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Construction and characterization of a *recA* mutant of *Xanthomonas campestris* pv. *citri*

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Abstract. The *recA* gene of *Xanthomonas campestris* pathovar *citri* (*X. c.* pv. *citri)* was cloned and sequenced. Nucleotide sequence analysis revealed an open reading frame of 1,032 bp that encodes a 344-amino acid protein and which shows a high degree of homology to *recA* genes from other bacteria. The cloned *recA* gene of *X. c.* pv. *citri* restored to a *recA* deletion mutant of *Escherichia coli* the ability to resist killing induced by methylmethane sulfonate or ultraviolet radiation, indicating that the cloned gene was functional in *E. coli*. A *recA* mutant of *X. c.* pv. *citri* was constructed by gene replacement and was shown both not to possess detectable DNA recombination activity and to be markedly more sensitive to DNA-damaging agents than is wild-type *X. c.* pv. *citri*. Transformation of *recA* mutants of *E. coli* and *X. c.* pv. *citri* with a plasmid containing *X. c.* pv. *citri recA* conferred the ability to produce a 37-kDa protein that reacted with antiserum to *E. coli* RecA protein. Two additional open reading frames were also identified in the *recA* region of the *X. c.* pv. *citri* genome: One located immediately upstream from *recA* that encodes a 213-amino acid protein with a high degree of similarity to the LexA protein of *E. coli*, and another located immediately downstream of *recA* that encodes a 153-amino acid protein with sequence similarity to the RecX protein of *Pseudomonas aeruginosa*.

Keywords: Complementation; DNA repair; Homologous recombination; RecA.

Abbreviations: ***X. c.* pv. *citri*,** *Xanthomonas campestris* pathovar *citri*; **LB**, Luria-Bertani; **PCR**, polymerase chain reaction; **UV,** ultraviolet; **CFU**, colony-forming unit; **MMS,** methylmethane sulfonate; **ORF**, open reading frame; ***Km*^{r}**, kanamycin-resistance gene; **RF**, replicative form.

Introduction

Xanthomonas campestris pv. *citri*, a Gram-negative, non-spore-forming bacterium, and one of approximately 140 pathovars of *X. campestris* (Verniere et al., 1993), causes citrus canker disease and thereby imposes an enormous economic burden worldwide. To date, only one gene, referred to as *pthA*, has been associated with the virulence of this organism (Lawson et al., 1989; Swarup et al., 1991, 1992); additional virulence genes remain to be identified. However, molecular studies of *X. c.* pv. *citri* have been hampered by the lack of recombination-deficient mutants. Thus, it has been difficult to maintain recombinant plasmids in *X. c.* pv. *citri* without the occurrence of deletions or rearrangements in the plasmid, which are likely the result of homologous recombination.

The RecA protein plays a major role in homologous recombination (Camerini-Otero and Hsieh, 1995; Clark, 1973; Cox, 1991; Kowalczykowski et al., 1994; Ogawa and Ogawa, 1986; Roca and Cox, 1990). More than 60 *recA* genes have been isolated and characterized from a wide variety of organisms, including *X. c.* pv. *oryzae* and *X. c.* pv. *campestris* (Karlin and Brocchieri, 1996; Lee et al., 1996; Lloyd and Sharp, 1993; Miller and Kokjohn, 1990; Rabibhadana et al., 1993). We have now cloned, sequenced, and characterized the *recA* gene of *X. c.* pv. *citri* as well as constructed a *recA* mutant of this organism.

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Materials and Methods

Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. Both *X. campestris* and *E. coli* strains were grown in Luria-Bertani (LB) medium at 28°C and 37°C, respectively. Chloramphenicol, kanamycin, and gentamycin were added to LB medium at final concentrations of 30, 50, and 15 μg/ml, respectively.

DNA Manipulation and Hybridization

Plasmid DNA was isolated from bacterial cultures by the method of Birnboim and Doly (1979), and was further purified by ethidium bromide-CsCl density gradient centrifugation. Chromosomal DNA was isolated from *X. c.* pv. *citri* as described by Pitcher et al. (1989). Restriction enzyme digestions and ligation were carried out according to standard methods described by Sambrook et al. (1989). The competent *X. c.* pv. *ciri* cells were prepared and subjected to electroporation by the method of Yang

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype or characteristics	Source or reference
Strain		
Escherichia coli		
JC10287	AB1157 derivative; D(*srl-recA*) 304	Czonka and Clark, 1979
AB1157	*recA*$^+$ strain	Bachmann, 1971
X. campestris pv. *citri*		
XW47	Virulent citrus canker type strain	Wu et al., 1985
XCK75	*recA* mutant derived from XW47	This study
Plasmid		
pBCSK$^+$	Phagemid derived from pUC19; used as cloning vector	Stratagene
pUC4-KIXX	*E. coli* gene cartridge vector	Pharmacia
PUCGM	Derivative of pUC19, *Gm*^{r}	Schweizer, 1993
pHC8	Derivative of pRK415, *oriV trfA Tc*^{r}	Chen, 1994
pUC4G	Plasmid pUC4-KIXX with *Km*^{r} replaced by *Gm*^{r}	This study
pXC560	pBCSK$^+$ with an inserted 560-bp PCR fragment containing a portion of *X. c.* pv. *citri recA*	This study
pAP74	pBCSK$^+$ with an inserted 7.4-kb *Apa*I fragment containing *X. c.* pv. *citri recA*	This study
pAP74K	pAP74 with the *recA* gene interrupted by *Km*^{r}	This study
pEG40	pHC8 with an inserted 4.0-kb *Eag*I fragment containing *X. c*. pv. *citri recA*	This study
pCF4G	pUC4G containing the 1.5-kb *NlaI*V fragment from RF DNA of bacteriophage cf	This study

Gm^r and *Tc*^{r}, gentamycin- and tetracyclin-resistance genes, respectively.

et al. (1991). Southern blot analysis was also performed as described previously (Southern, 1975; Yang and Yang, 1997). DNA probes were labeled with digoxigenin by random priming (Feinberg and Vogelstein, 1983) with the use of a DIG DNA labeling kit (Boehringer Mannheim). Colony hybridization was performed as described (Grunstein and Hogness, 1975).

PCR Amplification

Comparison of the nucleotide sequences of *recA* from various bacterial species revealed several highly conserved regions. Two oligonucleotides based on these conserved sequences were synthesized and used as primers for polymerase chain reaction (PCR) amplification of a portion of the *recA* gene of *X. c.* pv. *citri*. The forward primer (primer 1, 5'-CGGAATTCTCGGGCAAGACCACC-3') and reverse primer (primer 2, 5'-TAGCAAGCTTGTTCTT GACCACCTT-3') correspond to amino acids 67 to 73 (Asp-Ser-Ser-Gly-Lys-Thr-Thr) and 246 to 251 (Lys-Val-Val-Lys-Asn-Lys), respectively, of the RecA protein of *E. coli*; *Eco*RI and *Hin*dIII restriction sites were incorporated at the 5' ends of primers 1 and 2, respectively, to simplify cloning. PCR was performed in a total volume of 100 µl containing 100 ng of *X. c.* pv. *citri* genomic DNA, PCR buffer, 2.5 U of *Taq* DNA polymerase, 2.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, and 20 pmol of each primer for 35 cycles of 94°C for 1 min, 60°C for 1.5 min, and 72°C for 2 min.

DNA Sequencing and Nucleotide Sequence Analysis

DNA sequences were determined by the dideoxy chain termination method (Sanger et al., 1977) with *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Computer analysis of nucleotide and amino acid sequences was performed with the PC/GENE package version 6.85 (IntelliGenetics, Mountain View, CA).

Assay of Cell Survival After UV Irradiation or MMS Treatment

Sensitivity of bacteria to ultraviolet (UV) irradiation was determined by exposing 10 ml of a mid-log phase culture (optical density at 600 nm, 0.5) in a 100-mm petri dish to a GE germicidal lamp (256 nm) for 10 to 40 s at a distance of 15 cm. The UV dose was determined in each experiment with a UV radiometer (model VLX-254; Vilber Lourmat, Torcy, France). After irradiation, the bacterial cells were serially diluted, and 100 µl of each dilution were spread onto LB agar plates containing appropriate antibiotics. A sample of nonirradiated cells was also diluted and plated. After incubation for 24 h in the dark, the colonies on each plate were counted to determine the number of colony-forming units (CFU) per milliliter of sample. The survival rate at each UV dose was calculated by dividing the CFU/ml value of the irradiated sample by that of the non-irradiated sample.

Sensitivity to methylmethane sulfonate (MMS) was determined by plating bacterial cells directly onto LB agar containing various concentrations (0.6 to 3.6 mM) of MMS and incubating the cells overnight. The survival rate was calculated in the same manner as was that for UV sensitivity.

Detection of RecA Protein

Cell lysates prepared from *X. c.* pv. *citri* or *E. coli* were fractionated by SDS-polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane and subjected to immunoblot analysis with polyclonal antibodies to the *E. coli* RecA protein, as described previously (Towbin et al., 1979; Yang and Yang, 1997). These antibodies have been shown to be specific to the RecA protein (Lee et al., 1996).

Results

Cloning and Identification of the recA Gene of X. campestris pv. citri

With two oligonucleotide primers (primers 1 and 2) based on conserved sequences of *recA* genes from various bacterial species, a 560-bp DNA fragment was amplified from the *X. c.* pv. *citri* genome by PCR. After digestion with *Eco*RI and *Hin*dIII, this fragment was cloned into the plasmid pBCSK$^+$, thereby generating pXC560. The nucleotide sequence of the entire *X. c.* pv. *citri* fragment was determined and compared with that of the *E. coli recA* gene (Sancar et al., 1980). The two sequences shared 67% identity, suggesting that the 560-bp fragment corresponded to a portion of *X. c.* pv. *citri recA*.

The 560-bp fragment was then labeled with digoxigenin and used as a probe to identify a DNA molecule containing the entire *recA* gene of *X. c.* pv. *citri*. Genomic DNA from *X. c.* pv. *citri* was digested separately with each of the restriction enzymes *Apa*I, *Bam*HI, *Cla*I, *Eco*RI, and *Pvu*I, and the resulting fragments were subjected to electrophoresis on a 1% agarose gel and Southern blot analysis with the probe. An *Apa*I fragment of ~7 kb hybridized with the probe. Therefore, *Apa*I fragments of *X. c.* pv. *citri* genomic DNA of ~6 to 9 kb were eluated and cloned into pBCSK$^+$, and the colonies corresponding to the resulting partial genomic library were screened with the digoxigenin-labeled 560-bp fragment as probe. Of ~1000 colonies examined, four reacted with the probe. Restriction enzyme analysis revealed that all four positive colonies harbored a 7.4-kb *Apa*I fragment. A partial restriction map of the insert of the corresponding pAP74 plasmid is shown in Figure 1.

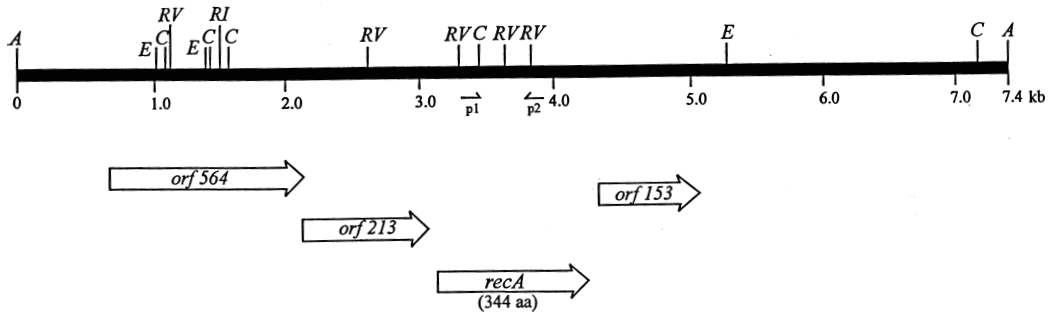

Figure 1. Partial restriction map of, and the locations of open reading frames in, the *recA* region of the *X. campestris* pv. *citri* genome. The positions of primer 1 (p1) and primer 2 (p2) used for PCR are indicated with half-arrows. Abbreviations for restriction enzyme sites: A, *Apa*I; C, *Cla*I; E, *Eag*I; RI, *Eco*RI; and RV, *Eco*RV. aa, amino acid.

Table 2. Percentage identity of nucleotide and predicted amino acid sequences of *recA* from various bacteria.

Organism[b]	Nucleotide and amino acid sequence identity (%)[a]							
	X. c. pv. *citri*	*X. c.* pv. *campestris*	*X. o.* pv. *oryzae*	*P. aeruginosa*	*E. coli*	*L. pneumophila*	*V. anguillarum*	*E. carotovora*
X. c. pv. *citri*	100	91	92	75	70	65	66	63
X. c. pv. *campestris*	97	100	91	76	69	65	64	61
X. o. pv. *oryzae*	97	97	100	77	71	64	65	60
P. aeruginosa	70	70	70	100	73	64	65	62
E. coli	67	70	67	71	100	67	75	76
L. pneumophila	72	76	72	76	73	100	69	69
V. anguillarum	68	71	69	72	83	71	100	75
E. carotovora	67	69	67	73	89	72	83	100

[a]Nucleotide and amino acid sequence comparisons are shown above and below the diagonal, respectively.

[b]Sequences of *recA* genes of the following organisms were compared: *X. c.* pv. *citri* (this study), *X. c.* pv. *campestris* (Lee et al., 1996), *X. o.* pv. *oryzae* Rabibhadana et al., 1998), *P. aeruginosa* (Kokjohn and Miller, 1985; Sano and Kageyama, 1987), *E. coli* (Horii et al., 1980; Sancar et al., 1980), *L. pneumophila* (Dreyfus, 1989), *V. anguillarum* (Singer, 1989), and *E. carotovora* (Keener et al., 1984).

DNA Sequence Analysis of the recA Gene of X. campestris pv. citri

The entire nucleotide sequence of the 7.4-kb *Apa*I fragment was determined. Sequence analysis revealed the presence of four open reading frames (ORFs) in the same translational orientation (Figure 1). The ORF designated *orf344* contains the sequence of the 560-bp fragment that was initially cloned. A database search for nucleotide and amino acid sequence similarities between *orf344* and other known genes revealed that *orf344* shows a high level of homology to *recA* genes of various bacteria, including *X. c.* pv. *campestris*, *X. oryzae* pv. *oryzae*, *Pseudomonas aeruginosa*, *E. coli*, *Legionella pneumophila*, *Vibrio anguillarum*, and *Erwinia carotovora* (Table 2). The greatest identity at both the nucleotide (92%) and amino acid (97%) levels was apparent with *recA* of *X. o.* pv. *oryzae.* These results suggest that *orf344* encodes the RecA protein of *X. c.* pv. *citri*.

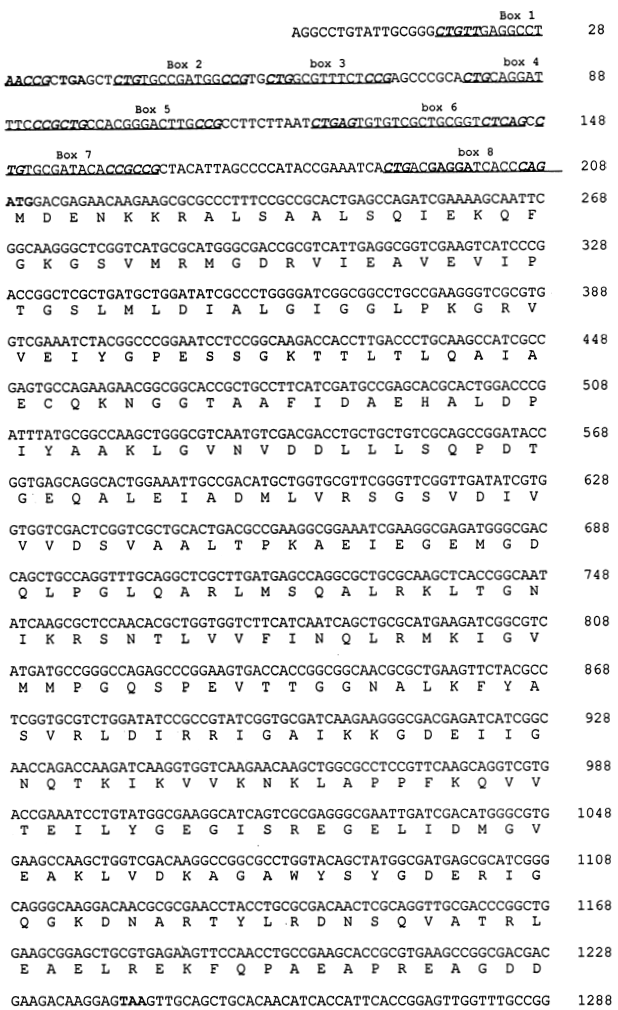

Figure 2. Nucleotide and deduced amino acid sequences of the *recA* gene of *X. campestris* pv. *citri*. The putative Shine-Dalgarno sequence (GAGGA) as well as the translation initiation (ATG) and termination (TAA) codons are indicated by bold type. The putative SOS boxes ($CTGN_{8-12}CCG$) are underlined, with the inverted repeat sequences indicated by bold letters. Nucleotide numbers are shown on the right. The GenBank accession number of the *recA* sequence is AF006590.

The ORF (*orf213*) located immediately upstream of *orf344* shares 76 to 82% sequence identity at the amino acid level with the *lexA* genes of *E. coli*, *P. aeruginosa*, *Pseudomonas putida*, *Salmonella typhimurium*, and *E. carotovora* (Garriga et al., 1992; Horii et al., 1981). The ORF (*orf153*) located immediately downstream of *orf344* shows 84% sequence identity at the amino acid level to the *recX* gene of *P. aeruginosa* (De Mot et al., 1994; Sano, 1993). The fourth ORF (*orf564*), which encodes a protein of 564 amino acids, shows no sequence homology to previously determined sequences in GenBank.

A putative Shine-Dalgarno sequence, GAGGA, is located 9 bp upstream from the putative ATG translational initiation codon of the *X. c.* pv. *citri recA* gene (Figure 2). No sequences similar to those of *E. coli* promoters were identified in the region immediately upstream of this gene. However, eight $CTGN_{8-12}CCG$ sequences, resembling those of the SOS box of DNA damage-inducible promoters of *E. coli* and other Gram-negative bacteria, are present within 200 bp upstream from the putative initiation codon (Figure 2).

Functional Analysis of the X. campestris pv. citri recA Gene

To determine whether the cloned *recA* gene of *X. c.* pv. *citri* was functional, we performed complementation assays. The pAP74 plasmid was introduced into a *recA* deletion mutant (JC10287) of *E. coli* (Czonka and Clark, 1979), and the resulting cells were assayed for their sensitivity to MMS treatment and UV irradiation. JC10287 cells containing the vector $pBCSK^+$ were assayed as a negative control, and *E. coli* strain AB1157, which contains wild-type *recA*, was used as a positive control. JC10287 cells containing $pBCSK^+$ were highly sensitive to MMS (Figure 3A). However, at 0.6 mM MMS, the survival rate of JC10287 cells containing pAP74 was five orders of magnitude greater than that of JC10287 cells containing $pBCSK^+$. At all concentrations of MMS tested (0.6 to 3.6 mM), the survival rate of AB1157 cells was 10 to 100 times that of JC10287 cells containing pAP74.

A similar pattern of survival rates was apparent for the three types of cells after UV irradiation (Figure 3B). Thus, at the lowest UV dose (20 J/m^2), the survival rate of JC10287 cells containing pAP74 was about three orders of magnitude greater than that of JC10287 cells containing $pBCSK^+$; no survivors were detected after exposure of the latter cells to UV doses of >20 J/m^2. At the highest dose (80 J/m^2), the survival rate of JC10287 cells containing pAP74 was $\sim 1 \times 10^{-3}$; although this survival rate is 2.5 log units below that of AB1157 cells, it still suggests that the *X. c.* pv. *citri recA* gene is functional to a certain extent in *E. coli*.

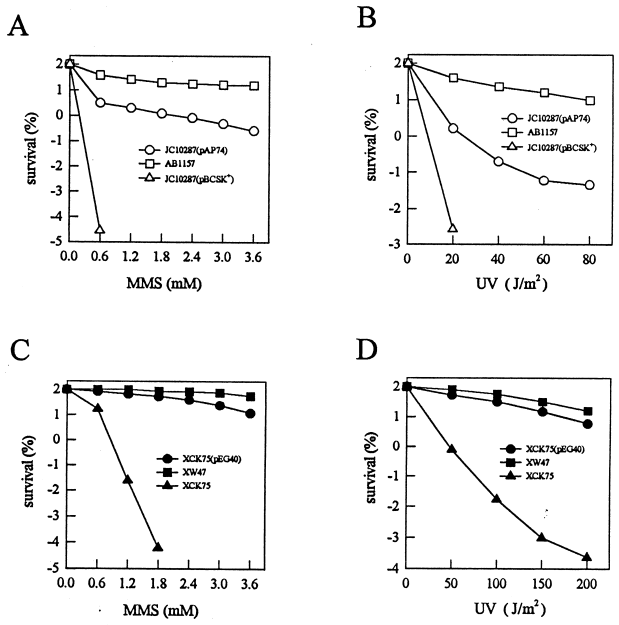

Figure 3. Sensitivity of *E. coli* and *X. campestris* pv. *citri* strains to MMS and UV. *Escherichia coli* strains AB1157 (*recA* wild type), JC10287 (*recA* deletion mutant) harboring pBCSK$^+$, and JC10287 harboring pAP74 (which contains the *recA* gene of *X. c.* pv. *citri*) were assayed for their sensitivities to MMS treatment (A) and UV irradiation (B). The sensitivities of the wild-type strain XW47 and *recA* mutant XCK75 of *X. c.* pv. *citri* to MMS (C) and UV (D) were also determined; complementation of the MMS and UV sensitivities of XCK75 by the cloned *recA* gene of *X. c.* pv. *citri* was examined by transformation with pEG40 (pAP74 cannot replicate in *X. c.* pv. *citri*). Data are expressed as the log of percentage survival values and are means of three independent experiments.

Construction and Characterization of a recA Mutant of X. campestris pv. citri

We subjected *X. c.* pv. *citri* to targeted homologous recombination in order to generate *recA* mutants. A 1.2-kb *Cla*I DNA fragment containing the kanamycin-resistance gene (*Km*^{r}) was isolated from pUC4-KIXX and then inserted into the internal *Cla*I site (Figure 2, nucleotide position 482) of the *X. c.* pv. *citri recA* gene in pAP74, thereby generating pAP74K. The latter plasmid was then introduced into the wild-type *X. c.* pv. *citri* strain XW47 by electroporation, and kanamycin-resistant cells were selected. Because pAP74K cannot replicate in *X. c.* pv. *citri*, the *Km*^{r} gene should have been integrated by a single or double crossover into the chromosome of resistant cells. A single crossover, occurring between the *recA* gene of pAP74K and that on the chromosome, might be expected to result in insertional inactivation of the chromosomal gene and integration of the entire plasmid, including its chloramphenicol-resistance gene. In contrast, a double crossover, occurring at the *recA* sequences flanking *Km*^{r}, may result in interruption of chromosomal *recA* by *Km*^{r}; the resulting mutants would therefore be kanamycin resistant but chloramphenicol sensitive.

Six kanamycin-resistant, chloramphenicol-sensitive colonies were picked and examined for the presence of an integrated *Km*^{r} gene. Chromosomal DNA was extracted from these cells and subjected to PCR using primers corresponding to both the N- and C- terminal sequence of RecA protein for characterization of *recA*. A PCR product of 1.3 -kb would indicate the presence of the wild-type *recA* gene, whereas a 2.5-kb product would suggest integration of *Km*^{r} into the chromosomal *recA* gene. All six colonies examined yielded a 2.5-kb PCR product, which was further shown to react with both *recA* (1.4-kb *Bgl*II fragment) and *Km*^{r} (1.2-kb *Cla*I fragment) probes on Southern hybridization analysis (data not shown). These results suggest that the six colonies comprise *recA* mutants of *X. c.* pv. *citri*.

One of these six potential *recA* mutants of *X. c.* pv. *citri*, designated XCK75, was further studied for its sensitivity to the DNA-damaging agents MMS and UV. At an MMS concentration of 1.8 mM, the survival rate of XCK75 cells was about six orders of magnitude smaller than that of the wild-type strain XW47 (Figure 3C). Similarly, at a UV dose of 200 J/m^2, the survival rate of XCK75 cells was four orders of magnitude smaller than that of XW47 cells (Figure 3D). We then examined whether the *recA* mutation in XCK75 could be complemented by the cloned *X. c.* pv. *citri recA* gene. For its introduction into XCK75, the *X. c.* pv. *citri recA* gene was first cloned into the *E. coli-X. campestris* shuttle vector pHC8 (Chen, 1994). A 4.0-kb *Eag*I fragment, containing the 3' half of *orf564* and all of *orf213*, *orf344* (*recA*), and *orf153* (Figure 1), was isolated from pAP74. The 5' overhangs of the fragment were converted to blunt ends with the Klenow enzyme, and the product was inserted into the unique *Xba*I site located among the multiple cloning sites of pHC8, thereby generating pEG40. This plasmid was then introduced into XCK75 by electroporation, and cells containing pEG40 were assayed for sensitivity to MMS and UV. After exposure to these agents, the survival rates of XCK75 cells harboring pEG40 were virtually identical to those of the XW47 wild-type strain.

We then examined XCK75 for a defect in homologous recombination, given that such defects are a prominent characteristic of *recA* mutants. The plasmid pCF4G, which contains the protein A gene of the *X. c.* pv. *citri* bacteriophage Cf, was introduced together with the replicative form (RF) of Cf DNA into XCK75 by electroporation. The frequency of homologous recombination between the protein A genes located on pCF4G and the RF DNA of Cf was then determined. In control experiments, both pCF4G and RF DNA of Cf were also introduced into the *X. c.* pv. *citri* wild-type strain XW47 and into XCK75 containing pEG40. The pCF4G plasmid was constructed by inserting the 1.5-kb *Nla*IV fragment containing the entire protein A gene of phage Cf into the unique *Hin*cII site of pUC4G, which itself was generated by replacing the 1.6-kb *Sma*I fragment containing the *Km*^{r} gene of pUC4-KIXX with the 0.85-kb *Sac*I fragment containing the gentamycin-resistance gene of pUCGM (Schweizer, 1993). Because pCF4G cannot replicate autonomously in *X. c.* pv. *citri*, the only

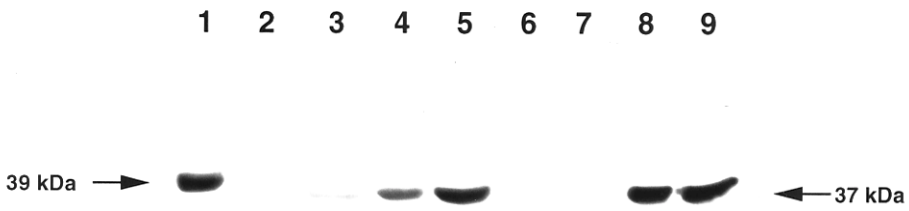

Figure 4. Expression of the cloned *recA* gene of *X. campestris* pv. *citri* in *E. coli* and *X. campestris* pv. *citri*. Cell lysates (30 μg of protein) of various *E. coli* or *X. c.* pv. *citri* strains were fractionated by SDS-polyacrylamide gel electrophoresis on a 12.5% gel and subjected to immunoblot analysis with antibodies to *E. coli* RecA protein. Samples were loaded as follows: lane 1, *E. coli* AB1157 (*recA*wild type); lane 2, *E. coli* JC10287 (*recA* deletion mutant); lane 3, JC10287 containing pAP74; lane 4, *X. c.* pv. *citri* wild-type strain XW47; lane 5, *X. c.* pv. *citri* XW47 treated for 2 h with mitomycin C (100 ng/ml); lane 6, *X. c.* pv. *citri recA* mutant XCK75; lane 7, *X. c.* pv. *citri* XCK75 treated with mitomycin C (100 ng/ml); lane 8, *X. c.* pv. *citri* XCK75 containing pEG40; lane 9, *X. c.* pv. *citri* XCK75 containing pEG40 and treated with mitomycin C (100 ng/ml). The positions of the 39-kDa *E. coli* RecA and the 37-kDa *X. c.* pv. *citri* RecA proteins are indicated.

cells that can grow in the presence of gentamycin are those in which Cf and pCF4G have cointegrated. Such cointegration would result from homologous recombination between the two protein A genes and replication by the phage replication machinery.

We plated 1×10^8 CFU (in 100 μl) of the various transformants and determined the number of gentamycin-resistant colonies. No gentamycin-resistant colonies were observed when pCF4G alone was introduced into XW47 or XCK75, confirming the inability of this plasmid to replicate in these two hosts. When both Cf DNA and pCF4G were introduced into XW47 or XCK75, only XW47 cells produced colonies (1.0×10^3 CFU/ml) that were resistant to gentamycin, suggesting that homologous recombination between the protein A genes in pCF4G and in Cf DNA did not take place in XCK75. A similar number (4.0×10^3 CFU/ml) of gentamycin-resistant colonies was observed with XCK75 containing pEG40, indicating that the defect in homologous recombination in XCK75 was corrected by the *X. c.* pv. *citri recA* gene in pEG40.

Expression of the X. campestris pv. citri recA Gene

The expression of the *X. c* . pv. *citri recA* gene in XCK75 cells containing pEG40 was examined by immunoblot analysis with antibodies to the *E. coli* RecA protein. Expression of the *X. c.* pv. *citri recA* gene was also examined in the *recA* deletion mutant of *E. coli* (JC10287) containing pAP74. Wild-type *E. coli* and *X. c.* pv. *citri* strains were also assayed as controls. A 39-kDa immunoreactive protein was apparent in the lysate of the wild-type *E. coli* strain AB1157 (Figure 4, lane 1) but not in that of the *recA* deletion mutant JC10287 (lane 2). An immunoreactive band (37-kDa, based on the deduced amino acid sequence) that migrated slightly faster than did the *E. coli* RecA protein was detected in the lysate of JC10287 cells containing pAP74 (lane 3) as well as in that of the wild-type *X. c.* pv. *citri* strain XW47 (lane 4). The intensity of this band was increased in the lysate prepared from XW47 cells treated with mitomycin C (lane 5). No such band was detected in lysates of either nontreated (lane 6) or mitomycin C-treated (lane 7) XCK75 cells, indicating that the *recA* gene in XCK75 was indeed completely inactivated. The 37-kDa band was apparent in the lysate of XCK75 cells containing pEG40 (lane 8), indicating that the *X. c* . pv. *citri recA* gene on the plasmid was expressed. The intensity of the 37-kDa band was not markedly affected by treatment of XCK75 cells containing pEG40 with mitomycin C (lane 9).

Discussion

The aims of this study were to clone the *recA* gene and to construct a *recA* mutant of *X. c.* pv. *citri*. A 7.4-kb *Apa*I fragment that contains the *recA* gene of *X. c.* pv. *citri* was isolated, and the gene was shown to encode a protein of 344 amino acids that shares a high degree of homology with RecA proteins of other bacterial species (97 and 67% sequence identity with RecA of *X. o.* pv. *oryzae* and *E. coli*, respectively). The cloned *X. c.* pv. *citri recA* gene partially restored the resistance of a *recA* deletion mutant of *E. coli* to treatment with MMS or UV radiation, confirming that the cloned gene is indeed *X. c.* pv. *citri recA*. The incomplete complementation of the *E. coli recA* deletion by the *X. c.* pv. *citri recA* gene might be due to a low effeciency of expression of the cloned gene in *E. coli*, or to reduced activity of the recombinant protein in *E. coli* relative to that of the native *E. coli* RecA protein.

The isolation of the *recA* gene of *X. c.* pv. *citri* enabled us to construct a *recA* mutant of this pathovar by homologous recombination. A *Km*^{r} gene was inserted into the cloned *recA* gene of *X. c.* pv. *citri*, and the resulting construct was introduced into *X. c.* pv. *citri* in order to replace the wild-type *recA* gene. The resulting *recA* mutant, designated XCK75, was shown by immunoblot analysis to have lost the ability to produce RecA protein. It also was highly sensitive to MMS and UV irradiation, suggesting that DNA repair function was impaired, and was incapable of mediating homologous recombination.

Eight $CTGN_{8-12}CCG$ sequences that are similar to the SOS box of DNA damage-inducible promoters of *E. coli* genes were detected in the promoter region of *X. c.* pv.

citri recA. The consensus sequence of the SOS box of *E. coli* is CTGTN$_8$ACAG (Wertman and Mount, 1985). Although not identical to the SOS box of *E. coli*, the sequence CTGN$_{8-12}$CCG may constitute the SOS box of *X. c.* pv. *citri*. The difference in sequence may reflect the evolutionary divergence of the two species. The SOS box is thought to function as an operator to which the LexA protein binds and thereby suppresses the expression of SOS genes (Walker, 1984). A gene homologous to *E. coli lexA* was detected upstream of *recA* in *X. c.* pv. *citri*. Furthermore, a gene similar to *recX* of *P. aeruginosa* was shown to be present downstream of *recA* in *X. c.* pv. *citri*. The LexA protein of *E. coli* is a transcriptional repressor of *recA* (Little and Mount, 1982; Wertman and Mount, 1985), and the RecX protein regulates the expression of *recA* in *P. aeruginosa* (Sano, 1993). Whether the products of the *lexA* and *recX* genes of *X. c.* pv. *citri* perform similar functions remains to be determined. The enhancement of *recA* expression in *X. c.* pv. *citri* by mitomycin C treatment suggests that *X. c.* pv. *citri* responds to DNA damage in a manner similar to that of *E. coli* and many other bacteria.

XCK75 is the first *recA* mutant of *X. c.* pv. *citri* to be constructed. It is also the first well-characterized *recA* mutant of any pathovar of *X. campestris*. Although *recA* mutants of *X. c.* pv. *campestris* (Lee et al., 1996) and *X. c.* pv. *oryzae* (Rabibhadana et al., 1993) have been constructed, characterization of these mutants has not been described. The availability of a *recA* mutant of *X. c.* pv. *citri* will allow more extensive molecular genetic studies of this organism. We have shown that XCK75 is completely defective in homologous recombination, a characteristic that will allow it to be used as a host for recombinant plasmids that contain portions of the *X. c.* pv. *citri* genome.

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Literature Cited

Bachmann, B.J. 1971. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:** 525-557.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:** 1513-1523.

Camerini-Otero, R.D. and P. Hsieh. 1995. Homologous recombination protein in prokaryotes and eukaryotes. Annu. Rev. Genet. **29:** 509-552.

Chen, S.C. 1994. Construction of Broad-Host-Range Promoter-proving Vector and Analysis of Promoter in *Xanthomonas campestris* pv. *campestris*. Masters thesis. Institute of Molecular Biology, National Chung Hsing University, Taichung, Taiwan.

Clark, A.J. 1973. Recombination deficient mutants of *E. coli* and other bacteria. Annu. Rev. Genet. **7:** 67-86.

Cox, M.M. 1991. The RecA protein as a recombinational repair system. Mol. Microbiol. **5:** 1295-1299.

Czonka, L.N. and A.J. Clark. 1979. Deletions generated by the transposon Tn10 in the *srl-recA* region of the *Escherichia coli* K-12 chromosome. Genetics **93:** 321-343.

De Mot, R., G. Schoofs, and J. Vanderleyden. 1994. A putative regulatory gene downstream of *recA* is conserved in Gram-negative and Gram-positive bacteria. Nucleic Acids Res. **22:** 1313-1314.

Dreyfus, L.A. 1989. Molecular cloning and expression in *Escherichia coli* of the *recA* gene of *Legionella pneumophila*. J. Gen. Microbiol. **135:** 3097-3107.

Feinberg, A.P. and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:** 6-13.

Garriga, X., S. Calero, and J. Barbe. 1992. Nucleotide sequence analysis and comparison of the *lexA* genes from *Salmonella typhimurium, Erwinia carotovora, Pseudomonas aeruginosa* and *Pseudomonas putida*. Mol. Gen. Genet. **236:** 125-134.

Grunstein, M. and D.S. Hogness. 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:** 3961-3965.

Horii, T., T. Ogawa, and H. Ogawa. 1980. Organization of the *recA* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **77:** 313-317.

Horii, T., T. Ogawa, and H. Ogawa. 1981. Nucleotide sequence of the *lexA* gene of *E. coli*. Cell **23:** 689-697.

Karlin, S. and L. Brocchieri. 1996. Evolutionary conservation of *recA* genes in relation to protein structure and function. J. Bacteriol. **178:** 1881-1894.

Keener, S.L., K.P. McNamee, and K. McEntee. 1984. Cloning and characterization of *recA* genes from *Proteus vulgaris, Erwinia carotovora, Shigella flexneri* and *Escherichia coli* Br. J. Bacteriol. **160:** 153-160.

Kokjohn, T.A. and R.V. Miller. 1985. Molecular cloning and characterization of the *recA* gene of *Pseudomonas aeruginosa* PAO. J. Bacteriol. **163:** 568-572.

Kowalczykowski, S.C., D.A. Dixon, A.K. Eggleston, S.D. Lauder, and W.M. Rehrauer. 1994. Biochemistry of homologous recombination in *Escherichia coli*. Microbiol. Rev. **58:** 401-465.

Lawson, R.H., M.M. Dienelt, and E.L. Civerolo. 1989. Histopathology of *Xanthomonas campestris* pv. *citri* from Florida and Mexico in wound-inoculated detached leaves of *Citrus aurantifolia*: light and scanning electron microscopy. Phytopathology **79:** 329-335.

Lee, T.C., N.T. Lin, and Y.H. Tseng. 1996. Isolation and characterization of the *recA* gene of *Xanthomonas campestris* pv. *campestris*. Biochem. Biophys. Res. Commun. **221:** 459-465.

Little, J.W. and D.W. Mount. 1982. The SOS regulatory system of *Escherichia coli*. Cell **29:** 11-22.

Lloyd, A.T. and P.M. Sharp. 1993. Evolution of the *recA* gene and the molecular phylogeny of bacteria. J. Mol. Evol. **37:** 399-407.

Miller, R.V. and T.A. Kokjohn. 1990. General microbiology of *recA*: environmental and evolutionary significance. Annu.

Rev. Microbiol. **44:** 365-394.

Ogawa, H. and T. Ogawa. 1986. General recombination: functions and structure of RecA protein. Adv. Biophys. **21:** 135-148.

Pitcher, D.C., N.A. Saunder, and R.J. Owen. 1989. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Lett. Appl. Microbiol. **8:** 151-156.

Rabibhadana, S., S. Chamnongpol, J.E. Trempy, N.P. Ambulos, and S. Mongkolsuk, 1993. Isolation and expression in *Escherichia coli* of a *Xanthomonas oryzae recA*-like gene. Gene **213:** 113-118.

Rabibhadana, S., S. Chamnongpol, R. Sukchawalit, J.E. Trempy, N.P. Ambulos, and S. Mongkolsuk. 1998. Characterization and expression analysis of *Xanthomonas oryzae* pv. *oryzae recA.* FEMS Microbiol. Lett. **158:** 195-200.

Roca, A.I. and M.M. Cox. 1990. The RecA protein: structure and function. Crit. Rev. Biochem. Mol. Biol. **25:** 415-456.

Sambrook, J., E. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sancar, A., C. Stachelek, W. Konigsberg, and W.D. Rupp. 1980. Sequences of the *recA* gene and protein. Proc. Natl. Acad. Sci. USA **77:** 2611-2615.

Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:** 5463-5467.

Sano, Y. and M. Kageyama. 1987. The sequence and function of the *recA* gene and its protein in *Pseudomonas aeruginosa* PAO. Mol. Gen. Genet. **208:** 412-419.

Sano, Y. 1993. Role of the *recA*-related gene adjacent to the *recA* gene in *Pseudomonas aeruginosa*. J. Bacteriol. **175:** 2451-2454.

Schweizer, H.P. 1993. Small broad-host-range gentamycin resistance gene casettes for site-specific insertion and deletion mutagenesis. BioTechniques **15:** 831-833.

Singer, J.T. 1989. Molecular cloning of the *recA* analogs from the marine fish pathogen *Vibrio anguillarum* 775. J. Bacteriol. **171:** 6367-6371.

Southern, E.M. 1975. Detection of a specific sequence among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503-517.

Swarup, S., R. De Feyter, R.H. Brlansky, and D.W. Garbriel. 1991. A pathogenicity locus from *Xanthomonas citri* enables strains from several pathovars of *X. campestris* to elicit cankerlike lesions on citrus. Phytopathology **81:** 802-809.

Swarup, S., Y. Yang, M.T. Kingsley, and D.W. Gabriel. 1992. An *Xanthomonas citri* pathogenicity gene, *pthA*, pleiotropically encodes gratuitous avirulence on nonhosts. Mol. Plant-Microbe Interact. **5:** 204-213.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:** 4350-4354.

Verniere, C., O. Pruvost, E.L. Civerolo, O. Gambin, J.P. Jacquemoud-Collet, and J. Luisetti. 1993. Evaluation of the biological substrate utilization system to identify and assess metabolic variation among strains of *Xanthomonas campestris* pv. *citri*. Appl. Environ. Microbiol. **59:** 243-249.

Walker, G.C. 1984. Mutagenesis and inducible responses to deoxyribonucleic acid damage in *Escherichia coli*. Microbiol. Rev. **48:** 60-93.

Wertman, K.F. and D.W. Mount. 1985. Nucleotide sequence binding specificity of the LexA repressor of *Escherichia coli* K-12. J. Bacteriol. **163:** 376-384.

Wu, W.C., Y.K. Hong, C.M. Huang, Y.Y. Ten, T. Lee, S.F. Chang, C.H. Wang, and M.H. Huang. 1985. Phage induced mutations in *Xanthomonas campestris* pv. *citri*. Plant Protect. Bull. (Taipei) **27:** 19-33.

Yang, M.K., W.C. Su, and T.T. Kuo. 1991. Highly efficient transfection of *Xanthomonas campestris* by electroporation. Bot. Bull. Acad. Sin. **32:** 197-203.

Yang, M.K. and Y.C. Yang. 1997. The A protein of the filamentous bacteriophage Cf of *Xanthomonas campestris* pv. *citri.* J. Bacteriol. **179:** 2840-2844.

柑橘潰瘍病菌 *recA* 突變株之建立與特性分析

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自柑橘潰瘍病菌（*Xanthomonas campestris* pv. *citri*）所選殖之 *recA* 基因，是由 1032 個核±Ò酸所組成，可合成含 344 個氨基酸之蛋白質。其序列與其他細菌之 *recA* 基因很類似，合成之蛋白在大腸桿菌內亦具有功能。利用插入性突變法，得到之 *recA* 突變株，對 DNA 突變劑極為敏感，也失去基因重組的能力。選殖之 *recA* 基因，可形成一 37 kDa 之蛋白，可與抗大腸桿菌 RecA 之抗體作用。另外，在 *recA* 基因之上游，還發現一可產生 213 氨基酸之蛋白的基因，相似於大腸桿菌之 *lexA* 基因；而在其下游，還有一由 153 氨基酸所組成之蛋白之基因，相當於綠膿桿菌之 *recX* 基因。

關鍵詞：基因重組；RecA；互補；DNA 修補。