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recA mutations reduce adherence and colonization by classical and El Tor strains of *Vibrio cholerae*

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Two *recA* mutants of *Vibrio cholerae* (classical and El Tor biotypes) were constructed by disruption of the wild-type *recA* gene with mutated *recA* sequences of *V. cholerae* cloned in the suicide vector pGP704. Mutants defective in the *recA* gene were compared with their respective RecA$^+$ parent strains with regard to their adherence to isolated rabbit intestine and colonization of intestine of infant mice. The *recA* mutation in *V. cholerae* was found to diminish adherence and markedly affected colonization.

Keywords: *Vibrio cholerae*, *recA*, adherence, colonization

INTRODUCTION

Vibrio cholerae O1 causes cholera, a diarrhoeal disease of man. After oral ingestion, bacteria enter the lumen of the gut where adherence to and colonization of intestinal epithelium occurs followed by secretion of cholera enterotoxin. It was reported several years ago that *V. cholerae* have a *recA* system analogous to that of *Escherichia coli* (Ghosh *et al.*, 1985). Subsequently, the *recA* gene of *V. cholerae* was cloned and shown to complement an *E. coli recA* mutant (Goldberg & Mekalanos, 1986a; Paul *et al.*, 1986). It was further shown that duplication and amplification of the cholera toxin gene was RecA-dependent (Goldberg & Mekalanos, 1986b). In volunteer studies, *recA* derivatives of a *V. cholerae* vaccine strain were found to show diminished immunogenicity (Ketley *et al.*, 1990). Since adherence and colonization are important virulence characteristics of *V. cholerae*, it prompted us to construct *recA* mutants of classical and El Tor biotypes and to study the effect of the mutation on adherence and colonization of vibrios in experimental animals.

METHODS

Media and buffer. Bacteria (Table 1) were routinely grown in L-broth (pH 6·5) containing tryptone (10 g), yeast extract (5 g) and NaCl (5 g) in 1 litre distilled water. For plates, L-broth was solidified with 1·2% (w/v) agar. All dilutions were made in phosphate-buffered saline (PBS; 1·21 g K_2HPO_4 l^{-1}, 0·34 g KH_2PO_4 l^{-1}, 8·0 g NaCl l^{-1}, pH 7·3). Filter-sterilized solutions of ampicillin (Ap) and streptomycin (Sm) were added, when required, at 50 μg ml^{-1}.

Construction of *recA* mutants. Ketley *et al.* (1990) reported the cloning of an approximately 7 kb *Eco*RI DNA fragment that contained the *recA* gene of *V. cholerae* O1 strain 569B. A frame-shift mutation was constructed in this *recA* gene by ligation of an 8 bp synthetic oligonucleotide containing the restriction site *Kpn*I and the plasmid containing the mutant gene was designated pCVD842 (Ketley *et al.*, 1990). The 7 kb *Eco*RI fragment containing the mutagenized sequence from pCVD842 was purified and ligated into the *Eco*RI site of the suicide vector pGP704 (Miller & Mekalanos, 1988) to generate plasmid pCVD845. pGP704 replicates only when its deficient R6K origin is complemented *in trans* by the *pir* function contained in the λ phage integrated in the chromosome of *E. coli* SM10 λ*pir* (Kolter *et al.*, 1978). This strain also contains the transfer genes of the broad host range plasmid RP4 integrated in its chromosome which allows mobilization of the suicide vector or its derivative pCVD845 (Simon *et al.*, 1983). Thus pCVD845 was stably maintained in strain SM10 λ*pir* and conjugally transferred into *V. cholerae* strains KB207 and CD81. Since pCVD845 cannot replicate autonomously in *V. cholerae*, recombination with homologous chromosomal DNA can occur, thereby generating *recA*-deficient derivatives. *V. cholerae* strains KB207 and CD81 were grown in L-broth and mated with *E. coli* SM10 λ*pir*(pCVD845) grown in L-broth containing ampicillin by mixing 4 ml of the donor and 6 ml of the recipient strains and filtering them through a 0·22 μm membrane filter. The membrane was then placed on L-agar and incubated overnight at 37 °C. The mating mixture was resuspended in L-broth and plated on ampicillin- and streptomycin-containing agar plates to allow growth of recombinant bacteria. Colonies were tested for the *recA* mutant phenotype by assessing their sensitivity to UV light and methyl methanesulphonate (MMS). Individual colonies were suspended in a drop of PBS and replica-spotted on two L-agar plates. One plate was exposed to a UV dose that did

Abbreviation: MMS, methyl methanesulphonate.

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference or source
V. cholerae		
KB207	El Tor, Sm^r	Srivastava & Srivastava (1980)
CD81	Classical, Sm^r derivative of strain 0395	This study
recA1	*recA* mutant of KB207, Sm^r Ap^r	This study
recA4	*recA* mutant of CD81, Sm^r, Ap^r	This study
CD192	Classical, Nal^r derivative of KB92	This study Srivastava *et al.* (1979)
E. coli		
SM10 *λpir*	*recA* RP4-2TC::Mu Km^r *thi thr leu su*III	Kolter *et al.* (1978)
Plasmid		
P::Tn1	P factor, Ap^r	Khan *et al.* (1985)
pGP704	Suicide cloning vector, Ap^r	Miller & Mekalanos (1988)
pCVD842	Mutant *recA* gene cloned in pBR322	Ketley *et al.* (1990)
pCVD845	Mutant *recA* gene cloned in pGP704, Ap^r	This study

not kill the parent strain. Both irradiated and unirradiated plates were incubated in the dark at 37 °C. UV-sensitive colonies were picked from the unirradiated plate and further tested for MMS sensitivity. MMS sensitivity was tested on plates on which 2 μl MMS (Sigma) suspended in 50 μl L-broth had been spread.

Southern analysis. A 2·5 kb *Bgl*II fragment containing the *recA* gene of *V. cholerae* strain 569B was labelled with α-^{32}P by the random primer method (Feinberg & Vogelstein, 1983) and used as a probe for hybridization (Southern, 1975) under stringent conditions (50 % formamide, 37 °C, 750 mM NaCl washing at 65 °C). Total DNA was extracted from each strain by SDS lysis, proteinase K treatment, phenol/chloroform extraction and ethanol precipitation and digested with restriction endonucleases *Bgl*II, *Kpn*I and *Bgl*II + *Kpn*I. After separation by agarose gel electrophoresis, Southern hybridization was performed with the probe mentioned above.

Adherence. Adherence of vibrios to rabbit intestinal discs were determined as previously described (Srivastava & Srivastava, 1980; Jacob *et al.*, 1993). Bacteria were grown overnight on L-agar slopes at 37 °C and harvested in PBS. Bacteria were diluted to about 10^7 cells ml^{-1} in 5 ml PBS in which freshly isolated rabbit intestinal discs of 10 mm diameter were incubated for 30 min. After two washings with 20 ml PBS, the discs were homogenized and the number of adherent bacteria was determined by enumerating colony-forming units plated on antibiotic-containing selective media. The adherence index is the number of adherent vibrios expressed as a percentage of the total number of vibrios to which the disc of intact rabbit intestinal mucosa was exposed (Srivastava & Srivastava, 1980).

Colonization. Colonization of vibrios was studied in 5-d-old suckling mice in two different ways, either by infection of mice with a single strain (Jacob *et al.*, 1993) or infection in a competition assay with a mixture of both parent and isogenic *recA* strains in equal numbers (Freter *et al.*, 1981). In the competition assay, the mice were infected with 0·1 ml inoculum that contained parent and *recA* strains in approximately equal numbers.

Briefly, colonization was studied as follows. Bacterial dilutions containing 10^7 vibrios ml^{-1} with 0·01 % Evans blue dye were prepared and 0·1 ml was given orally to each mouse. Three mice were sacrificed each day from each group; their intestines were removed and homogenized in 10 ml PBS, and viable counts were determined on antibiotic-containing selective agar media. For single infection studies, parent and *recA* strains were plated, respectively, on streptomycin- and ampicillin-containing plates. In double infections, plating on streptomycin gave total counts of both strains, whereas only *recA* strains grew on ampicillin plates. The results of the competition assay are expressed as a competitive index, defined as the ratio of viable counts of the parent strain to that of the *recA* mutant. Similarly, an *in vitro* competition assay was designed in which both strains were inoculated in L-broth at 37 °C and the viable count of each strain was determined at intervals.

SDS-PAGE and immunoblotting. SDS-PAGE (12%, v/v, polyacrylamide gels) was done as described previously (Studier, 1973; Hames, 1987; Jacob *et al.*, 1993). Cultures were grown overnight in L-broth at 37 °C. A 0·2 ml sample of the culture was collected by centrifugation, suspended in 0·2 ml of SDS-PAGE sample buffer, kept in a boiling water bath for 5 min and finally centrifuged for 5 min in a microfuge. A 20 μl vol. of the supernatant was loaded in each lane of the gel. After electrophoresis, proteins were transferred by electroblotting onto nitrocellulose paper and detected by ELISA (Harlow & Lane, 1988; Jacob *et al.*, 1993). The source of primary antibody was rabbit anti-*E. coli* RecA IgG (obtained from Dr G. Barcak, University of Maryland at Baltimore) whereas the secondary antibody was goat anti-rabbit IgG coupled with alkaline phosphatase.

RESULTS AND DISCUSSION

Construction and characterization of *recA* mutants

We obtained recombination-deficient mutants of *V. cholerae* strains KB207 and CD81 by conjugal mating with *E. coli* SM10 *λpir*(pCVD845), which results in transfer of the mutated *recA* gene present on pCVD845. The recombinants obtained in these bacterial crosses were scored on L-agar containing ampicillin and streptomycin. The Ap^r recombinants represent intermediate forms in which the whole plasmid is integrated into the chromosome through recombination in the *recA* region: they carry a wild-type and a mutant copy of the *recA* gene. When Ap^r recombinants were grown and tested for Ap^s derivatives lacking the integrated plasmid, none were obtained in more than 5000 colonies screened. Ap^r recombinants were therefore screened for *recA* mutants by testing UV and MMS sensitivities. Of the Ap^r recombinants, 0·1–0·3 % were phenotypically $RecA^-$. Two such strains (*recA1* and *recA4*) were selected for further study.

The survival of *recA* and parent strains at a single UV dose is given in Table 2. The survival of *recA* strains was

Table 2. UV sensitivity and frequency of recombination of the parent and *recA* strains

Strain	Survival (%) at a UV dose of 5 J m^{-2}	10^{-7} × No. of Nal^r recombinants
KB207	5	54
recA1	0·01	0
CD81	1	195
recA4	0·0035	3

found to be less than isogenic parent strains. The efficiency of homologous recombination of *recA* mutants was determined in P-factor-mediated transfer of a Nal^r marker (Khan *et al.*, 1985; Goldberg & Mekalanos, 1986a). Both *recA* and parent strains were mated with CD192(P::Tn*1*) and Nal^r recombinants were selected in media containing nalidixic acid (2 μg ml^{-1}) and streptomycin. As shown in Table 2, a higher frequency of recombination was observed with parent than with *recA* strains. The *recA* mutants are, therefore, greatly reduced in homologous recombination. Thus, the *recA* mutants isolated in this study exhibit the same characteristics described for *recA* mutants of *E. coli* (Clark, 1973) and *V. cholerae* (Goldberg & Mekalanos, 1986a). When parent and *recA* strains were probed with antibodies to *E. coli* RecA protein by immunoblotting, *recA* strains were found to lack RecA protein which was prominently in the parent strains (Fig. 1). The size of the RecA protein of KB207 and CD81 corresponds to the size reported for *V. cholerae* and *E. coli* (Paul *et al.*, 1986).

To explain the $RecA^-$ phenotype of the Ap^r derivatives, the genotypes of the *recA1* and *recA4* mutants were investigated by Southern blot analysis using as probe a 2·5 kb *Bgl*II fragment containing the *recA* gene of *V. cholerae* (Fig. 2). When the parent strains KB207 and CD81 were digested with *Bgl*II and *Bgl*II + *Kpn*I, the probe annealed to a 2·5 kb fragment, indicating that wild-type *recA* lacks a *Kpn*I site (Fig. 2, lanes A-C). The pattern obtained with the *recA4* mutant indicates that it contains only mutant copies of the *recA* gene, since *Bgl*II + *Kpn*I digestion generated a 2 kb fragment. In contrast, double digestion of the *recA1* mutant generated two fragments of 2·5 and 2·0 kb, which is consistent with this strain containing one wild-type and one mutant copy of the *recA* gene. However, since the *recA1* mutant did not synthesize RecA protein (Fig. 1) we concluded that the wild-type copy of the gene is not functional.

The suicide vector pCVD845, to which a mutant *recA* gene was ligated, required the *pir* gene product of λ for autonomous replication and could not replicate in *V. cholerae*. Selection of Ap^r transconjugants required integration of the plasmid into the chromosome, an event that takes place through homologous recombination at the site of the *recA* gene. The *recA1* mutation is probably due to a disruption of the wild-type *recA* gene in the chromosome as a result of integration.

Adherence and colonization studies

Adherence of strains KB207 and CD81 and their *recA* derivatives to rabbit intestine was measured. The adherence of parent strains was higher than that of *recA* mutants. The *recA* mutation caused about 65% decrease in adherence (Table 3). To assess the effect of *recA* mutation on colonization of intestine by parent and *recA* strains, survival of bacteria in the intestine of infant mice was determined. Results of an experiment in which mice were infected with a single strain and followed for up to 72 h are shown in Fig. 3. It was found that the *recA* mutants did not colonize the intestine as efficiently as the parent strains. KB207 persisted in the gut in high numbers up to 72 h following infection whereas cell numbers for the isogenic *recA* derivative were 1000-fold fewer. The *recA4* mutant of CD81 was eliminated from the gut in 24 h, whereas CD81 colonized the gut with resulting high viable counts up to 72 h. The results obtained from this

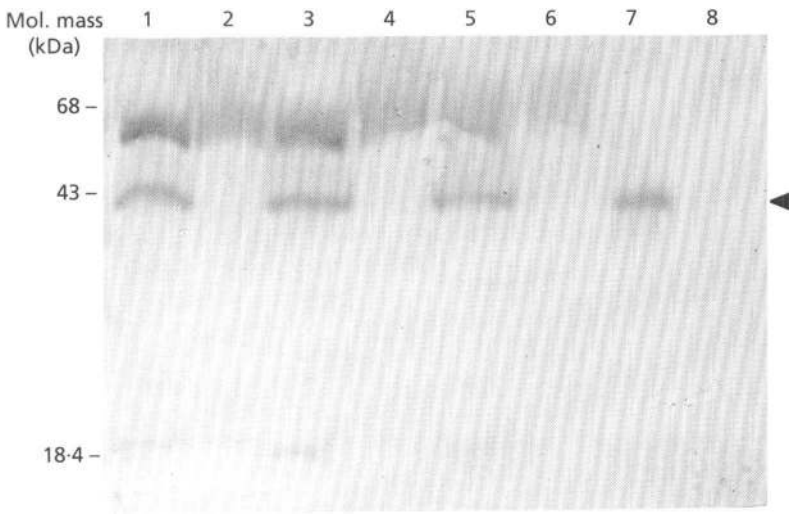

Fig. 1. Immunoblotting of SDS-PAGE resolved proteins of KB207 (lanes 1, 3), *recA1* (lanes 2, 4), CD81 (lanes 5, 7) and *recA4* (lanes 6, 8). Double loading of each strain represents protein samples prepared from two independent experiments. Horizontal bars indicate prestained molecular mass markers (BRL). The arrowhead indicates RecA protein.

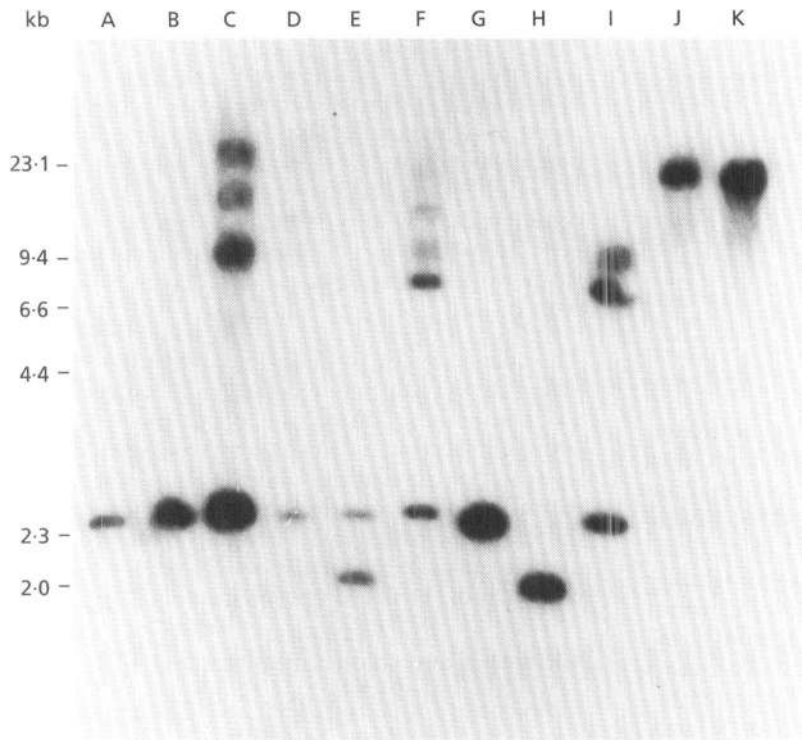

Fig. 2. Southern blot analysis of restriction endonuclease digested genomic DNA of the parent and *recA* mutant strains. Lanes: A, KB207 *BglII*+*KpnI*; B, CD81 *BglII*; C, CD81 *BglII*+*KpnI*; D, *recA1* *BglII*; E, *recA1* *BglII*+*KpnI*; F, *recA1* *KpnI*; G, *recA4* *BglII*; H, *recA4* *BglII*+*KpnI*; I, *recA4* *KpnI*; J, KB207 *KpnI*; K, CD81 *KpnI*. Horizontal bars indicate *Hin*dIII-digested λ DNA molecular size markers.

Table 3. Adherence and competitive index of the parent and *recA* strains

Strain	Adherence index ±SE	Competitive index ±SE	
		24 h	48 h
KB207	2·95±0·187	15±0·115	60±1·73
recA1	1·01±0·109		
CD81	1·67±0·086	250±6·99	> 2000
recA4	0·58±0·058		

Data are from a representative experiment; variation among similar experiments was < 10%; $P < 0{\cdot}01$. Adherence index is the number of adherent vibrios expressed as percentage of the total number of vibrios to which the discs of intact rabbit intestinal mucosa were exposed. Competitive index is the ratio of viable counts of the parent strain to that of the *recA* mutant.

experiment suggested that the colonizing ability of *V. cholerae* was significantly reduced by *recA* mutation. A similar conclusion could be drawn from an experiment in which mice were infected with both parent and *recA* strains. The results of this competition assay are given in Table 3. The competitive index increased with time, suggesting a greater rate of elimination of *recA* mutants. The reduced colonization by *recA* mutants was not due to differences in the growth rates of parent and mutant strains. In an *in vitro* competition assay in which parent and isogenic *recA* strains were grown together, the competitive index was only 2–4 (data not shown). Within 6 h, cultures were saturated and both strains were nearly equal in viable counts.

These results suggest that *recA* plays a significant role in colonization and adherence of *V. cholerae* in animal intestine. Indeed, a recombination-deficient derivative of a *V. cholerae* vaccine strain was found to be less immunogenic in volunteer studies. On the basis of positive stool cultures, this effect was attributed to diminished colonization by the *recA* vaccine strain as compared to the $recA^+$ vaccine strain (Ketley *et al.*, 1990). The mechanism by which *recA* affects adherence and colonization of *V. cholerae* in the intestine is not known.

In *E. coli*, a functional *recA* gene has been shown to have a number of cellular functions, namely homologous recombination (Clark, 1973) and repair of DNA damage induced by radiation, chemical agents and non-physiological growth conditions (Howard-Flanders, 1968; Bridges *et al.*, 1969). It is possible that a functional *recA* gene might be required by *V. cholerae* to overcome deleterious environmental conditions in the intestinal tract.

In *V. cholerae*, it has been reported that the *recA* gene is required in DNA rearrangements leading to the amplification of cholera toxin structural genes (Goldberg & Mekalanos, 1986b). Since amplification of *ctx* genetic element *in vivo* could enhance pathogenicity of *V. cholerae*, a mutation in *recA* would reduce the virulence of *V. cholerae*. It may be speculated that the *recA* mutation might affect expression of colonizing factors. Little is known about factors involved in adherence and colonization of *V. cholerae* and regulation of expression of these factors *in vivo*. Toxin-coregulated pili (Taylor *et al.*, 1987), fimbriae (Hall *et al.*, 1988), accessory colonization factors (Peterson

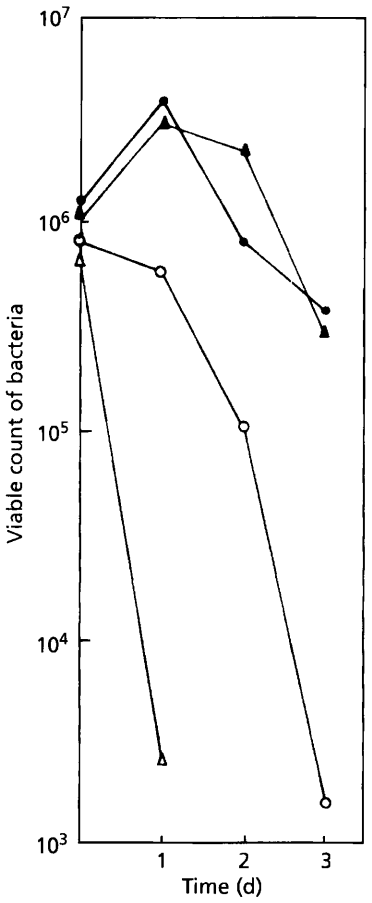

Fig. 3. Non-competitive colonization of infant mice. ●, KB207; ○, *recA1*; ▲, CD81; △, *recA4*.

& Mekalanos, 1988) and a 33 kDa protein antigen (Jacob *et al.*, 1993) with a possible role in adherence and/or colonization of *V. cholerae* have been reported. Of these, the toxin-coregulated pilus is the most extensively studied colonization factor, expression is linked to toxin biosynthesis under the control of the ToxR regulon. Therefore, the molecular mechanism governing the effect of RecA on vital steps in the pathogenesis of cholera, namely adherence, colonization and toxin production is of fundamental interest.

The results reported here and previously (Ketley *et al.*, 1990; Goldberg & Mekalanos, 1986b) on the possible role of the *recA* gene suggest that a recombination-deficient strain may have positive as well as negative applications in the development of a cholera vaccine. While a *recA* mutation may enhance the safety of a vaccine strain, it would, on the other hand, diminish adherence and colonization in the intestine.

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