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Gene sequence of *recA*⁺ and construction of *recA* mutants of *Vibrio cholerae*

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Abstract The *recA*⁺ gene of *Vibrio cholerae* O1 has been cloned, its nucleotide sequence determined and the product characterized. A deletion mutation was constructed in the *recA* gene and mutants showed the typical sensitivity to UV and to DNA-damaging agents, as well as an inability to mediate homologous DNA recombination. The chromosomal *recA* deletion mutants in *V. cholerae* do not show altered virulence in the infant mouse cholera model and are thus ideal strains for use in complementation studies.

Key words DNA repair · Recombination · Virulence Mutation

Introduction

Vibrio cholerae O1 is the causative agent of cholera, the acute diarrhoeal disease, in humans. The bacterial components responsible for the disease are multifactorial, and are often encoded by complex genetic determinants. Genetic complementation analysis of a number of these systems is significantly hindered due to recombination, and consequently such studies would be greatly facilitated by the availability of *V. cholerae* *recA* strains.

The RecA protein is a key component of homologous recombination and the SOS system associated with DNA repair (Clark 1973; Walker 1984). It is involved in the early steps of homologous recombination, allowing the alignment of DNA molecules before strand exchange. During the recombination process there is transfer of a single strand of DNA to the recipient duplex, forming a limited heteroduplex between the interacting DNA molecules; the extension of this heteroduplex involves the winding and unwinding of in-

coming and outgoing DNA chains, respectively. The RecA protein hydrolyses ATP during this process (Dressler and Potter 1982; Radding 1982). The role of *recA* is also pivotal in the SOS response of DNA repair and mutagenesis (Zeig et al. 1978). Many of the genes involved in this response are repressed by the LexA protein (Little and Mount 1982). The expression of these genes is derepressed by proteolysis of the LexA protein, which appears to be carried out by the RecA protein (Craig and Roberts 1980; Little et al. 1980).

RecA-like activity has been identified in a wide range of gram-positive and -negative bacteria, including *Streptococcus faecalis* (Yagi and Clewell 1980), *Proteus mirabilis* (Eitner et al. 1982), *Rhizobium meliloti* (Better and Helinski 1983), *Bacillus subtilis* (Lovett and Roberts 1985), and *V. cholerae* (Goldberg and Mekalanos 1986). More than 30 different *recA* sequences are included in the databases and the encoded proteins are very closely related, with the exception of that of *V. cholerae* reported by Gosh et al. (1992). Consequently we wished to establish whether this gene was in fact the *recA* gene and how it was related to the *recA* genes clones by others (Sancar et al. 1980; Tolmasky et al. 1992), since this would dramatically affect our strategy for constructing *recA* mutant strains.

Materials and methods

Bacterial strains, plasmids and growth media

The following *Escherichia coli* strains were used during this study: DS410 F⁻, *minA minB rpsL* (Dougan and Sherratt 1977); JC14604 F⁻, *lac⁻ hsdR recA* (Clark 1973); DH1 F⁻, *gyrA-96 relA-1 thiA-1 hsdR-1 supE-44 endA-1 recA-1 λ⁻* (B. Bachman); DH5 α *recA-1 endA-1 recA-1 hsdR-17 deoR thiA-1 supE-44 gyrA-96 relA-1 λ⁻* (BRL); DK1 Δ(*srl-recA*)306 *aroD139 Δ(ara, leu)7697 ΔlacX74 galU galK hsdR rpsL mcrA mcrB* (Casadaban and Cohen 1980); SM10 RP4-2Tc::Mu, *thi thr leu supE* (U. Priefer). *V. cholerae* strain O17 (El Tor, Ogawa) was used in the construction of both the cosmid bank and *recA* mutant. *E. coli* strains were routinely grown in nutrient broth (Oxoid); *V. cholerae* strains were grown in brain heart infusion broth (Difco). The following plasmids were employed: pHC79 (Tc^r, Amp^r, Hohn and

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Collins 1980), pSUP301 (KM^r; Simon et al. 1983) and pBluescript K/S (Amp^r; Stratagene).

Enzymes

Restriction endonucleases were purchased from either Boehringer Mannheim or New England Biolabs and used according to manufacturers' instructions. Sequencing kits for dye primer and dye terminator sequencing were obtained from Applied Biosystems.

Minicell procedures

Plasmid-encoded proteins were analysed in minicells using the *E. coli* strain DS410 (Dougan and Sherratt 1977). Minicells were purified and the plasmid-encoded proteins labelled with [³⁵S]-methionine as described by Kennedy et al. (1977) and modified by Achtman et al. (1978). Cells were initially collected by centrifugation (Sorvall GSA rotor, 7000 rpm, 10 min). Minicell purification involved separation of minicells from whole cells (500 ml overnight culture in LB) by centrifugation (Sorvall rotor HB4, 6000 rpm, 20 min) through two successive sucrose gradients generated by freezing 20% sucrose in 1× buffered saline gelatin [BSG; 85% (w/v) NaCl, 0.03% (w/v) KH₂PO₄, 0.06% (w/v) Na₂HPO₄, 0.1% (w/v) gelatin] and thawing overnight at 4° C. The minicells were removed by puncturing the centrifuge tube and withdrawing the cells into a 10 ml syringe. The minicells were subsequently washed in minimal medium. The optical density of the cell suspension was adjusted to 0.6 at 650 nm. Minicells were then pre-incubated for 90 min in minimal medium to permit the degradation of long-lived mRNAs derived from chromosomal genes. The cells were pulse-labelled with 12.5 µCi [³⁵S]methionine, subsequently washed and resuspended in 1 ml of nutrient broth and incubated at 37° C for 5 min. Minicells were collected by centrifugation and solubilized by heating at 100° C in 100 µl of 1× sample buffer (Lugtenberg et al. 1975) and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed on 11–20% polyacrylamide gradients (for proteins), using a modification of the procedure of Lugtenberg et al. (1975) as described previously by Achtman et al. (1978). Samples were heated at 100° C for 3 min in SDS-sample buffer [0.25 mM TRIS-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 15 (w/v) bromophenol blue] prior to loading. Gels were generally electrophoresed at 100 V for 5 h. Proteins were stained with gentle agitation overnight at room temperature in 0.06% (w/v) Coomassie Brilliant Blue G250 [dissolved in 5% (v/v) perchloric acid]. Destaining was accomplished with several changes of 5% (v/v) acetic acid, with gentle agitation for 24 h. Size markers (Pharmacia) were phosphorylase B (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Autoradiography

SDS-PAGE gels were dried on Whatman 3MM chromatography paper at 60° C for 2 h on a Bio-Rad gel drier. [³⁵S]methionine autoradiography was performed at room temperature for 1–7 days without intensifying screens, using Kodak XR-100 film.

Selection of *recA*⁺ colonies

Two microlitres of a stock solution (11.8 M) of the DNA-alkylating agent, methyl methane sulphonate (MMS) was added to 25 ml

of nutrient agar (NA, Oxoid), producing a final concentration of ~1 mM. After spreading the bacterial suspension, the plates were incubated for 18 h at 37° C.

UV sensitivity test

A 10 ml overnight (o/n) bacterial culture in stationary phase was poured into a glass petri dish with a plastic cover. A sample was taken before exposure to any UV light, and serially diluted 10⁻², 10⁻⁴, 10⁻⁵ and 10⁻⁶; 100 µl of each of these dilutions was plated out on agar plates. The culture was then exposed to a UV light source at a distance of 45 cm, while being gently agitated. An aliquot was removed every 10 s over a total exposure period of 60 s, and appropriate dilutions were plated.

DNA sequencing and analysis

Sequencing was carried out on a Model 373A Applied Biosystems automated sequencer. The sequence was collated and analysed using SeqEd (Applied Biosystems), DNAsis and PROsis (LKB). Further analysis of the sequence was carried out using CLUSTAL V (Higgins and Sharp 1989) and PHYLIP (Felsenstein 1993). Protein sequences were obtained from either GenBank, SwissProt or PIR databases.

Results and discussion

Using an approach which has been highly successful in a wide variety of species, we have cloned the *V. cholerae recA* gene based upon the ability of the gene to complement the *recA* defect of *E. coli* K12 strain DH1 (Better and Helinski 1983; Keener et al. 1984; Finch et al. 1986; Hamood et al. 1986; Koomey and Falkow 1987; Owtrim and Coleman 1987; Resnick and Nelson 1988; Dreyfus 1989; Farrand et al. 1989; Singer 1989; Kuhl et al. 1990). The DNA-alkylating agent MMS was utilised as a selecting agent to isolate a clone containing a functional *recA* gene, since *recA* mutants do not have the ability to grow on this alkylating agent. A gene bank of *V. cholerae* O17 was constructed using the cosmid vector pHC79 (Hohn and Collins 1980) and the *E. coli recA1* host (DH1). This was achieved by partial restriction digestion of whole *V. cholerae* chromosomal DNA with the restriction endonuclease *Sau3A*I. The chromosomal DNA fragments were ligated to pHC79 (Hohn and Collins 1980) cut with the restriction endonuclease *Bam*HI. The ligation mix was packaged into Packagene extracts (Promega) and used to transfect *E. coli* strain DH1. This gene bank was screened for colonies able to grow on MMS. Five hundred single colonies were patched out, in duplicate, onto NA plates contained 50 µg/ml ampicillin (Amp) or Amp and MMS (300 µl of 78 mM MMS was spread onto the surface of the plates). Colonies that grew on both selection media (Amp^r, MMS^r) were used for further analyses. One positive clone was designated L7, and the cosmid it contained pPM3201. A similar procedure has been previously used by Goldberg and Mekalanos (1986), except that they used a plasmid library of a classical *V. cholerae* strain to isolate a clone.

Fig. 1 A–C The plasmids pSUP301, pPM3210 and pPM3220 were transformed into the *Escherichia coli* strain JC14604 (Clark 1973) and colonies tested for the production of papillae after 48 h on lactose-McConkey agar. Panels A, B and C are JC14604 colonies containing plasmids pSUP301, pPM3210 and pPM3220 (*recA**) respectively

Restriction analysis of pPM3201 showed the presence of a 2.5-kb *Bgl*III fragment and a 3.5-kb *Pst*I fragment, both of which are diagnostic for the *V. cholerae* *recA* gene according to Goldberg and Mekalanos (1986) and Hamood et al. (1986) but not to Paul et al. (1986). In addition, UV irradiation analysis showed that pPM3201 was able to complement the *recA*1 mutation of *E. coli* DH1 (data not shown). Attempts were made to clone the 2.5-kb *Bgl*III fragment from pPM3201 into high-copy-number vectors, but these clones proved difficult to manipulate as the 2.5-kb *Bgl*III insert seemed prone to excision from the clone: *Bgl*III digestions of such clones suggested either the presence of mixed plas-

Fig. 2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the minicell preparations from *E. coli* DS410 containing the plasmids: lane A, pSUP301; lane B, pPM3210; and lane C, pPM3220. The sizes of the plasmid-encoded proteins detected were estimated (in kDa) from a plot of the size markers (see the Materials and methods)

mid populations or deletion of the insert. Similar instability has been observed by Fyfe and Davies (1990) in their attempts to clone the *recA* gene of *Neisseria gonorrhoeae* into high-copy-number vectors. Consequently, we selected pSUP301 (Simon et al. 1983), a pACYC177 derivative, with a copy number of only 10–12 copies per cell (Maniatis et al. 1989) as a vector, and successfully cloned the 3.5-kb *Pst*I fragment. No evidence for instability was subsequently observed.

The 3.5-kb *Pst*I subclone, pPM3210, was able confer MMS resistance to *E. coli* DK1, a strain deleted for the entire *recA* gene (Casadaban and Cohen 1980). The ability of strain DK1 containing pPM3210 to survive UV irradiation was ascertained and compared to that of the *E. coli* strains LE392 (*recA*⁺) and DK1 (*recA*). The results show that DK1 (pPM3210) demonstrates an increased ability to resist UV-mediated killing when compared to DK1. After 30 s of irradiation, no surviving DK1 colonies were detected, whereas DK1 (pPM3210) showed ca. 70% survival after 60 s. DK1 (pPM3210) does not survive UV irradiation as efficiently as LE392; however, these strains are not isogenic, and it is therefore possible that they display slightly different sensitivities due to unknown genetic differences. This problem was addressed by the use of *E. coli* C600 and a *recA* derivative. To determine quantitatively the ability of the

5'
 CTGCAGTTTACCGAGCAAAATGAAACACTAGTGACACAATGAAACAGAAAGCGAGGCAAGCGTATGAAACACTGCATTCTTGAGTGAGCAGTTAGGGGGGCTACTGGCTAACCGAGC 120
 AGCAGATCCTCGCTAGCGCAGAANTCTTGACCGGGCGGTGGTGTGGCTTATGGGTGACTGAAGTGGCGGAAGTTCGGGATGGTTGATCGTAGTTTCGTGACTTACAGTAATGAAGCC 240
 AAACAAGAAATGCTCGGTGTTCGGCAAGCAACATTACAACAATTTGGTGGGTTTCTGAACAGACCGTTGAAGAGATGGCGCTTGGTGGCTCTTGCATTCAAAAGGCTACTAGCGGTGT 360
 GATTAGTGGCATTGCTGGCCCCGGTGGCGGTAGTGACAGAAAGCCAGTTGGCACCGTCTGCTTTGGCTTGTCTAGTGTCAAGTTGGCTCAAAGTGGAAACGTGCACCTTTACGGGTGAT 480
 CGCGAACAAGTGCCTCAGCAAGCCATAGCGCATGTGTGCAAGCTTGATTGAGCATCTCGCAGCAGATCAGGTTTCAACAGTTAACTGAGCCAAACTTTTTTCTATACAGGGGTGGACA 600
CGTGACAAATCAACAGTATAATAAACCCCAACATGACAAATCAGCGTCGACCCGATTTCGACACAATTACCTATCACCTGAGAGTGACTATCCGGAGAGAGTAATGGACGAGAATAAACA 720
CTGTACAAATCAACAGTATAATAAACCCCAACATGACAAATCAGCGTCGACCCGATTTCGACACAATTACCTATCACCTGAGAGTGACTATCCGGAGAGAGTAATGGACGAGAATAAACA 840
 GAAGGCACTTGGCGCGCCCTCGGTCAAATTTGAAAGCAATTTGGTAAAGGCTCCATCATGCACTTAGGCGCAACCGCGCGATGGATGTAGAAACCATTTTCGACCGGTTCTCTGTCTCT 960
 K A L A A A L G Q I E K Q F G K G S I M H L G D N R A M D V E T I S T G S L S L 46
 GGATATCAGCTTGGGTTCTGGTGGTCTACCCATGGGACGTATCGTTGAGATCTCGCGCCTGAATCTTCGGGTAAAACGCACTGACTCTGGAAGTATTGCTGCTGCACACGTGAAGG 960
 D I T L G S G G L P M G R I V E I L G P E S S G K T T L T L E L I A A A Q R E G 86
 TAAACCTGTGCGTTTATCGATACCGAGCAGCACTGGATCCGGTTTATGCCAAAAATTTGGCGTGAATATCGATGAGTACTGGTTTCTCAGCCAGATACCGGCGAGCAAGCACTGGA 1080
 K, T C A F I D T E H A L D P V Y A K K L G V N I D E L L V S Q P D T G E Q A L E 126
 AATTGTGATGCATGGCTCGCTCTGGTGTGTGGATGTCTGCTGTTGACTCAGTAGCGGCTTTGACACCAAAAGCGGAAATTTGAAGGCGAAATGGGCGATAGCCACATGGGTCTGCA 1200
 I C D A L A R S G A V D V I V V D S V A A L T P K A E I E G E M G D S H M G L Q 166
 AGCGGTATGTTGTGCGAAGCAATGCGTAACTGACGGGTAACTCAAGCAATCCAAGTGTATGTTGATCTTCATCAACCAAAATTCGATGAAGATTGGTGTGATGTTGGTAAACCCAGA 1320
 A R M L S Q A M R K L T G N L K Q S N C M C I F I N Q I R M K I G V M F G N P E 206
 AACTACCACTGGCGGTAAACCACTGAAATTTCTACGCTTCTGTTGTTGATATTCGCGTACTGGCGCAATCAAAGAAGCGAAGTGGTGGGTAAACGAAACCGGTATCAAGTTGT 1440
 T T T G G N A L K F Y A S V R L D I R R T G A I K E G E E V V G N E T R I K V V 246
 GAAGAACAGATTGCTGCGCGGTTAAAGAAGCGAAGCACTCAGATCATGTATGGCCAAGGTTTCAATCGTGAAGGTGAATTTGATCGATCTTGGTGTGAACACAAAATGGTTGAGAAGTC 1560
 K N K I A A P F K E A N T Q I M Y G Q G F N R E G E L I D L G V K H K M V E K S 286
 AGCGCTTGGTACAGCTATAACCGCGATAAATCGGCCAAGGTAAAGCGAATGCCTGCAAGTATCTGAAAGAAAACCCAGAAATTCGAAACTCTAGATAAAAACTCGCTGAAATGCT 1680
 G A W Y S Y N G D K I G Q G K A N A C K Y L K E N P E I A K T L D K K L R E M L 326
 GTTAAACCCAGAAAACATGCGAGTGTCTGCTGAAACATCTTCTCGCGCGGATGACGTGCAATTTGGCGCGGTGCCAGAAAGATTAAATCGCGCGGCTGAATGCAAGAGGTTTCAGCCCTC 1800
 L N P E N M Q L I A E T S S A A D D V E F G A V P E E F * 354
 TCAATCTCGATGTTGATGAAGCCCTGCAATGCAAGGCTTCTTTTGGCTGCCAATTTGAGTGAGTGACTCAAAACTCACCGTTAGCCATATAGGAGTTCCATGAGTTTGCAGTTAGCCAC 1920
 TTGCCGCAAAAGTGCCTTACAATCTCTGAGCGTGCAGATCATAGCGAATATGAGCTATACCAAAAATTTAGCCCTCAAAGGTTCATCTGCTGAAGTGATTGATGAGTTGTGAAGTACGT 2040
 TTTAGAGCTAGGTTATTTGAGTGATGCCGTTACGCGAGGTCAAGGCTCGACAAATCGTTTACATAAAGGTTACGGTGAGCAGCGTTTTCGCCACAACTC 2140

Fig. 3 Complete nucleotide sequence of the *recA* gene from *Vibrio cholerae* strain O17 is shown, is lodged under the EMBL accession no. X71969. The sequence codes for a 354-amino acid peptide of approx. 39 kDa. The ribosome binding site (RBS) is indicated by double underlining and the -10 and -35 regions are overlined. The SOS regulatory element (not shown) has homology to the SOS regulatory element of a number of bacteria including *E. coli* (Ball et al. 1990) and *V. anguillarum* (Tolmasky et al. 1992). The putative transcriptional terminator is shown (inverted arrows). This terminator structure has a free energy of -20.2 kcal/mol calculated using the parameters described by Tinoco et al. (1973). This structure is typical of a rho-independent transcriptional terminator site (Rosenberg and Court 1979)

cloned *recA* gene to restore UV resistance, a number of *E. coli* C600 derivatives with or without various plasmids (pSUP301, pPM3210) were analysed. Plasmid pPM3210 restored the UV resistance of C600 (*recA*) to near wild-type levels, suggesting both good expression of the *V. cholerae recA*⁺ gene in *E. coli* and complete functional complementation.

Plasmids pPM3210 and pSUP301 were transformed into *E. coli* JC14604 (Clark 1973). This *recA* mutant contains a tandem chromosomal duplication of the *lacZ* gene, with each copy of the gene containing a different missense mutation (Keener et al. 1984). Consequently, this strain exhibits a Lac⁻ phenotype. The production of a functional β -galactosidase protein is possible only if a recombination event occurs between the missense alleles to produce a wild-type copy of the *lacZ* gene. Only cells in which such an event has occurred express the

Lac⁺ phenotype, which is manifest as the formation of papillae within colonies. After 48 h growth on lactose-MConkey agar (Fig. 1), colonies were screened for papilla formation. The transformants containing pSUP301 showed no evidence of papillae, whereas those containing pPM3210 showed the presence of Lac⁺ papillae in almost 100% of the colonies screened. This supports the notion that the *Pst*I fragment, cloned in pPM3210, is able to promote homologous recombination.

Mutagenesis of the *recA* gene was facilitated by restriction analysis of the 3.5-kb *Pst*I insert. Plasmid pPM3210 was digested with both *Xba*I and *Pvu*II, as previous workers have shown that the *Xba*I site resides within *recA* (Goldberg and Mekalanos 1986). Since the *Pvu*II site is adjacent to the *Xba*I site, there is a high probability that it too lies within the gene. The 5' *Xba*I overhang was removed using the Klenow fragment of DNA polymerase I, and the resultant linear plasmid was ligated and transformed into *E. coli* DK1, selecting for kanamycin resistance (Km^r). Fifty-three resultant clones were patched onto NA plates containing either Km or MMS. None of these Km^r colonies was able to grow successfully on MMS plates, indicating disruption of the *V. cholerae recA* gene residing on the 3.5-kb *Pst*I fragment. The plasmid from one of the Km^r MMS^s colonies was designated pPM3220. Restriction analysis of this construct showed the absence of both the *Pvu*II and *Xba*I restriction sites, which was subsequently con-

firmed by DNA sequencing. This mutated form of the *recA* gene was designated *recA**.

Several lines of evidence indicate that this deletion disrupts gene function. The plasmid pPM3220 was transformed into *E. coli* DK1, and the UV sensitivity of this strain was assessed relative to DK1 containing pSUP301, and DK1 containing pPM3210. Comparison of strain DK1 containing pPM3210 and DK1 containing pPM3220 reveals a marked decrease in the ability of the latter to survive UV-mediated killing (data not shown). The strain containing pPM3220 (*recA**) demonstrates a level of resistance comparable to that of DK1 with pSUP301. The reduced UV resistance of cells containing pPM3220 is consistent with the *XbaI-PvuII* deletion having disrupted the *recA* gene, thereby destroying the DNA repair function associated with the RecA protein.

Analysis of the plasmid-encoded proteins in *E. coli* DS410 minicells labelled with [³⁵S]methionine by SDS-PAGE and autoradiography identified a number of labelled proteins. The band seen at 40 kDa was correlated with the RecA protein, whereas minicells harbouring the *recA** plasmid (pPM3220) produced a 36 kDa protein (Fig. 2). In the *recA*⁺ strain the 40 kDa band has a similar mobility to that reported for the *E. coli* RecA protein (ca. 43 kDa; Gudas and Mount 1977). This band presumably represents the intact *V. cholerae* RecA protein (Fig. 2).

Sequence analysis (see Fig. 3) has revealed that the 40 kDa band is in fact the true RecA protein. The 36 kDa protein observed in strains carrying the mutant *recA** gene is presumably an altered RecA protein, resulting from the deletion of the *XbaI-PvuII* fragment from the wild-type gene. This leads to a truncation of RecA due to the in-frame deletion and the alteration of the variable carboxy-terminal region. The only conserved residue in the carboxy-terminal region in different RecA proteins is an arginine, which is deleted in the mutant; furthermore this region contains negatively charged residues in all RecA proteins, which may play an important role in determining how RecA monomers interact. The three plasmids, pSUP301, pPM3210 and pPM3220 were transformed into the *E. coli* strain JC14604 and analysed for their effects on recombination as described above (Fig. 1). Following growth at 37° C for 48 h, the transformants were scored for the presence of papillae. Transformants containing pPM3220 (i.e. *recA**), show no evidence of Lac⁺ conversion (see Fig. 1C). These data are consistent with the *XbaI-PvuII* deletion having destroyed the recombinational capacity of cells bearing the cloned *recA* gene.

Chromosomal mutations in the *recA* gene were made in a number of *V. cholerae* O1 strains. This was achieved by conjugating the mutant *recA* gene (pPM3220) into the *V. cholerae* strains. Plasmid pPM3220 contains the pACYC177 replicon (Simon et al. 1983), which is readily lost from *V. cholerae* (our unpublished data). Thus, it is possible to screen for strains which have lost pPM3220 and then test for MMS sensitivity. Those

Table 1 Survival of infant mice after oral challenge with *V. cholerae*, measured as the number of mice from a group of eight that were still alive at 48 h after receiving the challenge dose

Strain	Survival		
	Dose 2 × 10 ³	Dose 2 × 10 ⁵	Dose 2 × 10 ⁷
O17	2	0	0
V920	0	1	0

strains exhibiting sensitivity to kanamycin and MMS must have undergone a homologous recombination event between the intact *recA* gene in the chromosome and the *recA** gene in the plasmid. One such derivative of O17 was designated V920. The chromosomal mutations were confirmed by Southern hybridization (Southern 1975) using pPM3210 as a probe with DNA cleaved with *PvuII* and *XbaI*. Our mutation differs from that described by Goldberg and Mekalanos (1986) who constructed a 4 bp insertion leading to frameshift and termination at an early stop codon downstream. The mutation described here is located in frame and therefore is novel. It clearly shows that the deletion lies within a vital region of the RecA protein required not only for recombination but also for the SOS response, as indicated by the UV survival curve (not shown). Furthermore this mutation is unlikely to have either polar transcriptional or translational effects on downstream genes, since it uses the normal *recA* stop codon and terminator.

The *recA* mutants were tested for their virulence in the infant mouse cholera model (Ujiye et al. 1968; Chaicumpa and Rowley 1972). This involves the feeding of 2-day-old mice with a range of doses of bacteria. Survival of the mice was checked after 48 h to determine the virulence of the organism. Initial experiments were carried out to determine the dose of the parent strain O17 causing 50% lethality (LD₅₀); there appears to be no difference in the LD₅₀ between the *recA* strain and the parental strain (Table 1). Therefore, this set of strains is ideal for complementation studies especially with genetically complex systems, such as those encoding lipopolysaccharide O-antigen biosynthesis (Manning et al. 1986, 1993), the toxin co-regulated pilus (Taylor et al. 1987; Ogierman et al. 1993) and the mannose-fucose resistant haemagglutinin (Franzon et al. 1993), and can be used to eliminate the possibility of recombination events in *in vivo* studies.

The sequence of the *V. cholerae* *recA* gene was determined by subcloning various overlapping regions of the insert in pPM3210 into pBluescript K/S and S/K. Regions not covered by subclones were sequenced by chromosome walking using synthetic oligodeoxynucleotide primers. The complete sequence of the *recA* gene is presented in Fig. 3. The data confirm that the *XbaI* and *PvuII* sites, used in constructing the *recA** mutant, are both within the coding sequence and that the deletion would give rise to a 36.4 kDa truncated protein.

The RecA protein shows considerable homology to all other known bacterial RecA proteins, especially those from other gram-negative organisms (Fig. 4), in particular *V. anguillarum*. As expected, the homology between the RecA proteins extends over most of the protein but breaks down at the carboxy-terminus, as seen for all RecA proteins. We have found no homology to the *V. cholerae* RecA-like protein reported by Gosh and co-workers (Paul et al. 1986; Gosh et al. 1992). This leads us to conclude that the protein reported by these workers is not in fact the RecA protein of *V. cholerae* O1. Using many RecA protein sequences we have assembled

Fig. 5 Phylogenetic tree relating the *V. cholerae* O1 RecA protein to 29 known bacterial RecA proteins. Using the dendrogram obtained from a CLUSTAL V alignment (Higgins and Sharp 1989) the linkage map was set up using Protpass, which uses the parsimony method with Dayhoff matrix. This was then used to construct an unrooted phylogenetic tree (Felsenstein 1993). This clearly shows that the *V. cholerae* RecA protein is most closely related to *V. anguillarum*, followed by other gram-negative bacterial RecA proteins. The protein sequences used for this analysis can be obtained under the following accession numbers: *V. anguillarum*, P26348; *Enterobacter agglomerans*, S31481; *P. vulgaris*, P26346; *S. marcescens*, P17479; *E. coli*, P03017; *P. mirabilis*, P11406; *E. carotovora*, P26344; *Haemophilus influenzae*, L07529; *Pseudomonas aeruginosa*, P08280; *Azotobacter vinelandii*, P29246; *P. fluorescens*, Q01953; *P. putida*, L12684; *Methylobacillus flagellatum*, P18076; *Methylobacillus clara*, P24542; *P. cepacia*, P19690; *Bordetella pertussis*, P17740; *Thiobacillus ferrooxidans*, P16238; *Neisseria gonorrhoeae*, P21152; *Legionella pneumophila*, X55453; *N. meningitidis*, X64845; *B. subtilis*, S14684; *Acholeplasma laidlawii*, P29146; *Acetobacter polyoxogenes*, S60629; *Rhizobium sphaeroides*, S32210; *R. meliloti*, X59957; *R. leguminosarum* V, P24074; *R. leguminosarum* P, P24543; *Bordetella abortus*, L00679; *Agrobacterium tumefaciens*, JC1377

an evolutionary tree relating the *V. cholerae* O1 RecA protein to 29 other RecA proteins (Fig. 5).

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