Cloning and expression in *Escherichia coli* of a *recA* homologue from *Mycobacterium tuberculosis*

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(Received 8 March 1991; revised 29 May 1991; accepted 1 July 1991)

A 3·8 kb *Pst*I fragment of *Mycobacterium tuberculosis* was cloned in a *recA*-deleted *Escherichia coli* by selecting transformants with increased EMS resistance. The cloned fragment restored homologous recombination in Hfr crosses and conferred resistance to long wave (302 nm) but not short wave (254 nm) UV light. *E. coli* containing the 3·8 kb *Pst*I fragment produced a 38–40 kDa protein which cross-reacted with antibodies raised against the *E. coli* RecA protein. The cloned DNA thus probably encodes a RecA homologue.

Introduction

Tuberculosis is a destructive, debilitating disease that is estimated to afflict over 10 million people every year (Nordeen & Godal, 1988). The treatment of patients suffering from tuberculosis is prolonged and expensive, and requires the use of combinations of antibiotics to prevent the emergence of drug-resistant mutants of the causative organism *Mycobacterium tuberculosis*. At present, very little is known about the mechanisms by which spontaneous mutations occur in *M. tuberculosis* and what the role of DNA-modifying enzymes in these processes may be.

In *Escherichia coli* several enzymes are responsible for DNA repair and replication (Little *et al.*, 1980; McEntee *et al.*, 1979). One of these enzymes, RecA, has been shown to be associated with several different processes, including the mediation of recombination between homologous DNA fragments, recombination-repair of damaged DNA and the initiation of the SOS response (Walker *et al.*, 1987). *RecA* mutants are recombination-deficient, and extremely sensitive to UV radiation and to DNA-damaging chemicals (Walker, 1984). The SOS response is dependent on the *recA* and *lexA* gene products (Walker *et al.*, 1982). The role of RecA is to inactivate LexA, which is a repressor of SOS-regulated genes (Walker *et al.*, 1982), by proteolytic cleavage due to the formation of a RecA-dependent protease complex (Blanco *et al.*, 1982). The activated RecA protein is able to mediate the cleavage of several proteins including UmuD, which is required for UV and chemical mutagenesis (Nohmi *et al.*, 1988; Shinagawa *et al.*, 1988). DNA repair systems in *E. coli* are known to have many

genes in common with the processes controlling recombination (Howard-Flanders & Boyce, 1966).

Sancar *et al.* (1980) found the *E. coli recA* gene product to be a single polypeptide with a calculated molecular mass of 37·8 kDa. Genes encoding RecA-like activity have been cloned from many Gram-negative bacteria, including *Pseudomonas aeruginosa* (Kokjohn & Miller, 1985), *Shigella flexneri* (Keener *et al.*, 1984), *Vibrio anguillarum* (Singer, 1989), *Proteus vulgaris* (Keener *et al.*, 1984), *Agrobacterium tumefaciens* (Farrand *et al.*, 1989) and *Haemophilus influenzae* (Setlow *et al.*, 1988). However, there has been only one report of isolation from a Gram-positive organism, namely *Bacillus subtilis* (Marrero & Yasbin, 1988), and to date there has been no report of the cloning of a *recA* gene from a member of the Actinomycetes. The majority of these genes were cloned by complementation of *E. coli* mutants using either EMS or MMS selection.

Evidence for the existence of DNA repair mechanisms in the Actinomycetes, of which *M. tuberculosis* is a member, has been provided by the isolation of UV-sensitive mutants of *Streptomyces coelicolor* (Harold & Hopwood, 1970a), and several DNA repair pathways have been shown to exist in *Streptomyces fradiae* (Baltz, 1987). The interrelationship between the *rec* and *uvr* systems in *Streptomyces* may be essential to an understanding of genetic instability in these organisms (Usdin *et al.*, 1985). The homologous recombination system in *Streptomyces* appears to be distinct from the DNA repair system, since no UV-sensitive mutants of *Streptomyces* have been isolated which are recombination-negative (Harold & Hopwood, 1970b, 1972).

Although the cloned *E. coli recA* gene has been shown

to complement defects in DNA repair and mutagenesis in *Streptomyces fradiae* JS6 (*mcr-6*) (Matsushima & Baltz, 1987), it is not known if DNA repair systems in an organism such as *M. tuberculosis* can complement those of *E. coli*, and to address this possibility we have examined the functional complementation of *E. coli recA* mutants with a recombinant plasmid encoding a *M. tuberculosis* RecA homologue.

Methods

The bacterial strains and plasmids used in this study are listed in Table 1.

Media, culture conditions and DNA isolation. *E. coli* cultures were grown using Luria agar or M9 minimal media (Maniatis *et al.*, 1982) and, when required, ampicillin ($50 \mu\text{g ml}^{-1}$) or streptomycin ($25 \mu\text{g ml}^{-1}$) were added. Freeze-dried cultures of the *M. tuberculosis* strain H37RV were reconstituted and streaked onto Lowenstein-Jensen (Difco) slopes. After 6 weeks incubation at 37°C , the cells were harvested and suspended in SDS buffer [10 mM-Tris, 1 mM-EDTA, 0.15 mM-NaCl and 10% (w/v) SDS] for DNA isolation as described by de Wit *et al.* (1990). H37RV DNA was digested to completion with *Pst*I and ligated to *Pst*I-cut pEcoR252 (Zabeau & Stanley, 1982). *E. coli* DK cells were transformed (Hanahan, 1983) and plated onto Luria agar containing ampicillin and 0.1% (v/v) EMS.

Resistance to UV irradiation. Bacterial cultures (10 ml) were grown with aeration at 37°C for 2 h ($A_{600} = 0.2$) and the cells were harvested by centrifugation and suspended in physiological saline (1 ml). Aliquots ($100 \mu\text{l}$) of a series of 10-fold dilutions were exposed to UV light ($1 \text{ J m}^{-2} \text{ s}^{-1}$ at 254 nm or 302 nm). All dilutions were performed in the dark, and the plates were incubated overnight at 37°C to determine cell survival.

DNA hybridization. The DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane (Hybond N, Amersham) (Southern, 1975). Probe fragments were radiolabelled with [α - ^{32}P]dATP (Amersham) by nick translation (Boehringer Mannheim) and DNA hybridizations and post hybridization washes were performed as described by Johnson *et al.* (1984). Hybridizations were performed in hybridization buffer ($6 \times \text{SSC}$, $5 \times$ Denhardt's solution, 0.5% SDS, 1 mg salmon sperm DNA ml^{-1}) containing 50% formamide, at 42°C for 16 h. The membranes were then washed three times in $2 \times \text{SSC}$, 0.1% SDS at room temperature for 20 min, once in $1 \times \text{SSC}$, 0.1% SDS for 30 min at 56°C and once in $0.1 \times \text{SSC}$, 0.1% SDS for 30 min at 56°C . In hybridizations involving *Streptomyces coelicolor* DNA, the temperature of the post-hybridization washes was reduced from 56°C to 42°C . SSC ($1 \times$) is 150 mM-NaCl and 15 mM-sodium citrate.

Recombination studies. Homologous recombination experiments using *E. coli* HB101 containing pSNS201 or pSNS202 as recipients and the Hfr strain, CSH62, as donor, were performed as described by Ramesar *et al.* (1988). *E. coli* HB101 is a proline auxotroph and is streptomycin-resistant, while CSH62 is streptomycin-sensitive. The number of colony forming units per $50 \mu\text{l}$ CSH62 donor cells was determined on M9 agar supplemented with thiamine. Recombinants from the crosses were selected on M9 agar containing streptomycin ($25 \mu\text{g ml}^{-1}$), leucine ($40 \mu\text{g ml}^{-1}$) and thiamine ($3 \mu\text{g ml}^{-1}$). The recombination frequency is expressed as the number of recombinants obtained per 1000 donor cells.

Protein isolation and Western blot analysis. Cells were harvested from

Fig. 1. Restriction endonuclease cleavage maps of the cloned *M. tuberculosis* chromosomal 3.8 kbp DNA fragment encoding *recA*-like activity (pSNS201). Recognition sites for *Bam*HI, *Pst*I, *Hind*III, *Kpn*I, *Cl*aI, *Eco*RI, *Sac*I or *Eco*RV were not detected. The direction of *lacZ* transcription is indicated by the arrow.

cultures (1.5 ml , $A_{600} = 0.5$) of *E. coli* MC1060, *E. coli* DK and *E. coli* DK (pSNS201 and pSNS202) and were suspended in $300 \mu\text{l}$ of SDS/mercaptoethanol (10%, w/v) and placed in a boiling water bath for 5 min. Denaturing polyacrylamide gels (10%, w/v) (Laemmli, 1970) were used to separate the cellular proteins at a constant current of 35 mA for 2.5 h and the proteins were electroblotted onto nitrocellulose membranes (Schleicher & Schuell) at 200 mA for 3–4 h in transfer buffer (25 mM-Tris, 0.19 M-glycine, pH 8.3, 25% methanol), (Towbin *et al.*, 1979). The membrane was blocked for 2 h at 37°C with 10 mM-Tris/HCl, 150 mM-NaCl containing 1% low-fat milk powder and 0.05% Tween-20, pH 7.4 (Johnson *et al.*, 1984). Purified antiserum raised in rabbits against *E. coli* RecA protein (Goodman *et al.*, 1987) was diluted 1 in 20 and used for the detection of cross-reacting proteins with alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin as the reporter molecule (Rybicki & von Wechmar, 1982).

Results

Isolation of a *M. tuberculosis* chromosomal fragment that complements an *E. coli recA* deletion

H37RV DNA was digested to completion with *Pst*I and ligated to *Pst*I-cut pEcoR252 (Zabeau & Stanley, 1982). *E. coli* DK cells were transformed (Hanahan, 1983), and plated onto Luria agar containing ampicillin and 0.1% (v/v) EMS. One recombinant plasmid, pH15, was isolated which on retransformation allowed consistent growth of *E. coli* DK on EMS. pH15 contained a *Pst*I fragment of 3.8 kb (Fig. 1). Southern transfer and DNA hybridization were used to confirm that the insert originated from the *M. tuberculosis* chromosome (Fig. 2a). The insert also hybridized to a 3.8 kb fragment in *M. bovis* BCG (Fig. 2b) but not to *Streptomyces coelicolor* [strain A3(2)M130, John Innes Institute, Norwich] genomic DNA digested with *Pst*I even under moderate stringency conditions (Fig. 2a).

EMS resistance conferred by the *M. tuberculosis* chromosomal fragment

The plasmids pSNS201 and pSNS202 are pUC19 derivatives containing the 3.8 kb fragment of pH15 in

Fig. 2. Southern blots of DNA isolated from *M. tuberculosis* H37RV, the vaccine strain *M. bovis* BCG (WHO), and *Streptomyces coelicolor*, probed with labelled DNA of the insert from pSNS201. (a) Lanes: 1, *S. coelicolor* DNA (5 µg) digested with *Pst*I; 2, *M. tuberculosis* DNA (5 µg) digested with *Pst*I. (b) Lanes: 1–2, BCG DNA digested with *Pst*I and *Bgl*II, respectively; 3–4, *M. tuberculosis* DNA digested with *Pst*I and *Bgl*II, respectively.

Fig. 3. Effect of the recombinant plasmid pSNS201 on the survival of *E. coli* DK cells which were plated on various EMS concentrations. Symbols: ●, MC1060; ○, DK; ■, DK(pSNS201).

opposite directions. Both plasmids conferred sufficient EMS resistance on DK cells to allow growth on 0.1% EMS. Six percent of the cells containing pSNS201 but only 2% of those containing pSNS202 formed colonies on 0.1% EMS (data not shown). *E. coli* DK without plasmid did not grow on 0.1% EMS. Fig. 3 shows that *E. coli* DK(pSNS201) was less resistant to EMS than *E. coli* MC1060, the *recA*⁺ parent of *E. coli* DK. The percentage of EMS-resistant cells containing these recombinant plasmids was obtained from five independent experiments. Selection was always performed with both ampicillin and EMS to ensure that no loss of plasmid DNA occurred.

UV sensitivity

No significant difference in UV resistance at 254 nm (1 J m⁻² s⁻¹) was found between *recA*⁻ strains and strains carrying the *M. tuberculosis* recombinant plasmid pSNS201. Clear differences in UV sensitivity were detected at a longer wavelength. Exposure of *E. coli* DK(pSNS201) to UV light of wavelength 302 nm (1 J m⁻² s⁻¹) failed to kill these cells, whereas no survivors were detected from the plasmid free *E. coli* DK under the same conditions.

Complementation of homologous recombination in *E. coli recA* mutants

The recombinant plasmids pSNS201 and pSNS202 were tested for their ability to complement the homologous recombination functions of various *E. coli recA* mutants. *E. coli* HB101 containing the recombinant plasmid pSNS201 restored recombination to levels approaching those of *E. coli* RR1 (77–81 as opposed to 95–102 recombinants per 1000 donor cells), while the recombinant plasmid with the insert in the opposite orientation (pSNS202), produced fewer transconjugants (18–21 per 1000 donor cells). The ranges indicated were obtained from four individual experiments. No revertants were obtained when similar conjugation experiments were performed with HB101 cells containing pUC19.

Fig. 4. Detection of RecA protein in crude cell extracts by Western blot analysis. Lanes: 1, *E. coli* DK; 2, *E. coli* DK (pSNS201); 3, *E. coli* MC1060; 4, *E. coli* RecA protein (Sigma) (arrowed).

Western blotting

Rabbit antiserum prepared against *E. coli* RecA protein (Goodman *et al.*, 1987) was used to detect RecA protein in crude extracts of *E. coli* MC1060, *E. coli* DK and *E. coli* DK(pSNS201) (Fig. 4). As the antiserum was not preabsorbed to *E. coli* DK cell extracts, it reacted with several polypeptides in the control lysates as well as in the test samples. The antiserum reacted with new polypeptide bands with an apparent molecular mass of 38–40 kDa in extracts prepared from *E. coli* DK (pSNS201) as well as in extracts of *E. coli* MC1060 (*recA*⁺) cells. The *E. coli recA* gene product is a polypeptide with a molecular mass of 37800 Da. RecA proteins were not detected in DK (pSNS202), which has the 3.8 kbp insert in the opposite orientation to *E. coli* DK (pSNS201) (results not shown).

Discussion

Several lines of evidence indicate that the recombinant plasmid (pSNS201) contains a gene from *M. tuberculosis* that encodes activity similar to that of the *E. coli recA* gene. The cloned DNA fragment restored near-wild-type EMS resistance in *E. coli recA* mutants, promoted homologous recombination between a Hfr donor and *recA* mutant, encoded a protein which cross-reacts with antiserum against the *E. coli* RecA protein and conferred low-level resistance to UV light (302 nm) on *recA E. coli*. Southern hybridisation confirmed that the insert originated from the *M. tuberculosis* genome.

E. coli DK (pSNS201) cells had increased resistance to

Table 1. Bacterial strains and plasmids

Strain or plasmid	Relevant markers	Reference
Bacterial strains		
<i>E. coli</i> LK111	<i>E. coli</i> K514 derivative (<i>lacZ</i> Δ M15)	Zabeau & Stanley, 1982
<i>E. coli</i> HB101	<i>leuB6</i> , <i>proA2</i> , <i>recA13</i> , <i>rpsL20</i>	Boyer & Roulland-Dussoix, 1969
<i>E. coli</i> DK	Δ (<i>ara</i> , <i>leu</i>)7697	ATCC 35691
<i>E. coli</i> MC1060	Δ (<i>srt-recA</i>)306	Willis <i>et al.</i> , 1981
	<i>recA</i> ⁺ parent strain of DK	Casadaban & Cohen, 1980
<i>E. coli</i> RR1	<i>recA</i> ⁺ parent strain of HB101	Bolivar <i>et al.</i> , 1977
<i>E. coli</i> CSH62	HfrH (<i>thi</i> , Str ^S)	Miller, 1972
Plasmids		
pEcoR252	Ap ^R <i>EcoR</i> I ('suicide vector')	Zabeau & Stanley, 1982
pUC19	Ap ^R	Vieira & Messing, 1982
pSNS201	<i>M. tuberculosis recA</i> in pUC19	This study
pSNS202	<i>M. tuberculosis recA</i> in pUC19	This study
pH15	<i>M. tuberculosis recA</i> in pUC19	This study

the DNA-damaging agent EMS (0.1%), whereas cells without the recombinants were killed at EMS concentrations above 0.01%. Based on studies of DNA repair in *Streptomyces cattleya*, Hromic *et al.* (1987) suggested that two repair systems exist in the *Streptomyces*. The first was evident when the cells were exposed to low levels of EMS. This may represent a constitutive, error-free repair system, which may be sufficient when the integrity of the genome is not disrupted. At high levels of EMS, when extensive DNA damage occurs, a second DNA repair system can be demonstrated, which is error-prone and similar to that found in *E. coli* (Baltz & Stonesifer, 1985). Thus in *Streptomyces*, EMS mutagenesis occurs by induction of an error-prone repair pathway, while in contrast *E. coli* EMS mutagenesis occurs by inducing base pair mismatching. At present the type of system that may be induced by EMS in *M. tuberculosis* is unknown.

pSNS201-mediated homologous recombination in a *recA13* recipient following conjugation with a Hfr donor strain. There were quantitative differences in the number of recombinant prototrophs obtained when the insert was in opposite orientations in the cloning vector (pSNS201 vs pSNS202). *E. coli* DK (pSNS202) cells also had fewer survivors on EMS. This polarity may reflect the effects of plasmid promoters on the expression of the *M. tuberculosis recA* gene. Most mycobacterial promoters are recognized poorly, if at all, by the *E. coli* transcriptional machinery (Thole *et al.*, 1985). This could explain

the lack of UV induction of pSNS201 expression in *E. coli*. *Streptomyces* hosts have been recognised as excellent vehicles for the cloning of *Mycobacterium* genes (Hopwood *et al.*, 1988) as they recognise promoters from several other bacterial genera (Bibb & Cohen, 1982).

Based on Western analysis, the 3.8 kbp *M. tuberculosis* chromosomal fragment (pSNS201) encodes polypeptides of apparent molecular mass 38–40 kDa, which cross-react with antisera raised against *E. coli* RecA protein. With *E. coli* DK (pSNS202), however, we failed to detect polypeptides in this molecular mass range. This may reflect the relative insensitivity of the Western blot technique compared with the functional complementation of RecA deficiency *in vivo*. Previous studies on RecA proteins from different micro-organisms have shown evidence for polypeptides with apparent molecular masses of 37–38 kDa in *Anabaena variabilis* (Owtttrim & Coleman, 1989); 37, 39 and 40 kDa in *Bacteroides fragilis* (Goodman *et al.*, 1987) and 35.5 and 38 kDa in *Legionella pneumophila* (Zhao & Dreyfuss, 1990). According to Zhao & Dreyfuss (1990), the higher-molecular-mass form could correspond to the *E. coli* *recA* gene product and the lower-molecular-mass polypeptide to a proteolytic degradation product of the RecA protein.

Exposure of DNA to a UV-light source of 302 nm results predominantly in the formation of pyrimidine dimers, while exposure to a source of 254 nm results in damage due to the formation of ionising radicals (Parrish *et al.*, 1981). The effects of these two wavelengths on cells containing the cloned insert in *E. coli* DK were studied. Unlike cells exposed to the longer-wavelength UV light, *E. coli* mutants carrying the *M. tuberculosis* recombinant plasmid were sensitive when exposed to short-wavelength UV radiation. Similar results were observed for the cloned *recA* homologues from the Gram-negative bacterium *Vibrio anguillarum* (Singer, 1989) and the obligate anaerobe *Bacteroides fragilis* (Goodman *et al.*, 1987). The results obtained with *Vibrio anguillarum* indicate that complementation becomes less efficient when doses of UV radiation are higher. Thus the cloned RecA homologue from *M. tuberculosis* did not fully complement defects in *E. coli* *recA* strains.

In *E. coli*, repair to UV induced damaged DNA is due to the cleavage of the protein UmuD. Dutreix *et al.* (1989) have described *recA* mutants which do not cleave UmuD. They suggest that the RecA protein has different domains which interact to mediate protein cleavage or DNA recombination. It has been shown that *recA* mutagenesis functions are genetically separable from *lexA* and cI proteolysis and that the latter activities are also separable from each other (Ennis *et al.*, 1989). It is possible that the *M. tuberculosis* RecA protein is defective as regards UmuD cleavage or that a separate protein fulfills this role in *M. tuberculosis*. Tessman &

Peterson (1985) have also provided evidence for the separation of recombination and mutagenesis functions of the *E. coli* RecA protein. Evidence for the functional conservation of RecA has been provided by the complementation of *E. coli* *recA* mutants by cloned genes from a variety of bacteria. This study indicates that this functional conservation extends to members of the Actinomycetes as exemplified by *M. tuberculosis*. Cloned *recA* genes that have been sequenced show relatedness in their functional domains (Ramesar *et al.*, 1989). Thus comparison of the *M. tuberculosis* *recA*-like gene sequence with those of other sequences would provide a further indication of the degree of conservation of this gene.

This study has demonstrated the existence of a *recA* homologue in *M. tuberculosis*. At present it is not clear if all the *E. coli* RecA functions are contained within one protein in *M. tuberculosis* or whether these are performed by more than one enzyme in this organism.

We thank D. Rawlings for helpful discussions and editorial comments and H. Goodman for providing the *E. coli* RecA antibody. We are indebted to Professor A. A. Forster for his constant enthusiasm and optimism. This work was supported by grants from the University of Cape Town and Medical Research Council to L.M.S.

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