***Xenorhabdus bovienii* T228 phase variation and virulence are independent of RecA function**

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Colony pleomorphism, or phase variation, expressed by entomopathogenic bacteria belonging to the genus *Xenorhabdus*, is an important factor which determines the association of the bacteria with their nematode symbiont and the outcome of infection of susceptible insect larvae by the bacterium–nematode parasitic complex. The mechanism underlying phase variation is unknown. To determine whether RecA-mediated processes are linked to phase variation, the *recA* gene of *Xenorhabdus bovienii* was cloned and sequenced. When expressed in a *recA*-deleted strain of *Escherichia coli*, the *X. bovienii* *recA* clone was able to complement the loss of RecA function. *X. bovienii* chromosomal *recA* insertion mutants showed increased sensitivity to UV. Phase 1 forms did not show altered ability to convert to phase 2 and no significant differences in expression of other phase-dependent characteristics, including phospholipase C, haemolysin, protease, antibiotic activity and Congo Red binding, were noted. Furthermore, the LD₅₀ of the *X. bovienii* *recA* insertion mutant for *Galleria mellonella* larvae was not significantly different from that of wild-type strains. From these data the authors conclude that *recA* is unlikely to be involved in phase variation, the expression of phase-dependent characteristics, or virulence factors involved in killing of susceptible larvae.

Keywords: entomopathogen, recombination, *recA* mutation, *Galleria*, phylogeny

INTRODUCTION

Xenorhabdus spp. are entomopathogenic bacteria symbiotically associated with insect-pathogenic nematodes belonging to the family *Steinernematidae* (Akhurst, 1993; Akhurst & Dunphy, 1993; Forst & Nealson, 1996). These bacteria are carried monoxenically within the foregut of non-feeding infective-stage nematodes. Nematodes invade the haemocoel of susceptible insect larvae and release bacteria into the haemolymph. Growth of the bacteria leads to a fatal septicaemia and creation of nutrient conditions necessary for host nematode reproduction within the insect (Akhurst, 1982b; Poinar & Thomas, 1966). This process is aided by secretion of antibiotic substances which restrict the growth of other micro-organisms (Akhurst, 1982a).

Xenorhabdus spp. exhibit two colony forms, or variants, when cultured *in vitro*. Colonies of phase 1 (P1) variants are readily distinguished from those of phase 2 (P2) by the differential adsorption of dyes incorporated into the growth medium. Furthermore, P1 variants produce a range of extracellular products, including protease, lipase, phospholipase C, haemolysin, antibiotic substances and Congo Red binding proteins (Akhurst, 1982a; Boemare & Akhurst, 1988; Akhurst & Boemare, 1990; Nealson *et al.*, 1990). Cytoplasmic inclusion bodies composed of highly crystalline proteins are produced during stationary phase (Couche & Gregson, 1987; Couche *et al.*, 1987). P2 forms are unable to (or only weakly) express many of these putative virulence determinants (Akhurst & Boemare, 1990). However, the role of these determinants in the process of infection is unclear, since susceptible insect larvae are killed equally well by P1 and P2 forms of *Xenorhabdus* spp. Nevertheless, production of antibiotic substances by P1 forms prevents putrefaction of the cadaver by other micro-organisms, and this helps support an environment conducive to nematode growth and reproduction.

Abbreviations: Ap, ampicillin; Km, kanamycin; NA, nutrient agar; NB, nutrient broth; P1, phase 1; P2, phase 2; Sm, streptomycin; Tc, tetracycline. The GenBank accession number for the sequence reported in this paper is U87924.

P1 cultures readily convert to P2 during extended liquid subculture and during the process of infection of insect larvae (Akhurst & Boemare, 1990). The mechanism responsible for this pleomorphism is not yet understood. In some other bacteria, differential expression of virulence determinants and the process of phase variation are dependent on expression of functional RecA protein (Koomey *et al.*, 1987; Ball *et al.*, 1990; Zagaglia *et al.*, 1991). To determine the role of RecA in phase variation in *Xenorhabdus* spp., we cloned and characterized the *recA* gene of *Xenorhabdus bovienii* T228. The ability of P1 wild-type and *recA* insertion mutants to convert to P2 forms was then compared. To eliminate the possibility that RecA may independently affect virulence of these bacteria for larvae, we also examined the ability of *recA* insertion mutants to infect and kill susceptible larvae.

METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1. Unless otherwise specified, the P1 form of *X. bovienii* T228 was used in all experiments.

Growth media for bacterial cultures. All strains were stored in 15% (v/v) glycerol and 1% (v/v) peptone at -70°C . Fresh cultures of the P1 and P2 forms of *Xenorhabdus bovienii* were prepared by streaking a loopful of glycerol stock for single colonies onto a Nutrient Agar (NA) (Oxoid) plate and incubating for 36 h at 28°C prior to use. *Escherichia coli* and *Sarcina lutea* strains were plated on NA and incubated overnight at 37°C . MacConkey agar no. 3 was purchased from Oxoid. Phase variants of *X. bovienii* T228 were confirmed by growth on BTB agar [NA+0.0025% (v/v) bromothymol blue and 0.004% (v/v) tetrazolium chloride]. Where appropriate, antibiotics were added to broth and solid media at the following final concentrations: 100 μg ampicillin ml^{-1} (Ap), 50 μg kanamycin ml^{-1} (Km), 100 μg streptomycin ml^{-1} (Sm), 5 μg tetracycline ml^{-1} (Tc).

Media required for identification of expression of phospholipase C, haemolysin, protease and antibiotic activity were prepared using the standard techniques for *Xenorhabdus* spp. (Boemare *et al.*, 1997). Congo Red NA was prepared by addition of Congo Red dye (Sigma) to NA at a final concentration of 0.1% (v/v).

Chemicals, reagents and enzymes. Chemicals were Analar grade. Unless otherwise stated, all chemicals in this study were purchased from Ajax Chemicals, BDH Laboratory Supplies or Sigma. Lysozyme was obtained from Sigma. Proteinase K and T4 ligase were purchased from Boehringer Mannheim. Restriction enzymes were routinely purchased from Boehringer Mannheim, New England Biolabs, Pharmacia Biotech or Progen Industries.

Isolation of DNA. Plasmid DNA was prepared using a standard three-step procedure (Maniatis *et al.*, 1982). Chromosomal DNA was isolated by modification of a previously described method (Manning *et al.*, 1986). Sequencing-grade plasmid DNA was prepared using QIAGEN columns following protocols described by the manufacturer.

DNA manipulations. PCR products and DNA fragments used for subcloning or preparation of DNA hybridization probes were purified either by phenol extraction (Bewsey *et al.*, 1991), or using the Bresaclean DNA extraction kit (Bresatec). After restriction endonuclease digestion, enzymes were inactivated

with heat and/or phenol/chloroform extraction. This was followed by precipitation with 2 vols 100% ethanol, and a 70% (v/v) ethanol wash, before resuspension in an appropriate volume of purified water.

DNA sequencing and sequence analysis. DNA sequencing using dye-labelled primers and/or terminators was carried out using the PRISM Ready Reaction Cycle Sequencing Kit (Applied Biosystems). Searches for similarity to known DNA and protein sequences in databases were performed using the BLAST2 search service at EMBL (Altschul *et al.*, 1990). Similar DNA and amino acid sequences were aligned using CLUSTALW (Thompson *et al.*, 1994). The PHYLIP package (J. Felsenstein, University of Washington, USA) was used to determine the phylogenetic relationships of selected RecA amino acid sequences. Distance measures were obtained using the PROTDIST (Dayhoff PAM matrix) program. KITSCH was then used to estimate phylogenetic relatedness by a Fitch-Margoliash and least-squares method. KITSCH was configured to search for the best tree and randomize the input order of the sequences; a power setting of 2 was used. An unrooted phylogenetic tree was constructed using DRAWTREE.

Cloning and amplification of the *X. bovienii* *recA* gene. Preliminary sequence data were obtained through a strategy consisting of amplification of an internal *recA* fragment with the aid of degenerate oligonucleotide primers, followed by extension of the sequence data using a combination of inverse PCR and plasmid-assisted PCR rescue. These data were then used to amplify DNA encoding the entire *recA* gene from *X. bovienii* T228.

PCR amplification of a 363 bp internal fragment of the *recA* gene of *X. bovienii* was accomplished using degenerate oligonucleotide primers based on 27 aligned RecA amino acid sequences. The DNA sequence of the coding strand primer [5'-TT(TCA)AT(TC)GA(TCAG)GC(TC)GA(AG)CA(TC)GC-3'] has a 192-fold degeneracy and the complementary strand primer [5'-CC(AT)CC(AT)G(GT)(AT)GT(ATG)GT(CT)-TC(ATCG)GG-3'] has a 384-fold degeneracy. The PCR reaction mixture consisted of: 1 $\times$ PCR buffer, 2.0 mM MgCl_2 , 0.2 mM of each dNTP (Pharmacia Biotech), formamide (1.5%, v/v), 125 pmol of each primer and 1.25 units of AmpliTaq Gold II *Taq* polymerase (Perkin Elmer). Approximately 100 ng template chromosomal DNA from *X. bovienii* was used per reaction tube. The amplification cycle consisted of an initial 10 min at 95°C , 30 cycles of 1 min at 95°C , 2 min at 55°C and 3 min at 72°C , followed by a final 3 min extension at 72°C . The PCR product was cloned into pGEM-T Easy Vector System (Promega) and the resulting plasmid designated pCT300. DNA sequence analysis was used to confirm that pCT300 contained an insert containing DNA with homology to other *recA* sequences.

Inverse PCR was then used to amplify DNA flanking the internal *recA* fragment on the *X. bovienii* chromosome. Chromosomal DNA was digested to completion with *DraI*, and the fragments circularized with T4 DNA ligase. Circularized fragments containing the internal *recA* region were linearized by digestion with *NdeI*. This enzyme has a single recognition site within the internal *recA* fragment cloned as an insert in pCT300. Primers complementary to the 5' and 3' ends of the internal *recA* fragment (5'-CACAGC-AGGTTATCAATATC-3' and 5'-TTATCAACCAGATCC-GTATG-3') were then used to amplify DNA containing the 5' and 3' flanking DNA. The PCR reaction mixture consisted of: 1 $\times$ PCR buffer, 2.5 mM MgCl_2 , 2.0 mM of each dNTP, 50 pmol each primer, 100 ng template DNA and 1.25 units AmpliTaq Gold II *Taq* DNA polymerase. The amplification

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Source or reference
<i>E. coli</i>		
DH5 α	Cloning strain	BRL
DK1	$\Delta(sr1-recA)$	Casadaban & Cohen (1980)
JC14604	F ⁻ <i>lac</i> ⁻ <i>hsdR</i> $\Delta(sr1-recA)$ 306	Clark (1973)
SM10(λ pir)	<i>thi-1 thr leu tonA lacY supE</i> <i>recA::RP4-2-Tc::Mu</i> , Km ^r , λ pir	Miller & Mekalanos (1988)
SY327(λ pir)	$\Delta(lac-pro)$ <i>argE</i> (Am) <i>rif</i> <i>nalA</i> <i>recA56</i>	Donnenberg & Kaper (1991)
<i>Sarcina lutea</i>		IMVS, Adelaide, South Australia
<i>X. bovienii</i>		
T228	Ap ^r Sm ^r , P1 and P2 variants	R. J. Akhurst, CSIRO Entomology, Canberra, Australia
XB001	T228 <i>recA::Km</i> , Km ^r	This study
XB002	XB001 containing pCT303, Tc ^r	This study
Plasmids		
pBSL15	Source of Km ^r cassette	Alexejev (1995)
pCVD442	Suicide vector, Ap ^r , <i>sacB</i> <i>mob</i> RP4	Donnenberg & Kaper (1991)
pGEM-T, pGEMT Easy	PCR cloning vector, Ap ^r	Promega
pSUP203	Mobilizable vector, Ap ^r Cm ^r Tc ^r	Simon <i>et al.</i> (1983)
pWKS130	Cloning vector, Ap ^r	Wang & Kushner (1991)
pCT300	pGEM-T Easy with a 363 bp PCR product insert containing part of the <i>X. bovienii recA</i> gene	This study
pCT301	pGEM-T with a 962 bp PCR product insert containing part of <i>X. bovienii</i> <i>recA</i> gene	This study
pCT301.1	pCT301 with a Km ^r cassette inserted into the unique <i>Eco</i> RI site	This study
pCT301.2	pCVD442 with the 962 bp <i>recA</i> fragment containing a Km ^r cassette, removed from pCT301.1 as an <i>Sph</i> I– <i>Sal</i> I fragment	This study
pCT302	pGEM-T with the <i>recA</i> gene cloned as a 2064 bp PCR product	This study
pCT302.1	pWKS130 containing the <i>recA</i> gene, cloned as a <i>Not</i> I fragment, in the same orientation as the <i>lacZ</i> promoter	This study
pCT302.2	pWKS130 containing the <i>recA</i> gene, cloned as a <i>Not</i> I fragment, in the opposite orientation to the <i>lacZ</i> promoter	This study
pCT303	pSUP203 containing the <i>recA</i> gene cloned as a <i>Pst</i> I– <i>Not</i> I fragment	This study

cycle consisted of an initial 10 min at 95 °C, then 30 cycles of 1 min at 95 °C, 1 min at 55 °C and 5 min at 72 °C, followed by a final 7 min extension at 72 °C. The ~ 1.5 kbp PCR fragment produced was subjected to sequence analysis and this was used to extend the known *recA* DNA sequence.

Additional DNA sequence data 5' to the known *recA* sequence were obtained by plasmid-assisted PCR rescue based on a method described by Luo & Cella (1994). Briefly, *X. bovienii* chromosomal DNA was digested with *Eco*RI and *Bam*HI, followed by ligation to *Eco*RI- and *Bam*HI-digested pBR322.

This preparation of DNA was then used in a PCR using oligonucleotide primers specific for the tetracycline-resistance gene of pBR322 (5'-TCTTCCCCATCGGTGATG-3') and known 5' *recA* DNA sequence (5'-CAGACGCATGATAGACC-3') for *X. bovienii*. The PCR reaction mixture consisted of: 1 × PCR buffer, 2.5 mM MgCl₂, 50 pmol of each primer, 2.0 mM of each dNTP, 100 ng template DNA and 1.25 units *Taq*Bead Hot Start Polymerase (Promega). The mixture was overlaid with mineral oil. The amplification cycle consisted of an initial 2 min at 94 °C, then 30 cycles of 1 min at 94 °C, 30 s

at 55 °C and 3.5 min at 72 °C, followed by a final 3.5 min extension at 72 °C. The PCR product was subjected to sequence analysis.

Compilation of the sequence data obtained allowed design of oligonucleotide primers which would allow amplification of the complete DNA sequence of the *recA* gene from *X. bovienii*. The entire *X. bovienii* *recA* gene, plus flanking DNA, was cloned as a PCR fragment prepared using oligonucleotide primers P227 (5'-CAGCTTAGTATTGAACTTGG-3') and P2293 (5'-AGTGAAGCTTGGTACTAT-3') as follows. The PCR reaction mixture consisted of: 1×PCR buffer, 2.5 mM MgCl₂, 50 pmol of each primer, 2.0 mM of each dNTP, 100 ng chromosomal DNA and 1.25 units *Taq*Bead Hot Start Polymerase. The mixture was overlaid with mineral oil. The amplification cycle consisted of an initial incubation for 2 min at 94 °C, 30 cycles of 1 min at 94 °C, 30 s at 55 °C and 2.5 min at 72 °C, followed by a final 2.5 min incubation at 72 °C. PCR products were cloned using the pGEM-T Vector System (Promega) and the resulting construct designated pCT302.

A 962 bp DNA fragment internal to *recA*, used as a probe for Southern hybridization analysis and construction of an insertion mutant, was amplified using the oligonucleotide primers RecAF (5'-AGGTTCTATCATGCGTCTGG-3') and RecAR (5'-TCCAGAGACTGCACTACTGA-3'). PCR was carried out as described above and the 962 bp product cloned into pGEM-T. The resulting construct was designated pCT301. The 962 bp PCR product cloned in pCT301 was prepared for use as a DNA probe by incorporation of digoxigenin-labelled dNTPs (Boehringer Mannheim) in place of normal dNTPs in a PCR reaction using primers RecAF and RecAR. Southern hybridization was performed as previously described (Southern, 1975) and modified (Reed, 1990). Digoxigenin-labelled probe DNA was detected colorimetrically as recommended by the manufacturer (Boehringer Mannheim).

High-efficiency electrotransformation of *E. coli* strains. Electrotransformation was performed using an *E. coli* Gene Pulser (Bio-Rad) as described by the manufacturer.

Recombination proficiency assay. A recombination proficiency assay was used to demonstrate the ability of the cloned *recA* gene to facilitate recombination. *E. coli* strain JC14604 (Clark, 1973) is a *recA* mutant containing a tandem duplication of the *lacZ* gene, with each copy of the gene containing a different missense mutation. In the presence of RecA a recombination event can produce a functional copy of *lacZ*. Cells in which this event occurs can give rise to colonies capable of producing Lac papillae on MacConkey agar.

Plasmids pCT302, pCT302.1 and pCT302.2 were used to transform *E. coli* strain JC14604. Plasmid pCT302.1 contained the *recA* gene cloned into pWKS130 as a *NotI* fragment in the same orientation as the *lacZ* promoter. Plasmid pCT302.2 was identical to pCT302.1 except that *recA* was in the opposite orientation. Control transformations with pGEM-T and pWKS130 were also performed. All transformants were plated on MacConkey agar, incubated at 37 °C for 48 h and examined for presence of Lac papillae.

Bacterial conjugation. Filter matings were carried out with modification as previously described (Achtman *et al.*, 1978). Donor and recipient strains were grown overnight with gentle shaking (to avoid damage to the sex pili of the donor) in NB containing appropriate antibiotics. Cells were collected by centrifugation, gently washed and resuspended in fresh NB. These broth suspensions were mixed to achieve a donor:

recipient ratio of 1:10, and 100 µl was then plated on a sterile nitrocellulose filter laid on the surface of a pre-warmed NA plate. Plates were incubated at 28 °C overnight; the filter was then removed and placed in 1 ml NB to recover the cells. A 100 µl sample of the suspension was plated onto the appropriate selective media.

Construction of a *recA* mutant. The suicide delivery vector required for allelic-exchange mutagenesis was constructed as follows. An *EcoRI* fragment encoding a kanamycin-resistance gene cartridge from pBSL15 was inserted into *EcoRI*-digested pCT301. This insertion resulted in a *recA::Km* construct which was composed of 706 and 256 bp of *Xenorhabdus recA* DNA flanking the 5' and 3' ends of the kanamycin-resistance gene cartridge respectively. The resulting plasmid (pCT301.1) was then digested with *SphI* and *Sall* to isolate the *recA::Km* construct, and this was ligated to *SphI/Sall*-digested vector pCVD442 to produce pCT301.2. *E. coli* SY327(*λpir*) carrying pCT301.2 was selected by growth on NA supplemented with Ap and Km. Plasmid pCT301.2 was then used to transform *E. coli* SM10(*λpir*). Conjugal transfer of pCT301.2 between this transformant and P1 *X. bovienii* was then performed. Co-integrates were selected by their resistance to Ap, Km and Sm, and resolved by plating on NaCl-free NA containing 6% (w/v) sucrose (Blomfield *et al.*, 1991). A single sucrose-resistant colony containing a putative *recA::Km* mutation was selected and designated *X. bovienii* XB001. Allelic replacement of *recA* with the *recA::Km* insertion mutation was confirmed by PCR and Southern hybridization analysis.

Construction of a complemented *X. bovienii recA* insertion mutant. The *recA* gene from pCT302 was isolated as a *PstI*-*NotI* fragment and subcloned into the conjugative vector pSUP203. This construct, designated pCT303, was tested in an *E. coli* JC14604 background to confirm that a functional copy of the *recA* gene was present. Plasmid pCT303 was then transferred from *E. coli* SM10(*λpir*) into *X. bovienii* XB001 by conjugation. Exconjugants were selected on NA containing Sm and Tc. A single isolate, designated *X. bovienii* XB002, was selected for use in complementation analysis. P2 variants of XB002 were obtained by serial broth culture and selection on BTB agar.

UV sensitivity studies. Bacteria to be tested were grown overnight at the correct temperature, with appropriate antibiotic selection. Cultures were washed twice in saline before being resuspended in saline to an OD₆₀₀ of 1.0 (LKB Ultraspec Plus Spectrophotometer, Pharmacia). A 4 mm diameter loop was used to streak a standard inoculum of each culture over a distance of 5 cm on NA plates containing appropriate antibiotics. Inoculated plates were allowed to dry for 15 min before exposure to a UV source for 0–50 s. UV-treated plates were immediately incubated in the dark. The extent of bacterial growth on each plate was compared to that on control plates which had not been exposed to UV light.

Maintenance of *Galleria mellonella* larvae. *G. mellonella* eggs were obtained from Dr Kerry Davis (Department of Crop Protection, Waite Campus, University of Adelaide). Eggs were hatched on growth medium mixture consisting of wheatgerm (100 g), Heinz High Protein Baby Cereal (125 g), honey (100 ml), 100% glycerol (100 ml), *Torula* yeast (1.0 g), Nipagen (Sigma) (0.15 g) and the contents of five 500 mg multivitamin capsules (Cenovis). Larval cultures were incubated at 28 °C for 5–6 weeks. Sixth-instar larvae were used for all experiments described.

LD₅₀ of *X. bovienii* for *G. mellonella*. P1 and P2 cultures of *X. bovienii* T228 and XB001 were incubated for 12 h at 28 °C with shaking. Bacterial cells were harvested by centrifugation

(4000 g for 5 min), resuspended in PBS and kept on ice. Serial dilutions of these suspensions were used to infect larvae. Viable counts of bacteria per dose were estimated by triplicate plating of 100 µl volumes of appropriate dilutions on NA.

Sixth-instar larvae were infected with bacteria as follows. Larvae weighing 120–160 mg were selected and surface sterilized with 70% (v/v) ethanol prior to infection. Using a Hamilton syringe, each larva in a group of 10 was then injected with 10 µl of either a bacterial suspension or PBS. Treated larvae were incubated at 28 °C for up to 72 h. At regular intervals, each treatment group was examined and the number of dead or moribund larvae recorded. Estimates of the LD₅₀ of *X. bovienii* strains for *G. mellonella* larvae were then determined (Reed & Muench, 1938).

RESULTS

Cloning and sequence analysis of the *X. bovienii* T228 *recA* gene

Preliminary experiments enabled us to identify *recA*-associated DNA sequences which permitted amplification of a 2064 bp fragment using the oligonucleotide primer pair P227 and P2293. The PCR product was cloned into pGEM-T and the resulting plasmid designated pCT302. The *X. bovienii recA* gene, plus flanking DNA, contained in the 2064 bp insert in pCT302 was sequenced (GenBank accession U87924) and compared with the *recA* sequence of *X. nematophilus* AN6 (GenBank accession AF127333).

The entire *X. bovienii recA* gene, including regulatory sequences, is contained in a 1676 bp *DraI* fragment. The *recA* genes of both *X. bovienii* and *X. nematophilus* consist of an ORF of 1077 bp (including the stop codon) which is preceded by a putative ribosome-binding site (-AAGGAG-). Putative promoter sequences were found 76 bp and 47 bp upstream from the initiation codons of the *recA* gene from *X. bovienii* (TTGATG--16 bp--TATAAT) and *X. nematophilus* (TTTATC--17 bp--TTCATT) respectively. Putative stem-loop structures were found 35 and 21 bp downstream from the stop codons of the *X. bovienii* and *X. nematophilus recA* genes respectively. Both *recA* genes encode two putative

ATP-binding domains located at positions 178–234 bp (inclusive) and 769–843 bp (inclusive) relative to the start of the ORF. Analysis of the translated sequence data identified the critical arginine and glycine residues required for homologous recombination (Müller & Kokjohn, 1990), encoded in both genes at 181–183 bp (inclusive) and 898–900 bp (inclusive) respectively.

The *recA* genes from *X. bovienii* and *X. nematophilus* were 85% identical at the nucleotide level and 95% identical at the amino acid level. Analysis of the *X. bovienii recA* gene revealed extensive identity at the amino acid level to the *recA* genes of *Erwinia carotovora* (92% identity), *Proteus mirabilis* (87% identity), *Serratia marcescens* (88% identity) and *Yersinia pestis* (91% identity). Furthermore, the LexA binding site region in both *X. bovienii* and *X. nematophilus* is almost identical to that of the *E. coli recA* gene (Fig. 1).

Phylogenetic studies of *recA* from *Xenorhabdus* spp.

Fig. 2 shows the phylogenetic relatedness of the full-length RecA amino acid sequence of *X. bovienii* and *X. nematophilus* and those of 44 other bacteria. This phenogram showed that the two complete *Xenorhabdus* spp. RecA sequences were most closely related to one another, and furthermore grouped together with RecA sequences derived from other members of the family *Enterobacteriaceae*.

Assessment of *X. bovienii recA* function in *E. coli*

A recombination proficiency assay was used to determine whether *E. coli* JC14604 carrying the *X. bovienii recA* gene on plasmid pCT302 could promote recombination. When grown on MacConkey agar, this strain did not express Lac papillae indicative of recombination events leading to formation of a functional LacZ protein. Two possible explanations for this observation were that the *X. bovienii* gene was not efficiently expressed in an *E. coli* host, or that the copy number of the expression plasmid was too high, which in turn resulted in gene dosage effects. The former was unlikely since other

<i>X. bovienii</i> T228 <i>recA</i>	ctt gatg CTG atgattata CAG tataatt...64... AGGA gtaaac ATG gctaa
<i>X. nematophilus</i> AN6 <i>recA</i>	ctt gatg CTG atgattata CAG tataatc...64... AGGA gtaaac ATG gctaa
<i>E. coli recA</i>	ctt gata CTG atgagcata CAG tataatt...44... AGGA gtaaaa ATG gctat
<i>S. marcescens recA</i>	ctt gata CTG atgaccata CAG tataatt...65... AGGA gcaaaa ATG gctat
	SOS Box RBS Met

Fig. 1. Upstream regulatory sequences of the *recA* genes from *X. bovienii* T228, *X. nematophilus* AN6, *E. coli* and *S. marcescens*. Conserved bases from the consensus SOS box (LexA binding site) are highlighted (CTG and CAG), along with the ribosome-binding site (AGGA) and start codon (ATG).

Fig. 2. Unrooted radial tree produced using distance methods to compare the RecA amino acid sequence of *X. bovienii* T228 and *X. nematophilus* AN6 to the RecA sequences of 44 Gram-positive and Gram-negative bacteria. No outgroup was selected.

Xenorhabdus genes [for example *fliC* and *fliD* for *X. nematophilus* flagella synthesis (Givaudan *et al.*, 1996)], have been successfully expressed in *E. coli*. To investigate the effect of copy number on *recA* expression, the *recA* gene from pCT302 was subcloned as a *NotI* fragment into the low-copy-number vector pWKS130. Clones pCT302.1 and pCT302.2, with *recA* in the same

or opposite orientation as the *lacZ* promoter of pWKS130 respectively, were then used to transform *E. coli* JC14604. When plated on MacConkey agar, both clones produced colonies with Lac papillae and hence expressed functional RecA. These data implicated plasmid copy number as a reason for the failure of pCT302 to promote recombination in *E. coli* JC14604.

Fig. 3. Comparison of resistance to UV killing by strains of *X. bovienii* and *E. coli*. Resistance to UV is measured as exposure time required to completely inhibit growth of an inoculum of bacteria on a nutrient agar plate. Results shown are the means of four independent observations per experimental group. Error bars represent 95% confidence intervals about the mean. A, *X. bovienii* T228 P1; B, *X. bovienii* T228 P2; C, XB001 P1; D, XB001 P2; E, XB001(pCT303) P1; F, XB001(pCT303) P2; G, XB001(pSUP203) P1; H, XB001(pSUP203) P2; I, *E. coli* DK1; J, *E. coli* DK1(pSUP203); K, *E. coli* DK1(pCT303).

Further confirmation of a functional RecA was obtained by examining the UV sensitivity of the *E. coli* *recA* deletant strain, DK1, after transformation with plasmid pCT303. DK1(pCT303) demonstrated increased ability to resist UV-mediated killing when compared to DK1 alone, or DK1 carrying the vector pSUP203. After 8 s exposure, no surviving colonies of either DK1 or DK1(pSUP203) were detected, whereas DK1(pCT303) was able to survive up to 44 s exposure (Fig. 3).

Characterization of an *X. bovienii* *recA* insertion mutant

A *X. bovienii* *recA* insertion mutant (XB001) was constructed by allelic exchange. PCR analysis using primers RecAF and RecAR confirmed an insertion in the *recA* gene. A 962 bp fragment was amplified from DNA isolated from the wild-type parent, whilst a 2.1 kb fragment was amplified from XB001 DNA. This corresponded to the insertion of a 1.2 kb kanamycin-resistance cassette in *recA* (data not shown). Furthermore, Southern hybridization analysis of *Dra*I-digested chromosomal DNA showed that a 962 bp *recA*-specific DNA probe (derived from pCT301) hybridized with 1.6 kb and 2.8 kb fragments for the wild-type and mutant strains respectively (Fig. 4). These data indicated XB001 contained a *recA*::Km insertion mutation which was not the result of co-integrate formation.

No difference in the UV sensitivity of P1 and P2 strains of *X. bovienii* was detected (Fig. 3), although these strains were clearly more sensitive to UV exposure than the complemented *E. coli* strain DK1(pCT303). P1 and P2 variants of the *X. bovienii* *recA* insertion mutant XB001 were very sensitive to UV and were unable to survive even brief (<3 s) UV exposure. However,

Fig. 4. Southern hybridization analysis of plasmid and chromosomal DNA prepared from *X. bovienii* strains T228 and the *recA* insertion mutant XB001. Plasmids pCVD442, pGEM-T, pSUP203 and pCT303 were digested with *Dra*I; plasmids pCT301, pCT301.1 and pCT301.2 were digested with *Sph*I and *Sal*I; chromosomal DNA from T228 and XB001 was digested with *Dra*I. The filters were probed with a digoxigenin-labelled 962 bp internal *recA* fragment PCR amplified from pCT301. The sizes of DNA fragments hybridizing with the probe DNA are shown. SPP1 marker is *Bacillus subtilis* phage SPP1 DNA digested with *Eco*RI.

complemented P1 and P2 variants (which harboured pCT303) demonstrated partial restoration of resistance to UV-mediated killing, while transformants harbouring the vector pSUP203 alone remained UV sensitive.

Expression of phase-dependent phenotypic characteristics by the *recA* insertion mutant and the *recA*-complemented strain

No differences were detected between the wild-type *X. bovienii* strain T228, the *recA* insertion mutant XB001 and the *recA*-complemented strain XB001(pCT303) in the expression of phospholipase C, haemolysin or protease activity. In all three strains, P1 variants were positive and P2 variants negative for all these characters (results not shown). Similarly, antibiotic production and Congo Red binding ability were unaffected by mutations in *recA*. When P1 forms of the *recA* mutant were subcultured onto BTB agar, P2 forms were readily isolated. Furthermore, PCR and restriction endonuclease analysis of P1 and P2 variants of wild-type and *recA* mutants were unable to identify any alteration or rearrangement in *recA* (data not shown).

Virulence of *recA* mutants for *G. mellonella*

In order to determine whether *recA* plays a role in the pathogenicity of *X. bovienii* for susceptible insect larvae, LD₅₀ analysis was used to compare wild-type P1 and P2 bacteria and the *X. bovienii* *recA* insertion mutant, XB001. Sixth instar larvae were infected with known doses of *X. bovienii* T228 or XB001. Control groups of larvae were injected with PBS. The viability of the bacterial suspensions used did not change significantly

Fig. 5. Selected dose-response curves for *G. mellonella* larvae infected with P1 and P2 *X. bovienii* wild-type bacteria or the *recA* insertion mutant (XB001). $\blacktriangle$, *X. bovienii* T228 P1; $\bullet$, *X. bovienii* T228 P2; $\blacksquare$, *X. bovienii* XB001 P1.

Fig. 6. Proportion of larvae dying over a 60 h period following infection with doses of bacteria approximating that of the calculated LD_{50} for P1 *X. bovienii* T228 (~4 cells per larva). Larvae were infected with P1 and P2 *X. bovienii* wild-type bacteria or the *recA* insertion mutant (XB001). Error bars represent the standard error about the mean. $\blacktriangle$, *X. bovienii* T228 P1; $\bullet$, *X. bovienii* T228 P2; $\blacksquare$, *X. bovienii* XB001 P1.

over the time required to infect groups of larvae. Treated larvae were incubated at 28 °C for up to 72 h and the numbers of dead or moribund larvae recorded at regular time intervals.

All strains killed larvae irrespective of the dose applied. Doses greater than 5 bacteria per larva consistently killed all larvae within 24–36 h, whereas lower doses killed only a proportion of infected larvae. No deaths were recorded for control groups of larvae. LD_{50} values were determined directly from plots of percentage dosage mortality vs mean dose of bacteria (Reed & Muench, 1938). The mean LD_{50} values obtained from three independent experiments using P1 and P2 wild-type strains and the *recA* mutant were 3.1 ± 0.05 , 3.4 ± 1.5 and 2.7 ± 0.9 respectively. One-way analysis of variance indicated these were not significantly different ($P > 0.05$). These estimates of the LD_{50} are similar to those reported elsewhere (Pinyon *et al.*, 1996). Typical plots of percentage mortality ratio vs dose of bacteria were also very similar (Fig. 5). Furthermore, when doses

of bacteria approximating the calculated LD_{50} (~4 cells per larva) were used, the proportion of larvae dying over a 60 h period was not significantly different for the wild-type strain and the *recA* mutant ($P > 0.05$, analysis of variance; Fig. 6).

DISCUSSION

In this study we used PCR-based techniques to clone and sequence the *recA* gene from *X. bovienii* T228. The translated sequence was 95% identical to that of the related species, *X. nematophilus* AN6, and showed considerable similarity to RecA proteins from Gram-negative bacteria, in particular *Erwinia carotovora*, *Proteus mirabilis*, *Serratia marcescens* and *Yersinia pestis*. We constructed an evolutionary tree relating *Xenorhabdus* RecA peptide sequences to those of 44 other RecA proteins. This tree was similar to those constructed previously (Eisen, 1995) and supported the position of *Xenorhabdus* spp. within the family *Enterobacteriaceae* of the γ 1 *Proteobacteria*.

UV sensitivity, complementation analysis and recombination proficiency assays were used to demonstrate expression of a functional *X. bovienii* RecA protein from plasmids in RecA-deficient *E. coli* backgrounds. However, expression was apparently dependent on plasmid vector copy number. RecA protein instability due to expression from high-copy-number plasmid vectors has been observed previously (Fyfe & Davies, 1990; Stroehrer *et al.*, 1994).

Construction of a chromosomal *recA* mutation in *X. bovienii* was achieved by allelic exchange of a *recA*::Km insertion mutation from a pCVD442-based suicide delivery plasmid. Loss of RecA function by *X. bovienii* XB001 was established using UV sensitivity studies. Since a functional relationship exists between homologous recombination and the repair of DNA damage, mutations in the *recA* gene sensitize cells to killing by UV light. Although no difference in the UV sensitivity of P1 and P2 wild-type *X. bovienii* strains was noted, the *recA* insertion mutant XB001 had significantly reduced ability to repair DNA lesions caused by exposure to UV radiation. Furthermore, resistance to UV-induced damage of this mutant could not be restored to wild-type levels by complementation with a functional copy of *recA* from plasmid pCT303, even though introduction of pCT303 into *E. coli* DK1 (which has a deletion of the *recA* gene) completely restored UV resistance.

Previous *in vitro* work has shown that the single-stranded DNA-dependent ATPase activity of RecA is a property of the tetrameric form of the protein (Ogawa *et al.*, 1978). Thus ATPase activity is required for the *in vitro* recombination functions of purified RecA (Radding, 1982; Weinstock *et al.*, 1979), although this is not the case for the proteolytic activity of RecA involved in SOS induction (Phizicky & Roberts, 1981). ATP binding is also required for formation of RecA tetramers and filamentous aggregates of the RecA protein (Ogawa *et al.*, 1978). When RecA1 missense protein and wild-type RecA protein of *E. coli* form mixed tetramers in the

presence of ATP, a dramatic reduction in ATPase activity is observed (Ogawa *et al.*, 1978). Phenotypically, the *recA1* mutation, Gly-160 to Asp-160, is indistinguishable from a *recA* deletion (Kowalczykowski *et al.*, 1994). Therefore the extent of the reduction of ATPase activity is dependent on the ratio of the two polypeptides. *In vivo*, the recombination activity of wild-type RecA protein is also significantly reduced in the presence of a non-functional RecA1 polypeptide. This phenomenon is known as negative complementation and has been well documented for cases such as the *lacZ* gene (Müller-Hill *et al.*, 1968), and the *capR* gene (Charette *et al.*, 1982), where the active form of the protein is a tetramer.

Formation of mixed tetramers of RecA may explain the reduced ability of pCT303 to complement the *recA::Km* insertion mutation present in XB001. Mixtures containing truncated proteins constituting up to 79% of the wild-type protein have been shown to promote strong negative complementation (Yarranton & Sedgwick, 1982). Since the *recA::Km* mutation in XB002 has the potential to express 72% of the wild-type protein (starting at the N-terminus), this peptide has the potential to facilitate negative complementation in the presence of wild-type RecA (the C-terminal end of this putative truncated 265 amino acid protein is predicted to comprise proline, glycine, isoleucine and arginine derived from the kanamycin-resistance cassette). This hypothesis is supported by fact that the truncated RecA protein encoded by the *recA::Km* mutation would not include the ATP-binding site containing a tyrosine residue necessary for co-protease activity and recombination (Cotteril *et al.*, 1982; Knight & McEntee, 1986). Thus co-protease activity and recombination potential should be either reduced or abolished in strain XB002.

Interestingly, wild-type, *recA* and complemented *recA* strains of *X. bovienii* were identical in terms of expression of known phase-dependent characteristics. Furthermore, P1 variants of the *X. bovienii recA* insertion mutant were able to convert to P2 forms on extended serial subculture. When these results are taken together with Southern hybridization analyses which showed no obvious large rearrangements in DNA isolated from P1 and P2 forms (Akhurst & Boemare, 1990), it is very unlikely that *recA* plays a role in phase variation or regulation of phase-dependent characteristics of *Xenorhabdus* spp. This conclusion was further supported by larval infection studies, which showed that the *recA* insertion mutant XB001, was able to kill larvae as well as P1 and P2 forms of the wild-type derivative.

Finally, the construction of recombination deficient mutants is a commonly used approach which aids genetic analysis of virulence factors and other aspects of bacterial physiology. Results from this study will allow progress in molecular analysis of the basis for pathogenesis of *Xenorhabdus* spp. for susceptible insect larvae as well as other phenotypic characteristics. RecA-deficient mutants not only facilitate genetic complemen-

tation analyses, but also increase the stability of recombinant plasmids and retention of foreign DNA.

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