Cloning of the *Serratia marcescens* *recA* Gene and Construction of a *Serratia marcescens* *recA* Mutant

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(Received 5 July 1989; revised 21 August 1989; accepted 11 September 1989)

A recombinant plasmid, pSM2513, containing an 8.5 kb DNA insert was isolated from a genomic library of *Serratia marcescens* by using interspecific complementation. This plasmid conferred resistance to methyl methanesulphonate and UV irradiation upon *recA* mutants of *Escherichia coli* and enhanced recombination proficiency, as measured by Hfr-mediated conjugation, in *recA* mutants of *E. coli*. Furthermore, when *recA* mutants of *E. coli* harbouring pSM2513 were subjected to UV irradiation, filamentation of the cells was observed. This did not occur upon UV irradiation of the same mutants harbouring the cloning vector alone. These results imply that the *S. marcescens* *recA* gene on pSM2513 is functionally similar to the *E. coli* *recA* gene in several respects. Restriction enzyme analysis and subcloning studies revealed that the *S. marcescens* *recA* gene was located on a 2.7 kb *Bgl*II–*Kpn*I fragment of pSM2513, and its gene product of approximately 39 kDa resembled the *E. coli* RecA protein in molecular mass. Using transformation-mediated marker rescue, a *recA* mutant of *S. marcescens* was successfully constructed; its proficiency both in homologous recombination and in DNA repair was abolished compared with its parent.

INTRODUCTION

The *recA* gene of *Escherichia coli* was first reported by Clark & Margulies (1965); it was cloned by McEntee & Epstein (1977). The RecA protein is a single polypeptide with a molecular mass of approximately 38 kDa (McEntee *et al.*, 1976; Sancar *et al.*, 1980). This protein is vital for homologous recombination (Clark, 1973; Radding, 1982), DNA repair (Hanawalt *et al.*, 1979), and the induction of SOS responses to DNA damage in *E. coli* (Radman, 1975). RecA protein is also responsible for catalysing DNA-dependent hydrolysis of nucleoside triphosphates (Weinstock *et al.*, 1979), proteolysis of the repressors of temperate bacteriophages such as λ , P22 and ϕ -80 (Irbe *et al.*, 1981; Little & Mount, 1982; Walker, 1984), synapsis of homologous DNA molecules, and promotion of DNA strand exchanges (Cox & Lehman, 1987). *In vitro* studies have shown that the multifunction of RecA protein is based on its remarkable dual enzyme nature. RecA protein is both a specific DNA-dependent protease which can cleave certain repressors, including LexA and those of bacteriophages, and a specific DNA-dependent ATPase which promotes homologous pairing of DNA molecules and therefore directly affects recombination and DNA repair (Radding, 1982). However, recent studies have suggested that RecA may not be a protease, but may act indirectly as a positive factor of an autodigestion reaction performed by LexA and phage λ repressors (Little, 1984; Slilaty & Little, 1987).

Recently, various investigators have sought an analogous *recA* gene in bacterial species other than *E. coli*, for example to study the pathogenicity of medically important bacteria (Goldberg & Mekalanos, 1986), to explore the pathogenicity and ecological fitness of plant bacteria (Hickman *et al.*, 1987), to enhance the stability of merodiploid genes in a desirable host (Finch *et*

Abbreviation: MMS, methyl methanesulphonate.

al., 1986; Murphy *et al.*, 1987), to simplify the merodiploid analysis of the gene products expressed in maxicell systems (Hickman *et al.*, 1987), and to facilitate genetic analysis of a particular bacterium (Resnick & Nelson, 1988). Interspecific complementation of well-defined *recA* mutants of *E. coli* (Better & Helinski, 1983) has been used to isolate the *recA* genes from a number of Gram-negative bacteria (Better & Helinski, 1983; Goldberg & Mekalanos, 1986; Keener *et al.*, 1984; Kokjohn & Miller, 1985; Koomey & Falkow, 1987; Resnick & Nelson, 1988).

Serratia marcescens, which has many properties in common with other enteric bacteria, has been used for manufacturing various amino acids, and for basic research (Takagi & Kisumi, 1985; Von Graevenitz & Rubin, 1980). In order to develop recombinant DNA techniques in *S. marcescens*, a mutant deficient in RecA functions is needed as a suitable host for these applications, although it is also desirable that the host cell should be deficient in extracellular nuclease and restriction endonuclease activity (Takagi & Kisumi, 1985). In this investigation, using interspecific complementation, we have successfully cloned the *recA* gene of *S. marcescens* and constructed a defined *recA* mutant of *S. marcescens* which may serve as a good host for manipulation of recombinant DNA and facilitate the study of the molecular biology of *S. marcescens*.

METHODS

Bacterial strains and recombinant DNA techniques. The bacterial strains and their relevant genotypes used in this study are listed in Table 1. LB medium and M9 minimal medium (Maniatis *et al.*, 1982) with or without antibiotics were routinely used to culture bacteria. *S. marcescens* chromosomal DNA was isolated as described by Marmur (1961). For the construction of a genomic library of *S. marcescens*, chromosomal DNA and plasmid pBR322 DNA were manipulated as described by Owttrim & Coleman (1987); ligation with T4 DNA ligase, with a vector-to-insert molar ratio of 1:2 (total DNA concentration, 50 µg ml⁻¹), was performed at 14 °C for 18 h. The resultant ligation mixture was used to transform *E. coli* HB101 (Maniatis *et al.*, 1982). Approximately 6000 ampicillin-resistant colonies were obtained; these were screened for MMS-resistant transformants by positive selection on LB agar plates containing 0.01% MMS. Plasmid DNA for further characterization was isolated from transformants by the alkaline extraction method of Birnboim & Doly (1979); large-scale plasmid DNA purification was done as described by Maniatis *et al.* (1982). Insertional inactivation of the cloned *recA* gene was achieved by cloning in a 1.3 kb fragment carrying a kanamycin-resistance determinant from plasmid pUC-4K (Pharmacia).

MMS resistance and UV sensitivity. Bacterial strains to be tested were grown to a density of approximately 10⁸ cells ml⁻¹ in LB broth and collected by centrifugation. MMS resistance was determined as described by Owttrim & Coleman (1987), and UV sensitivity was determined as described by Kokjohn & Miller (1985). Percentage survival is defined as [(c.f.u. counted after treatment with DNA-damaging agent)/(untreated c.f.u.)] × 100.

Bacterial conjugation. Cells were grown at 37 °C in LB broth to a density of approximately 10⁸ cells ml⁻¹, washed and resuspended in LB broth at a donor-to-recipient ratio of 1:10. Conjugation was allowed to proceed for 2 h at 37 °C without shaking. After incubation, the cell mixtures were vigorously vortexed, serially diluted, spread on M9 minimal medium plates containing stated amino acid and antibiotics, and incubated at 37 °C for 48 h. Transconjugants were selected on the basis of their acquisition of the ability to ferment lactose; counterselection against parental cells was by selection for a Lac⁻ phenotype (against recipient strains) and sensitivity to ampicillin and streptomycin (against donor strains). Platings were made on LB agar plates to determine donor and recipient c.f.u. and on M9 minimal-ampicillin-streptomycin agar plates to determine transconjugant c.f.u.

Filamentation analysis in response to UV irradiation. Bacterial cultures were incubated overnight in LB broth, and subcultured and grown to a density of approximately 10⁸ cells ml⁻¹. Cells were collected by centrifugation and resuspended in 0.85% saline, and the suspensions were subjected to UV irradiation as described by Geoghegan & Houghton (1987). The cells were collected again by centrifugation, resuspended in fresh LB broth and incubated in the dark for 60 min at 37 °C. Samples were examined for filamentation by microscopy (1000× magnification).

Transformation. *S. marcescens* cells were grown in 10 ml LB broth at 30 °C with aeration to a density of 5–10 × 10⁸ cells ml⁻¹. The culture was centrifuged for 10 min at 4 °C. The pellet was suspended in 10 ml ice-cold 0.1 M-MgCl₂, centrifuged for 10 min at 0 °C, and then suspended and incubated for 1 h at 0 °C in 10 ml 0.1 M-CaCl₂/0.25 M-sucrose. The cells were collected by centrifugation at 0 °C, resuspended in 2 ml ice-cold 0.1 M-CaCl₂/0.25 M-sucrose, and incubated at 4 °C for 48 h. Plasmid or chromosomal DNA and 5 mg salmon sperm DNA (as carrier) were added to 0.2 ml CaCl₂-treated cells, and the mixture was incubated at 0 °C for 1 h, followed by a 2 min heat pulse at 42 °C. The cells were plated onto selective plates after 90 min incubation in LB broth. The plates were incubated for 1 d at 30 °C.

Table 1. *Bacterial strains*

Strain	Genotype	Reference or source*
<i>E. coli</i>		
HB101	<i>hsdS20</i> (r^- m_k^+) <i>recA13</i> <i>ara-14</i> <i>proA2</i> <i>lacY1</i> <i>galK2</i> <i>rpsL20</i> <i>xyl-5</i> <i>mtl-1</i> <i>supE44</i> λ^-	Maniatis <i>et al.</i> (1982)
RR1	F ⁻ ; as HB101 except <i>recA</i> ⁺	Maniatis <i>et al.</i> (1982)
SE5000	<i>araD139</i> Δ (<i>arg-lac</i>) <i>U169</i> <i>flbB5301</i> <i>relA1</i> <i>recA56</i> <i>rpsL150</i> <i>rbsR</i> <i>ptsF25</i> <i>deoC1</i>	Silhavy <i>et al.</i> (1984)
BNN45	<i>supF</i> <i>supE</i> <i>hsdR</i> <i>galK</i> <i>trpR</i> <i>metB</i> <i>lacY</i> <i>tonA</i>	Silhavy <i>et al.</i> (1984)
ATCC 23739	K12 Hfr (Hayes), prototrophic	ATCC
<i>S. marcescens</i>		
SM25	Wild-type	This study
SM25NA	Spontaneous nalidixic-acid-resistant mutant of SM25	This study
SM2501	As SM25, except <i>recA</i>	This study
<i>S. liquefaciens</i>		
ATCC 14460	Wild-type	ATCC
<i>S. odorifera</i>		
ATCC 33077	Wild-type	ATCC
<i>S. plymuthica</i>		
ATCC 183	Wild-type	ATCC

* ATCC, American Type Culture Collection.

In vitro transcription-translation. Plasmids were subjected to transcription-translation in an *in vitro* prokaryotic DNA-directed transcription-translation system as specified by the supplier (Amersham). Reaction mixtures were incubated at 37 °C for 60 min with [³⁵S]methionine and then for an additional 5 min with excess unlabelled methionine. The reactions were terminated by placing the mixtures on an ice bath. The mixtures were diluted by the addition of 1:1 loading buffer (1.5%, w/v, Tris base, 10%, v/v, β -mercaptoethanol, 4.6%, w/v, SDS, 20%, v/v, glycerol, 0.01% bromophenol blue) and heated to 100 °C for 5 min. A portion of the sample was subjected to SDS-PAGE (15%, w/v, acrylamide). The gel was dried and the newly synthesized polypeptides were visualized by autoradiography. [¹⁴C]Methylated proteins (Amersham) were used as standard markers.

Southern hybridization. Genomic DNA was digested with a 10-fold excess of the indicated restriction enzyme. Digested DNA fragments were separated by overnight electrophoresis in 0.8% agarose gel, denatured *in situ* and transferred to a nitrocellulose membrane as described by Maniatis *et al.* (1982). Fragments were isolated from agarose as described by Silhavy *et al.* (1984). The 2.7 kb *Bgl*III-*Kpn*I fragment of pSM2513 was labelled *in vitro* with [α -³²P]dCTP (Amersham) by using a nick-translation kit (Bethesda Research Laboratories). The specific activity of the DNA probe was approximately 10⁷ c.p.m μ g⁻¹. The nitrocellulose membrane was hybridized with ³²P-labelled DNA probe and washed to remove non-specific binding as described by Maniatis *et al.* (1982). Autoradiography was performed for 24-72 h at -70 °C with X-ray film and intensifying screens.

RESULTS AND DISCUSSION

Isolation of the S. marcescens recA gene

A genomic library of *S. marcescens* was constructed by inserting size-fractionated fragments of chromosomal DNA of strain SM25, partially digested with *Sau*3AI, into the *Bam*HI site of pBR322 and then transforming *E. coli* HB101 with these recombinant plasmids. After selection on agar plates containing ampicillin, a library of approximately 6000 independent clones was obtained, which had a greater than 99% probability of covering the entire *S. marcescens* genome (Maniatis *et al.*, 1982).

Since *E. coli* HB101 (*recA*) is unable to grow in media containing MMS, the method of interspecific complementation (Better & Helinski, 1983) was used to isolate clones containing the *S. marcescens recA* gene. All clones of the genomic library were pooled and suspended in LB broth, and 10⁴ cells were plated onto agar plates containing MMS and ampicillin to screen MMS-resistant clones. After overnight incubation at 37 °C, MMS-resistant colonies appeared at a frequency of approximately 1.4 $\times$ 10⁻³; control plates with cells containing the cloning vector pBR322 alone showed no MMS-resistant colonies. Twenty-two of these MMS-resistant colonies

Fig. 1. (a) Survival of various *E. coli* strains after MMS treatment. Bacterial cells were selected on LB agar plates containing the stated amount of MMS. (b) Survival of various *E. coli* strains after UV irradiation. Results shown are the means of three independent experiments. ●, RR1(pBR322); ○, HB101(pBR322); ▲, HB101(pSM2513); □, SE5000(pBR322); ■, SE5000(pSM2513).

Table 2. *Recombinational proficiency in Hfr-mediated conjugation*

E. coli ATCC 23739 was used as the donor strain and was counterselected with ampicillin plus streptomycin. Cells were mixed in a donor-to-recipient ratio of 1:10. Matings were performed for 2 h at 37 °C. Recombination frequency is defined as transconjugant c.f.u./donor c.f.u. Transconjugants were selected as Lac⁺ colonies by plating on lactose-proline-ampicillin-streptomycin-M9 agar plates (for RR1 and HB101) or on lactose-arginine-ampicillin-streptomycin-M9 agar plates (for MC4100 and SE5000) after 48 h incubation. Results are the means of three independent tests.

Recipient <i>E. coli</i> strain	Genetic background	Recombination frequency
RR1(pBR322)	<i>recA</i> ⁺	6.7×10^{-2}
HB101(pBR322)	<i>recA13</i>	4.2×10^{-7}
HB101(pSM2513)	<i>recA13</i>	2.4×10^{-3}
MC4100(pBR322)	<i>recA</i> ⁺	1.7×10^{-2}
SE5000(pBR322)	<i>recA56</i>	1.4×10^{-7}
SE5000(pSM2513)	<i>recA56</i>	1.0×10^{-3}

were chosen at random and were examined for the existence of recombinant plasmids: all were found to contain the same-sized (8.5 kb) insert in the *Bam*HI site of pBR322. Plasmid DNA isolated from one of the 22 identical clones was used to retransform *E. coli* HB101, and all the resultant transformants were MMS resistant. This plasmid, termed pSM2513, was also able to restore MMS resistance in another *E. coli recA* mutant, SE5000 (Table 1). The quantitative effect of pSM2513 on the MMS resistance of *E. coli recA* strains HB101 and SE5000 was determined (Fig. 1a); both *recA* mutants were significantly more resistant to MMS when harbouring pSM2513 than when harbouring cloning vector alone.

To ensure that the MMS-resistant phenotype was due to heterologous complementation with the *S. marcescens recA* gene product of pSM2513, the following RecA-associated phenotypes were examined. First, the ability of the MMS-resistant clones to survive after treatment with another DNA-damaging agent, UV irradiation, was determined (Fig. 1b). All MMS-resistant

Fig. 2. Restriction endonuclease map of pSM2513 and its derivatives. The ability or inability of each subclone to confer resistance to MMS and UV irradiation upon *E. coli* HB101 (*recA*) is indicated on the right, by RecA (+) and RecA (-), respectively.

clones were more UV resistant when harbouring pSM2513 than when harbouring the vector alone. Plasmid pSM2513 was thus able to complement various *recA* mutations, such as *recA13* and *recA56*, in *E. coli* so that resistance to MMS and UV irradiation was restored, and the complemented mutants could elicit the SOS responses induced by DNA-damaging agents and overcome the DNA damage. Thus it is suggested that the *S. marcescens* RecA protein is similar to the *E. coli* RecA protein in DNA-repair function. Second, the ability of pSM2513 to restore the function of homologous recombination in *E. coli* *recA* mutants was quantitatively assayed by measuring genetic recombination frequency after conjugation with the *E. coli* Hfr strain ATCC 23739 (Table 2). The recombinational proficiency of various *E. coli* *recA* mutants was enhanced by the presence of pSM2513, whereas the same mutants harbouring the cloning vector pBR322 alone showed relatively low recombinational proficiency. This result suggests that the *S. marcescens* RecA protein functions in homologous recombination in a manner similar to the *E. coli* RecA protein. Third, the morphology of *E. coli* *recA* mutants, as well as those harbouring cloning vector alone, was unresponsive to UV irradiation; in contrast, when these strains harbouring pSM2513 were subjected to UV irradiation, filamentation occurred (data not shown). This result implies that the *S. marcescens* RecA protein may replace the *E. coli* RecA protein function in *recA*-*lexA* circuitry (Walker, 1984) during SOS responses in *E. coli*.

The *recA*-associated phenotypes produced by the *E. coli* and *S. marcescens* *recA* gene products were quantitatively different from each other (Table 2, Fig. 1), indicating that the *S. marcescens* RecA protein, when expressed from a recombinant plasmid, is less competent than the *E. coli* RecA protein expressed from the chromosome. This might stem from a difference in regulation of gene expression at a transcriptional or translational level, for example, the *E. coli* RNA polymerase being unable to transcribe the *S. marcescens* *recA* gene efficiently.

Mapping and subcloning of the *S. marcescens* *recA* gene

A physical map of pSM2513 was determined by digestion of the plasmid with a variety of restriction enzymes (Fig. 2). To locate the *recA* gene in the insert of pSM2513 more precisely,

Fig. 3. Estimation of the molecular mass of *S. marcescens* RecA protein by using an *in vitro* transcription-translation system (see Methods). Newly synthesized polypeptides directed by plasmid DNA were analysed by SDS-PAGE and autoradiography. Lane 1, standard markers; lane 2, control, no plasmid DNA; lanes 3 and 5, 2 µg and 5 µg of plasmid pUC19, respectively; lanes 4 and 6, 2 µg and 5 µg of plasmid pSM2553, respectively. The positions of the putative *S. marcescens* RecA protein and of β-lactamase (doublet) are indicated.

various restriction fragments were subcloned and their ability to complement the RecA⁻ phenotype of *E. coli* *recA* mutants was determined. Two *Eco*RI fragments of pSM2513 were ligated to *Eco*RI-digested pACYC184 to construct pSM2523 and pSM2533; only pSM2523 retained the ability to complement the *E. coli* RecA⁻ phenotype (Fig. 2). The *Bgl*II-*Eco*RI fragment of pSM2523 was then subcloned into the *Bam*HI-*Eco*RI site of pBR322 to generate pSM2543. The *Bgl*II-*Kpn*I fragment of pSM2523 was cloned into the *Bam*HI-*Kpn*I sites of pUC18 and pUC19, to create pSM2553 and pSM2553R, respectively, having the insert in opposite orientations (Fig. 2). These three recombinant plasmids were all capable of complementing the *E. coli* RecA⁻ phenotype, indicating that the *S. marcescens* *recA* gene was expressed from its own promoter in *E. coli*. Plasmid pSM2563, constructed from pSM2513 by deletion of the 0.9 kb *Bam*HI fragment, could not complement the *E. coli* RecA⁻ phenotype. On the basis of the above data, the *S. marcescens* *recA* gene was located within the 2.7 kb *Bgl*II-*Kpn*I fragment of the pSM2513 insert (see Fig. 2).

Estimation of the molecular mass of the S. marcescens recA gene product

To estimate the molecular mass of the putative *S. marcescens* *recA* gene product, plasmid pSM2553 was used to prime an *in vitro* transcription-translation system, and the newly synthesized polypeptides labelled by [³⁵S]methionine were detected by autoradiographic analysis after SDS-PAGE. A major protein of approximately 39 kDa encoded by pSM2553 was considered to be the *S. marcescens* *recA* gene product (Fig. 3); it was similar in molecular mass to the *E. coli* *recA* protein (Sancar *et al.*, 1980), and to other bacterial *recA* gene products (Better & Helinski, 1983; Keener *et al.*, 1984; Owtrim & Coleman, 1987; Sancar & Rupp, 1979).

Fig. 4. Survival of *S. marcescens* strains SM25 and SM2501 after UV irradiation. Results shown are the means of three independent experiments. ●, SM25; ○, SM2501.

However, comparison of restriction maps from various *recA* genes did not reveal any similarities with the *S. marcescens* *recA* gene. It is interesting that the amount of RecA protein was higher than that of β -lactamase (doublet) (Fig. 3, lanes 4 and 6), suggesting that the promoter of the *recA* gene might be stronger than that of the β -lactamase gene.

Southern hybridization

Hybridization experiments using the *Bgl*II-*Kpn*I fragment of pSM2513 as a probe showed strong homology among *S. marcescens*, *S. plymuthica* and *S. odorifera* (data not shown), suggesting that there is a homologous gene in these *Serratia* species. It should therefore be possible to construct *recA* mutants of other *Serratia* species by using plasmid pSM2513 in a marker exchange experiment (Ruvkun & Ausubel, 1981). Moderate homology between the *recA* genes of *E. coli* and *S. marcescens* was evident from hybridization experiments (data not shown), and preliminary sequencing data of pSM2553 also indicate that nucleotide sequence homology exists between the *S. marcescens* and the *E. coli* *recA* genes (data not shown).

Construction of a *recA* mutant of *S. marcescens*

A well-characterized *S. marcescens* *recA* mutant deficient in homologous recombination could most easily be obtained by mutation of the cloned DNA in *E. coli*, covalent association with a selective marker, and reintroduction of this sequence into the *S. marcescens* chromosome by means of transformation-mediated marker rescue (Kooimey & Falkow, 1987). Initially, using plasmid pSM2536, a derivative plasmid, pSM2563::Km, was constructed by the insertion of a restriction fragment bearing a kanamycin-resistance gene into the *Bam*HI site of pSM2563 (Fig. 2). Like pSM2536, pSM2563::Km failed to suppress the Rec⁻ phenotype of *E. coli* HB101. After transformation with *Bgl*II-digested pSM2563::Km kanamycin-resistant transformants of *S. marcescens* SM25 were selected at a frequency of 3.7×10^{-6} transformants per recipient cell; the transformants were further replica-plated onto LB agar plates containing 0.01% MMS to screen for MMS-sensitive colonies. One representative kanamycin-resistant and MMS-sensitive SM25 transformant was chosen and designated as strain SM2501. This strain was a defined *recA* mutant, as confirmed by Southern blot hybridization (data not shown).

The effects of the mutated allele on strain SM2501 were determined to two criteria. First, the proficiency of homologous recombination was investigated. Strains SM25 and SM2501 were

examined for their ability to acquire antibiotic resistance by transformation with chromosomal DNA from strain SM25NA, an isogenic nalidixic-acid-resistant mutant of strain SM25 (Table 1). Strain SM25 (Rec⁺) was transformed at much higher frequency (1.6×10^{-4} transformants per recipient cell) than strain SM2501 (5.3×10^{-8} transformants per recipient cell). Second, we investigated resistance to UV irradiation (Fig. 4): strain SM2501 was more UV sensitive than its parental strain SM25.

Since the defined *recA* mutant of *S. marcescens* behaved similarly to *recA* mutants of *E. coli*, which lack proficiency both in homologous recombination and in DNA repair function, the analysis of this mutant and its wild-type progenitor should allow a clear assessment of the roles that homologous recombination and the SOS response may play in DNA metabolism and gene expression in *S. marcescens*. We hope that this mutant will be useful in the development of recombinant DNA techniques and genetic approaches for studying the biology of *S. marcescens*.

We are grateful to Dr Shou-Hwa Han for his indispensable help, and thank Dr Janise Meyertons at Cold Spring Harbor Laboratory and Dr George M. Luedemann for reviewing this manuscript before submission. We also wish to thank Ms Shu-Jen Wei and Mr Yen-Hom Lee for their technical assistance. This study was supported by National Science Council Grant NSC 78-0412-B016-56, Republic of China.

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