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Isolation and Characterization of the *recA* Gene of *Rhizobium meliloti*

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Interspecific complementation of an *Escherichia coli recA* mutant with plasmids containing a gene bank of *Rhizobium meliloti* DNA was used to identify a clone which contains the *recA* gene of *R. meliloti*. The *R. meliloti recA* protein can function in recombination and in response to DNA damage when expressed in an *E. coli recA* host, and hybridization studies have shown that DNA sequence homology exists between the *recA* gene of *E. coli* and that of *R. meliloti*. The isolated *R. meliloti recA* DNA was used to construct a *recA R. meliloti*, and this bacterium was not deficient in its ability to carry out symbiotic nitrogen fixation.

The *recA* gene product of *Escherichia coli* is involved in several fundamental aspects of DNA metabolism. It has been established that the *recA* product is essential for DNA repair (9), homologous recombination (3, 14), and the expression of SOS functions in response to DNA damage (15). The purified *recA* protein of *E. coli* is a multifunctional enzyme which can catalyze DNA-dependent hydrolysis of nucleoside triphosphates (23), proteolysis of specific regulatory proteins (17), and DNA strand exchanges analogous to steps in homologous recombination in vivo (5, 6). The protein which catalyzes these activities is a single polypeptide with a molecular weight of approximately 40,000.

It is clear that proteins analogous to the *recA* gene product of *E. coli* are important in the DNA metabolism of other bacterial species as well. Evidence has been presented that a *recA*-like protein functions in bacteria as diverse as *Haemophilus influenzae* (11), *Salmonella typhimurium* (12), *Streptococcus faecalis* (24), *Proteus mirabilis* (8), and *Agrobacterium tumefaciens* (10). In fact, the *recA* protein of *P. mirabilis* can function efficiently when expressed from a plasmid in *E. coli* (8).

We have used interspecific complementation to identify a DNA fragment containing the *recA* gene of *Rhizobium meliloti*, a species important in symbiotic nitrogen fixation. A functional similarity exists between the *recA* protein of *E. coli* and that of *R. meliloti*, and hybridization experiments suggest that considerable sequence homology exists between these two species and also among other bacteria related to *R. meliloti*. Once identified, the DNA fragment containing the *recA* gene was used to generate a *recA* mutant of *R. meliloti*. This mutant bacterium was not impaired in its ability to carry out symbiotic nitrogen fixation.

MATERIALS AND METHODS

Bacterial and phage strains. The bacterial strains used are listed in Table 1. Phage λ *bio*252 *c*I857 contains a biotin substitution for λ genes *int* to *N*.

Media. *E. coli* strains were grown in LB medium. *Rhizobium* strains were grown in either yeast manitol medium (21) or *Rhizobium* minimal medium (13). Antibiotics used for selection were at the following concentration: tetracycline, 15 μg/ml for *E. coli* and 5 μg/ml for *R. meliloti*; kanamycin and neomycin, 50 μg/ml each; gentamicin, 60 μg/ml; nalidixic acid, 10 μg/ml. To test for growth of bacteria in the presence of methyl methanesulfonate (MMS), 100 μl of a 2% solution was spread on the surface of an LB plate.

Plasmids. Plasmid pRK290 (7) is a broad-host range cloning vector derived from the P1 incompatibility group plasmid RK2 and confers tetracycline resistance; pRK2013 is a helper plasmid of pRK290 which carries the conjugal transfer genes of RK2 and confers kanamycin resistance (7). pRK2073 is a derivative of pRK2013 which carries a Tn*7* insertion in the kanamycin resistance gene. pPH1JI belongs to the incompatibility group P1 and confers gentamicin resistance (1).

pBEU14 is an amplifiable derivative of an R1 replicon which carries the *recA* gene of *E. coli* (19). pUC9 is a high-copy-number ColE1 derivative which confers ampicillin resistance (20).

Bacterial conjugation. Hfr mating tests of *E. coli* strains were performed in L-broth with a male-to-female ratio of 1:10 and a total cell concentration of about 2×10^8 per ml. Conjugal transfer of P1 plasmids from *E. coli* to *R. meliloti* or between *E. coli* strains was performed by mixing loopfuls of donors and recipients together on nonselective yeast mannitol or LB plates, respectively. After overnight incubation, transconjugants were selected on appropriate media.

Transposon mutagenesis. Plasmid pRK290 containing cloned *R. meliloti* DNA was transformed into HB101::Tn*5*. Several transformants were pooled, and plasmid DNA was mated into the *polA* strain C2110 in

TABLE 1. Bacterial strains

Strain	Relevant genotype/ phenotype	Source or reference
E. coli Ymel(P2)	*supIII* P2 lysogen	D. Friefelder
E. coli HB101	*pro leu thi lacY* Str^r *endA recA hsdR hsdM*	(2)
E. coli MB2	*thr leu met* Str^r *thyA supII endA*	(1a)
E. coli MB4	MB2 *srl-300*::Tn*10* *recA306*	(1a)
E. coli C2110	*polA* Nal^r	G. Ditta
E. coli HB101::Tn*5*	Kn^r	G. Ditta
E. coli M-20	HfrH Str^s	H. Echols
R. meliloti 102F34		T. Ruiz-Argueso
R. meliloti 41		T. Ruiz-Argueso
R. leguminosarum 791		T. Ruiz-Argueso
R. japonicum 778		T. Ruiz-Argueso
A. tumefaciens B6		T. Ruiz-Argueso

a triparental mating with the helper strain HB101 (pRK2073), with subsequent selection for colonies resistant to nalidixic acid, tetracycline, and kanamycin. Plasmid DNA was then isolated and used to transform strain HB101, selecting for tetracycline and kanamycin resistance.

Site-specific exchange of Tn*5* insertions with the *R. meliloti* chromosome. A plasmid with an insertion of *R. meliloti* DNA carrying the transposon Tn*5* in the *recA* gene was homogenotized with the *R. meliloti* genome by the technique of Ruvkun and Ausubel (18). Plasmid pPH1JI, which was incompatible, was used as the incoming plasmid, and selection was made on *Rhizobium* minimal media supplemented with neomycin and gentamicin. Tetracycline-susceptible bacteria were identified, and the physical location of Tn*5* in the genome was verified by hybridization analysis as previously described (4).

Hybridization probe and Southern blotting. The nick-translation procedure described by Rigby et al. (16) was used to label purified DNA fragments. Transfer of DNA from agarose to nitrocellulose paper for use in hybridization experiments was done as described by Wahl et al. (22). Purified total genomic DNA of *Rhizobium* and *Agrobacterium* strains was kindly provided by T. Ruiz-Argueso.

Plant tests. Plant tests were carried out as previously described (4). Twelve seeds were planted in each apparatus, and the *Rhizobium* inoculum was from a culture grown to stationary phase in yeast mannitol medium.

RESULTS

Isolation of the *R. meliloti recA* gene. In this section, we demonstrate that a gene from *R. meliloti*, expressed in *E. coli*, can complement an *E. coli recA* mutant. *E. coli* HB101 is *recA* and incapable of growth on media supplemented with MMS. *E. coli* HB101 which express a Rec$^+$ phenotype (and hence grow in the presence of MMS) were sought in a population of cells containing a plasmid gene bank of *R. meliloti* DNA.

Ditta et al. (7) have described the construction of a gene bank containing greater than 98% of the *R. meliloti* genome by cloning *Bgl*II restriction fragments of *R. meliloti* DNA into the broad-host range plasmid pRK290. The gene bank, maintained in *E. coli* HB101 as 1,400 clones, was grown on LB tetracycline plates overnight, and the colonies were then scraped off the plates and pooled. A sample containing approximately 6.5×10^4 cells was plated on an LB plate supplemented with MMS. An equal number of bacteria harboring plasmid pRK290 alone were plated as a control. After overnight growth, colonies appeared from the gene bank containing cells at a frequency of 3×10^{-3}, whereas none appeared among the controls.

Restriction analysis of the plasmid DNA from six MMS-resistant colonies revealed that each contained the same insert into the plasmid pRK290. To demonstrate that the MMS resistance phenotype was plasmid encoded rather than due to bacterial mutation, the plasmid DNA was retransformed into strain HB101. All transformants became MMS resistant. Thus, heterologous complementation allowed us to identify a segment of *R. meliloti* DNA which can express one *recA* gene-like function.

Restriction mapping, Tn*5* mutagenesis, and construction of an *R. meliloti recA* mutant. A plasmid, designated pRK290.34, which can complement the Rec phenotype of strain HB101 was described above. A restriction map of the 34-kilobase (kb) insert in this plasmid is shown in Fig. 1. Tn*5* mutagenesis was used as one criterion to localize the presumptive *R. meliloti recA* gene. The transposon Tn*5* was randomly inserted into pRK290.34 as described above. *E. coli* HB101 harboring pRK290.34::Tn*5* were then tested for MMS resistance. Of 50 colonies tested, 1 containing the plasmid designated pRMB1002 was no longer MMS resistant. Restriction mapping and subsequent Southern hybridization (see below) revealed that the *R. meliloti* gene capable of Rec complementation is centrally located within the 4.8-kb *Bam*HI fragment A; the position of this insertion is shown in Fig. 1. This fragment when subcloned into the plasmid pUC9 confers MMS resistance upon strain HB101.

The plasmid pRMB1002 is deficient in the complementation of all *E. coli recA* functions tested (see below); hence, we concluded that a

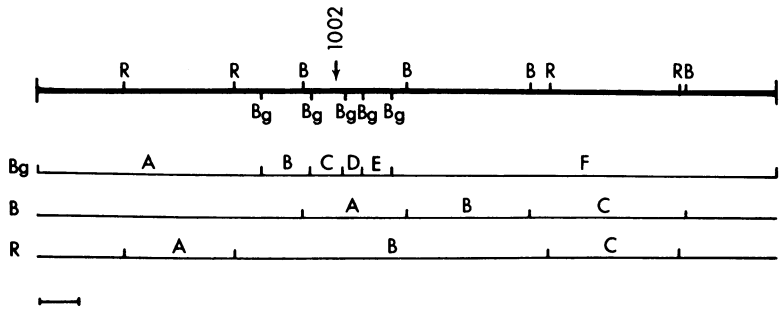

FIG. 1. Restriction endonuclease map of *R. meliloti* DNA in plasmid pRK290.34. This 34-kb DNA fragment was cloned into plasmid pRK290 after partial digestion of total *R. meliloti* DNA with *Bgl*II (7). Restriction enzyme sites for the following enzymes are shown: Bg, *Bgl*II; R, *Eco*RI; B, *Bam*HI. The individual restriction patterns are shown below the composite map, and each restriction fragment is labeled. Three small *Eco*RI fragments (<3 kb) which lie to the left of *Eco*RI fragment A and to the right of fragment C are not shown. The vertical arrow marked 1002 shows the position of a Tn*5* insertion which inactivates the *recA* gene. The horizontal bar (bottom left) indicates 2 kb.

Tn*5* insertion at this position inactivated the *R. meliloti recA* gene. A *recA* derivative of *R. meliloti* was generated from the wild-type strain Rm102F34 with pRMB1002 by the marker exchange technique described by Ruvkun and Ausubel (18). Replacement of the wild-type gene with the Tn*5*-inserted allele was verified by Southern hybridization (data not shown). The *recA R. meliloti* was quite UV sensitive compared with the wild type, as is expected of a *recA* strain (Fig. 2a).

Since very few data are available concerning the possibility of genetic exchange between *Rhizobium* DNA and the DNA of the plant symbiont, it was of interest to examine whether a recombination-deficient *R. meliloti* strain was affected in its symbiotic potential. Alfalfa plants inoculated with *R. meliloti recA*::Tn*5* grew as well as plants inoculated with the wild-type strain Rm102F34. There was no difference in the acetylene reduction from nodules of 6-week-old plants inoculated with wild-type or *recA* bacteria.

Expression of the *R. meliloti recA* gene in *E. coli*. The *recA* gene product of *E. coli* is a multifunctional protein. We have, therefore,

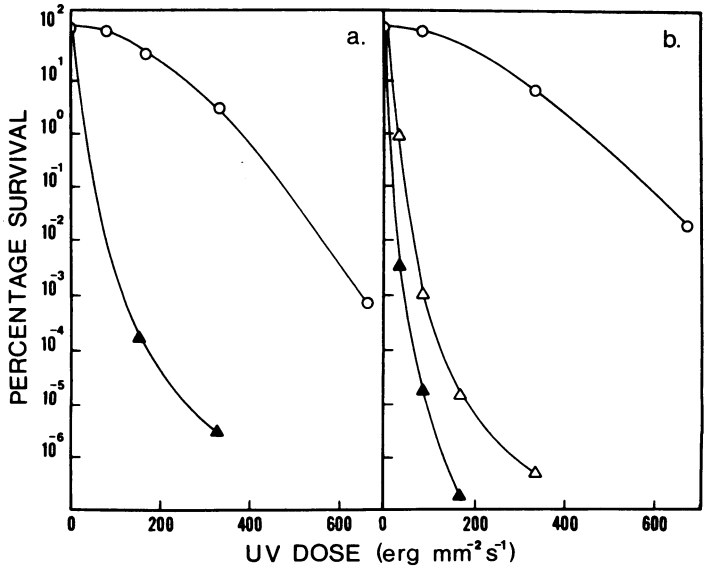

FIG. 2. UV sensitivity curves for *recA*$^{+}$ and *recA* bacteria. Bacteria from a log-phase liquid culture were spread onto the surface of dried LB plates and irradiated with UV light. (a) UV survival curve of wild-type *R. meliloti* 102F34 (○) and the *recA*$^{-}$ derivative (▲). (b) UV survival curves of *E. coli* strains MB2 (○), MB4 (▲), and MB4 harboring plasmid pRMB1008 (△).

TABLE 2. Recombination proficiency of *E. coli* strains

Recipient	Yield of recombinants to donors (%)[a]
MB2	2.0
MB4	3×10^{-3}
MB4(pRMB1008)	1.0
MB4(pRMB1002)	3×10^{-3}

[a] *E. coli* M-20 was mated with each of the recipients for 60 min as described in the text. Selection was for *leu*$^+$ recombinants. Each percentage is the average of three recombination tests.

characterized the activity of the presumptive *R. meliloti recA* gene in *E. coli* to study those activities which have been functionally conserved.

The ability of the *R. meliloti recA* gene product to catalyze genetic recombination in an Hfr cross was measured, and the results are shown in Table 2. A derivative of plasmid pRK290.34 containing a Tn*5* insertion in the vector DNA, pRMB1008, permitted 50% of the wild-type level of recombination.

In *E. coli*, the plasmid pRK290.34 also allows a λ *red*$^-$ *gam*$^-$ phage to plate on a lawn of *recA* bacteria (Table 3). In a *recA* host, a λ *red*$^-$ *gam*$^-$ phage is incapable of growth (the Fec phenotype [25]). We also examined the ability of plasmid pRMB1008 to suppress the UV sensitivity phenotype of an *E. coli recA*. Figure 2b shows that there is approximately a twofold UV resistance conferred by pRMB1008.

In conclusion, the plasmid pRK290.34 and its derivative pRMB1008 contain a fragment of *R. meliloti* DNA which is capable of being expressed in *E. coli*. The plasmid can fully or partially suppress several Rec$^-$ phenotypes, including recombination deficiency in an Hfr cross, inability to plate a λ *red*$^-$ *gam*$^-$ phage, UV sensitivity, and MMS sensitivity.

Sequence homology between the *E. coli* and *R. meliloti recA* genes. The previous sections demonstrated that a segment of *R. meliloti* DNA was expressed in *E. coli* and suppressed a Rec$^-$ phenotype. In this section, we show that considerable sequence homology exists between the *recA* gene of *E. coli* and that of *R. meliloti*.

We have used *E. coli recA* gene DNA as a heterologous hybridization probe to plasmid pRK290.34 and to total genomic *R. meliloti* DNA. A 3-kb *Bam*HI fragment which contains the entire *recA* gene of *E. coli* was purified from plasmid pBEU14 and nick-translated for use as a probe for hybridization. The results are shown in Fig. 3. In addition to the hybridization to pRK290.34 (lane a) and an analogous *Bam*HI fragment from *R. meliloti* 102F34 chromosomal DNA (lane b), it was shown (lanes c through f) that the *E. coli* probe has considerable sequence homology to other species closely related to Rm102F34, including *Rhizobium leguminosarum*, *Rhizobium japonicum*, and *A. tumefaciens*. In each case, the *E. coli recA* probe hybridized to a distinct *Bam*HI fragment of chromosomal DNA. Additional hybridization experiments (data not shown) revealed that the *E. coli recA* probe hybridized to *Eco*RI fragment B, *Bam*HI fragment A, and *Bgl*II fragments C and D of plasmid pRK290.34 DNA (Fig. 1).

DISCUSSION

We have identified a cloned segment of *R. meliloti* DNA which encodes a protein analogous to the *recA* gene product of *E. coli*. DNA hybridization experiments have shown that se-

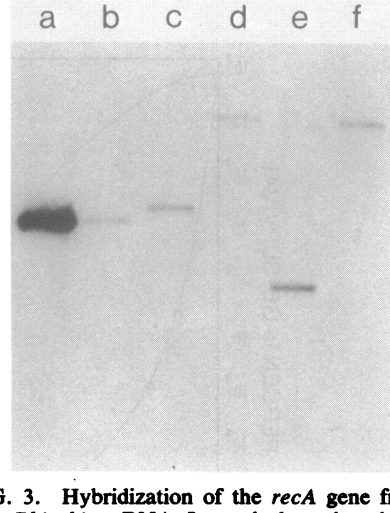

FIG. 3. Hybridization of the *recA* gene from *E. coli* to *Rhizobium* DNA. Lanes b through e show the autoradiograph of a Southern blot of *Bam*HI-digested total chromosomal DNA (3 μg of DNA per lane) hybridized with ^{32}P-labeled DNA containing the *recA* gene of *E. coli*. Lane a shows hybridization to 1 μg of *Bam*HI-digested pRK290.34 DNA. Lanes b through f show the hybridization with *R. meliloti* 102F34, *R. meliloti* 41, *A. tumefaciens*, *R. japonicum*, and *R. leguminosarum* DNAs, respectively.

TABLE 3. Growth of phage λ *bio*252 *c*I857[a]

Host strain	% of titer on Ymel(P2)
Ymel(P2)	100
HB101	0.01
HB101(pRMB1008)	30
HB101(pRMB1002)	0.006

[a] Phage from a stock at approximately 2×10^9 PFU/ml were plated on each of the host strains.

quence homology exists between the *recA* gene of *E. coli* and that of *R. meliloti* as well as to DNA of the related species *R. leguminosarum*, *R. japonicum*, and *A. tumefaciens*. In vivo assays for the function of the *R. meliloti recA* gene in *E. coli* have allowed us to characterize those protein activities which have been conserved.

UV sensitivity, characteristic of an *E. coli recA* mutant, was partially suppressed (twofold) by the *R. meliloti recA* protein expressed from the plasmid pRMB1008. Expression of this plasmid and of its parent pRK290.34 also allowed growth of *E. coli* in the presence of the DNA-alkylating agent MMS. Both UV irradiation and exposure to MMS elicit the SOS response in wild-type *E. coli* and allow the bacteria to survive the DNA damage induced by these agents. Since the expression of the *R. meliloti recA* gene in *E. coli* increases cell viability in response to these DNA-damaging agents, it appears that the *R. meliloti* protein is able to allow increased survival of *E. coli* cells in response to DNA damage.

In addition, the *recA* proteins of *R. meliloti* and *E. coli* appear similar in their ability to catalyze genetic recombination. Expression of plasmid pRMB1008 in the *recA* strain MB4 restored recombination proficiency in an Hfr cross and allowed a λ *red⁻gam⁻* phage to plate on strain HB101. Thus, the *recA* protein of *R. meliloti* phenotypically resembles the functional *E. coli recA* protein in recombination. Quantitative differences between the *E. coli* and *R. meliloti recA* proteins in their ability to catalyze *recA*-dependent functions may reflect a difference in promoter expression or protein level regulation in different genera.

After the identification of a clone containing the *recA* gene of *R. meliloti*, a *recA* derivative of *R. meliloti* was constructed. Several important questions were then asked concerning Rec function in bacterial growth and development. We found that a *recA R. meliloti* is proficient in symbiotic nitrogen fixation, suggesting that *recA*-dependent recombination is not required for symbiosis with the root nodules of alfalfa. By comparison, Klapwijk et al. (10) found that a recombination-deficient strain of the closely related species *A. tumefaciens* is not altered in its ability to induce plant tumors. We also found that the megaplasmid of Rm102F34, which carries genes essential for symbiotic nitrogen fixation, could still be detected in the *R. meliloti recA* mutant (unpublished observations).

The *recA* strain described here will be of further interest in genetic studies of *R. meliloti*. Since considerable conservation of the *recA* sequence between *E. coli* and *R. meliloti* is evident, it may be possible to generate *recA* mutants of other *Rhizobium* species by using the plasmid pRMB1002. In addition, heterologous complementation among gram-negative bacteria may be an efficient way to identify the *recA* gene of a variety of bacterial species.

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