. coli* but also performs functions in *V. cholerae* analogous to those described for the *E. coli* RecA protein. Our results thus expand the observations of Better and Helinsky (3) and Keener et al. (9) and confirm their conclusion that the RecA protein has been functionally conserved among a variety of bacterial species.

The *recA* mutant described here will find several applications in the study of *V. cholerae* genetics. For example, *recA* mutants of *V. cholerae* should facilitate the isolation of P′ factors (analogous to F′ plasmids in *E. coli*) by using *recA* strains as recipients in P-factor conjugative crosses. In the following paper (6), we report that the *recA* gene plays an essential role in DNA rearrangements which lead to the amplification of the cholera toxin structural genes. Since these rearrangements potentially enhance the pathogenicity of *V. cholerae* (15), one can conclude that the *recA* gene may

play an indirect role in the virulence of *V. cholerae*. As discussed further in the accompanying paper (6), this conclusion together with other considerations suggest that *recA* mutations may have practical applications in the development of stable and safe life attenuated cholera vaccines.

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