MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 1, No. 2, pp. 80–86, 1988

Construction and Characterization of *Pseudomonas syringae recA* Mutant Strains

David K. Willis,[1,2] Estelle M. Hrabak,[2] Steven E. Lindow,[3] and Nickolas J. Panopoulos[3]

[1]USDA/ARS Plant Disease Resistance Research Unit and [2]Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706; and [3]Department of Plant Pathology, University of California, Berkeley, California 94720 U.S.A.
Received 3 December 1987. Accepted 20 January 1988.

We have constructed *recA* mutant derivatives of both pathogenic and nonpathogenic strains of *Pseudomonas syringae* using two methods of site-directed mutagenesis. Two plasmids were constructed, one nonreplicative (pKW11) and one replicative (pEMH1) within *P. syringae*, each containing a 19.2-kb *Eco*RI fragment carrying the *P. syringae recA4*::Tn*5* mutation. Using pKW11, the nonpathogenic strain Cit7 (source of the *P. syringae recA* gene) was mutagenized by the apparent creation and resolution of a *cis*-merodiploid intermediate. The resultant strain, Cit7 (*recA4*::Tn*5*), exhibited sensitivity to ultraviolet irradiation characteristic of an *Escherichia coli recA* mutant. In order to test the effect of a *recA* genetic background on pathogenicity and *in planta* growth, we used pEMH1 to mutagenize, by the transplacement method, the heterologous *P. syringae* pv. *syringae* isolate B728a, a causal agent of bacterial brown spot disease of bean (*Phaseolus vulgaris*). The resultant mutant, B728a (*recA4*::Tn*5*), exhibited a degree of UV-sensitivity similar to Cit7 (*recA4*::Tn*5*) by quantitative assay. Strain B728a (*recA4*::Tn*5*) was not altered in its ability to grow in culture or *in planta* or in its ability to cause disease in the leaves or pods of bean. The ability to mutagenize heterologous strains of *P. syringae* by gene replacement without any apparent effect on *in planta* growth and pathogenicity indicates that a *recA* deficient background should be generally useful in the genetic analysis of phytopathogenic pseudomonads.

Additional key words: *Phaseolus vulgaris*, *Pseudomonas syringae*, *recA*, UV sensitivity

Bacterial mutants that are defective in the *recA* gene are important tools for the analysis of genes and their protein products. The inhibition of DNA repair in *recA* mutants (Little and Mount 1983; Walker 1984; Witkin 1976) has been utilized in the development of the maxi-cell technique for identification of the protein products of cloned genes in *Escherichia coli* (Sancar *et al.* 1979). The *recA* protein plays a central role in the cellular process of homologous recombination (Dressler and Potter 1982; Radding 1982). A *recA* deficient background allows the determination of the number and location of genes by complementation analysis and is essential for the stabilization of repetitive foreign DNA molecules in *E. coli*. Ice nucleation genes previously cloned from *Pseudomonas syringae* (Orser *et al.* 1985) contain tandemly repeated sequences through most of the coding region (Green and Warren 1985; M. Mindrinos, N. J. Panopoulos, and S. E. Lindow, unpublished data). Insertion mutants in these genes are unstable and are lost by what appears to be a homologous recombinational excision event leading to variable restoration of ice nucleation activity (Corotto *et al.* 1986; D. Geis, S. E. Lindow, and N. J. Panopoulos, unpublished data). The instability of these mutations has prevented fine structure and regulation studies with these genes within the presumably recombination proficient *P. syringae* parental strains. In addition, the lack of a suitable recombination deficient genetic background compromises the complementation analysis of cloned genes. To establish a reproducible pathogenic response *in planta*, an inoculum of 10^5 to 10^8 viable bacteria per milliliter is required when cosmid clones containing pathogenicity determinants are introduced into corresponding non-pathogenic mutants (T. M. Barta, E. M. Hrabak, and D. K. Willis, unpublished data). Inoculation of such large bacterial populations does not allow us to distinguish between the restoration of the pathogenic phenotype due to plasmid-encoded gene products produced *in trans* or restoration due to the marker rescue of mutant chromosomal sequences by homologous but incomplete DNA sequences contained on the restoring cosmid. A recombination deficient genetic background will prevent the latter possibility.

We have recently isolated a *recA*-like gene from the nonpathogenic *P. syringae* isolate Cit7 by the ability of a cosmid clone (pCUV8) to restore resistance of a *recA*$^-$ *E. coli* to DNA damaging agents (Hickman *et al.* 1987). Cosmid pCUV8 was found to quantitatively complement both the recombination deficiency (*Rec*$^-$) and sensitivity to ultraviolet light (UV^s) of an *E. coli recA* deletion mutant. This suggests that pCUV8 contains the *P. syringae recA* gene. Tn*5* mutagenesis yielded several derivatives of pCUV8 that had lost the ability to complement *recA*$^-$ *E. coli*. Preliminary restriction mapping localized this gene to a 13.4-kb *Eco*RI fragment of pCUV8 DNA.

In this paper, we report the construction of *recA* mutant strains of both nonpathogenic and pathogenic isolates of *P. syringae* by site-directed mutagenesis using both replicative and nonreplicative plasmids containing the *P. syringae recA4*::Tn*5* mutation. The analysis of these mutants allows us to establish that pCUV8 contains the *P. syringae recA* gene and to partially characterize the phenotype of a *P. syringae recA* mutant. Characterization of the *in planta* growth and pathogenic response of a *recA4*::Tn*5*-containing derivative of a *P.s.* pv. *syringae* isolate pathogenic on bean (*Phaseolus vulgaris*) demonstrates that a *recA* mutant genetic background can be utilized for the molecular genetic analysis of pathogenicity genes in this bacterium.

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and relevant genotypes of bacterial isolates and plasmids are listed in Table 1. The composition of restriction fragments and replicons used to construct recombinant plasmids are listed in Table 2.

Bacterial growth media. *E. coli* strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.* 1982) with aeration at 37° C. *P. syringae* isolates were grown in King's B (KB) medium (King *et al.* 1954) with aeration at 28° C. Antibiotics were used at the following concentrations for *E. coli*: ampicillin (amp), 50 μg/ml; tetracycline (tet), 15 μg/ml; and kanamycin (kan), 30 μg/ml. For selection of resistant *P. syringae*, the antibiotic concentrations were as follows: tet, 10 μg/ml; kan, 10 μg/ml; and rifampicin (rif), 100 μg/ml. Stocks of rif and tet were made in 100% methanol at 10 mg/ml and stored at 4° C. The kan and amp were dissolved at 30 and 50 mg/ml, respectively, in water, filter sterilized, and stored at 4° C.

DNA manipulations. Procedures for the isolation of plasmid DNA, restriction endonuclease digestion, ligation, isolation of restriction fragments from agarose gel by electroelution, transfer of DNA fragments to nitrocellulose membranes, labeling of DNA with ^{32}P by nick translation, and hybridization conditions were as described by Maniatis *et al.* (1982). Isolation of total bacterial DNA from *E. coli* and phytobacteria has been described previously (Peet *et al.* 1986).

Construction of pKW11, pEMH1, pEMH4, and pEMH5. Plasmid pKW11 was constructed by subcloning the 19.2-kb *Eco*RI fragment (Fig. 1A) that contains the *P. syringae recA4*::Tn*5* mutation from the wide host-range recombinant cosmid pCUV802 into the narrow host-range vector pLVC18 (Table 2). Plasmid pEMH1 consists of this same fragment subcloned from pCUV802 into the wide host-range cosmid pLAFR3. Plasmid pEMH4 was constructed by subcloning the 13.4-kb *Eco*RI fragment that contains the wild-type *P. syringae recA* gene (Fig. 1A) into pLAFR3. pEMH5 was constructed by subcloning the 5.1-kb *Bgl*II fragment (Fig. 1B) from pEMH4 into the *Bam*HI site of pLAFR3.

Site-directed mutagenesis. For the site-directed mutagenesis of the *P. syringae recA* gene in the nonpathogenic isolate Cit7, pKW11 was transferred from *E. coli* strain HB101 into Cit7 by triparental mating using HB101 (pRK2013), a conjugation proficient donor (Figurski and Helinski 1979). In a typical mating, 375 μl of a stationary culture of each *E. coli* donor was mixed with 750 μl of a stationary culture of the *P. syringae* recipient, washed once with 1 ml of KB, resuspended in 100 μl of KB, placed as a spot on a KB plate, dried briefly in a Biosafety hood, and incubated at 28° C overnight. Transconjugants containing the tetracycline resistance marker (tet^r) of pKW11 were selected by resuspending the mating mix in sterile water and plating on KB medium containing rif and tet at 28° C. The kanamycin resistance (kan^r) marker of the Tn*5* in pKW11 was not selected at this time to avoid the potential accumulation of transposition events in the transconjugant population. Then, the selection for the tet^r marker of the vector was removed and tetracycline sensitive (tet^s) recombinants were allowed to accumulate. This was accomplished by repeated cycling (i.e., inoculation and growth to stationary phase) of a *cis*-merodiploid culture at 28° C in 50 ml of KB media containing rif (KB-rif) (four cycles), 50 ml of KB-rif kan (two cycles), and then a round of cycloserine enrichment in the presence of tet (Miller 1972). Survivors were plated on KB-rif kan agar at 28° C and replica plated onto KB-rif kan tet agar at 28° C.

We mutagenized the pathogenic *P. s.* pv. *syringae* strain B728a using the transplacement technique (Gutterson *et al.* 1986). Plasmid pEMH1 consists of the 19.2-kb *recA4*::Tn*5* *Eco*RI fragment ligated into the *Pseudomonas* replicative vector pLAFR3 (Table 2). Plasmid pLAFR3 can be maintained in *P. s.* pv. *syringae* in culture by selection for the

Table 1. Bacterial strains and plasmids

Strain or Plasmid	Markers[a]	Source or reference
Escherichia coli		
HB101	F⁻ *recA13 rspL hsdS20 (hsdR hsdM) thi-1 leuB6 proA2 ara-14 lacY1 galK2 xyl-5 mtl-1 supE44* λ⁻	Boyer and Roulland-Dussoix 1969
HB102	HB101 *rpo*	Hickman *et al.* 1987
DH5α	F⁻ *recA1 hsdR17 endA1 thi-1 gyrA96 relA1 supE44*φ80*dlacZ*ΔM15λ⁻	Bethesda Research Laboratories
Pseudomonas syringae		
Cit7	Rif^r	Orser *et al.* 1985
P. syringae pv. *syringae*		
B728a	Rif^r	J. Lindeman
P. syringae pv. *phaseolicola*		
NPS3121	Rif^r	Peet *et al.* 1986
P. syringae pv. *glycinea*		
Pgs0	Rif^r race 0	B. J. Staskawicz
Pgs4	Rif^r race 4	B. J. Staskawicz
P. syringae pv. *tabaci*		
BR2		R. D. Durbin
ATCC11528		R. D. Durbin
Xanthomonas campestris pv. *glycines*		B. J. Staskawicz
Bradyrhizobium japonicum		B. J. Staskawicz
Plasmids		
pCUV8	*recA*⁺ Tc^r	Hickman *et al.* 1987
pCUV802	*recA4*::Tn*5* Tc^r Km^r	Hickman *et al.* 1987
pCUV808	*recA8*::Tn*5* Tc^r KM^r	Hickman *et al.* 1987
pLAFR1	Tc^r	Friedman *et al.* 1982
pLAFR3	Tc^r	B. J. Staskawicz
pRK2013	Km^r	Figurski and Helinski 1979
pLVC18	Ap^r Tc^r *mob* (RSF1010)	G. J. Warren
pKW11	*recA4*::Tn*5* Tc^r Ap^r Km^r	This work
pEMH1	*recA4*::Tn*5* Tc^r Km^r	This work
pEMH4	*recA*⁺ Tc^r	This work
pEMH5	*recA*⁺ Tc^r	This work

[a] Rif^r, Tc^r, Km^r, and Ap^r indicate resistance to rifampicin, tetracycline, kanamycin, and ampicillin, respectively.

Table 2. Composition of plasmid constructs

Plasmid	Vector	Genotype	Insert[a]
pCUV8	pLAFR1	*recA*⁺	A[b]
pEMH4	pLAFR3	*recA*⁺	A
pEMH5	pLAFR3	*recA*⁺	B
pCUV802	pLAFR1	*recA4*::Tn*5*	A[c]
pCUV808	pLAFR1	*recA8*::Tn*5*	A[d]
pKW11	pLVC18	*recA4*::Tn*5*	A[c]
pEMH1	pLAFR3	*recA4*::Tn*5*	A[c]

[a] Letters in this column refer to restriction maps presented in Figure 1.
[b] pCUV8 is a cosmid clone of Cit7 DNA that contains an additional 19-kb *Eco*RI fragment not associated with the *recA* gene (Hickman *et al.* 1987).
[c] The insert consists of the 13.4-kb fragment containing a Tn*5* inserted at the position shown by the white triangle in Figure 1A.
[d] The insert consists of the 13.4-kb fragment containing a Tn*5* inserted at the position shown by the black triangle in Figure 1A.

plasmid tet^r marker, but pLAFR3 is rapidly lost in the absence of selection (T. M. Barta and D. K. Willis, unpublished data). We introduced pEMH1 into B728a by triparental mating with *E. coli* donors HB101 (pEMH1) and HB101 (pRK2013) as described above. Tet^r transconjugants representing *trans*-merodiploids were selected on KB-rif kan tet plates at 28° C. The transconjugants were cycled five times in KB-rif and two times in KB-rif kan media as described above. This process allowed the accumulation of bacteria that had lost pEMH1 due to its instability in the absence of selection but had retained the *recA4*::Tn*5* mutation through homologous recombination. We identified potential exchange mutants by plating the culture on KB-rif kan plates at 28°C and screening by replica plating onto KB-rif kan tet plates at 28° C.

Qualitative and quantitative UV survival. The ability of a recombinant plasmid to restore UV^r to a *recA* mutant was ascertained by transformation into HB101 or DH5α and analysis by replica plating for resistance to increasing UV dosages (Miller 1972). We also used this replica assay for the routine analysis of the UV sensitivity of exchange mutants. Quantitative analysis of UV survival was modeled according to Kushner (1974) and has been described previously (Hickman *et al.* 1987).

Pathogenicity assays and determination of growth rates in culture and *in planta*. Pathogenicity of *P. s.* pv. *syringae* isolates was determined by inoculation beneath the epidermis of 8- to 10-wk-old pods (10–12 cm long) of *P. vulgaris* 'Bush Blue Lake 274' (Northrup King Seed Company) following the method of Daub and Hagedorn (1979). Briefly, overnight cultures were washed in sterile water, adjusted to an OD_{600} of 0.1 (approximately 1×10^8 colony-forming units [cfu] per milliliter), and diluted in 10-fold increments in sterile water. Bean pods were harvested and surface sterilized by immersion in a 10% solution of bleach for 2 min, followed by 10 rinses with tap water. Approximately 20-μl aliquots of the dilutions were injected just beneath the epidermis of a bean pod using a 25-gauge needle attached to a 1-ml tuberculin syringe. Inoculated pods were placed either in Whirl-Pak (Nasco) sterile bags and incubated in a growth chamber at 24° C with a 12-hr photoperiod or placed on moistened paper towels in plastic storage boxes and incubated at room temperature. Symptom development was recorded from 3 to 6 days after inoculation. For the determination of the pathogenic response in leaf tissue, we infiltrated bacterial dilutions into primary leaves of an intact bean plant (midrib length 8–10 cm) using a disposable plastic Pasteur pipet to force inoculum through a slight nick in the upper leaf surface made with a sterile razor blade. The plants were incubated in growth chambers as described above and reactions were scored after 5–6 days.

Growth rate in liquid culture was determined by growth in KB medium containing appropriate antibiotics with aeration at 28° C. Cell density was determined by monitoring absorbance at OD_{600} and by serially diluting cultures on KB plates containing appropriate antibiotics. We determined the epiphytic growth rate *in planta* by the following method (S. S. Hirano, unpublished). *P. vulgaris* 'Bush Blue Lake 274' plants were grown for 3 wk in a plant growth chamber (Percival, Boone, IA) at 24° C with a 14-hr photoperiod and light flux of 200 $\mu E/m^2/sec$. After 3 wk growth, the first and second trifoliate leaves were fully expanded. Bacterial inoculum was prepared from 2½-day-old, plate-grown cells that were suspended in sterile water and adjusted to a concentration of approximately 10^6 cfu/ml. Using an atomizer, approximately 10 ml of inoculum was applied to all upper and lower leaf surfaces of each plant. Sixty plants were inoculated with each bacterial suspension. Plants were immediately transferred to enclosed

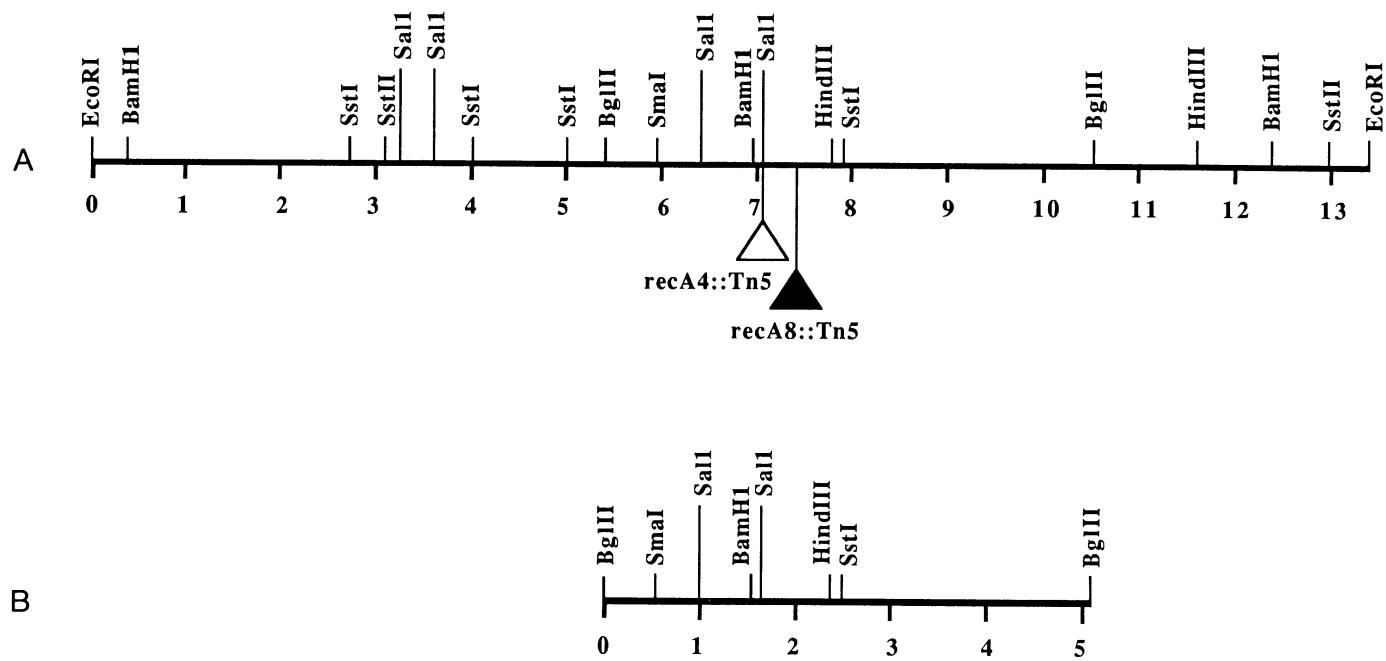

Fig. 1. Restriction maps of Cit7 DNA inserts within various plasmid constructs referenced in Table 2. Restriction sites of enzymes mapped are indicated above the line. The length of fragments in kilobases (kb) is indicated below the lines. **A,** 13.4-kb *Eco*RI fragment in pEMH4 containing the wild-type *Pseudomonas syringae recA* gene. The position of the Tn*5* inserts in the *recA4*::Tn*5* and the *recA8*::Tn*5* mutations are shown below the line. The orientation of the Tn*5* in the *recA4*::Tn*5* mutation is left-to-right IS*50R* (containing the active transposase gene)-unique DNA-IS*50L* (containing the inactive transposase and the promoter for kanamycin resistance) (Berg *et al.* 1980; Rothstein and Reznikoff 1981). In the *recA8*::Tn*5* mutation, the Tn*5* insertion is in the opposite orientation. **B,** 5.1-kb *Bgl*II fragment subcloned from the 13.4-kb fragment to yield pEMH5. This fragment contains active *recA* gene function as determined by the ability of pEMH5 to restore UV^r to DH5α.

Plexiglass chambers that were continuously humidified using commercial humidifiers. The Plexiglass chambers were kept inside a walk-in Percival growth chamber at 24° C with a 12-hr photoperiod and light flux of 130 $\mu E/m^2/sec$. For sampling, 15 random trifoliate leaflets were detached and transferred to a moist chamber for storage before processing. Leaflets were held in these chambers for a maximum of 2 hr. Each leaflet was cut into small pieces with sterile scissors and homogenized in 20 ml of grinding buffer (0.1 M KPO_4, pH 7.0, and 0.1% Bacto-Peptone [Difco]) using a Polytron (Brinkmann) at setting 5 for 30–60 secs. Samples were diluted in 10 mM KPO_4 buffer and plated on KB medium containing appropriate antibiotics.

RESULTS

Localization of the *P. syringae recA* gene. Figure 1A shows the position of two independent Tn*5* insertions into the 13.4-kb *Eco*RI fragment of pCUV8 (designated as *recA4*::Tn*5* from pCUV802 and *recA8*::Tn*5* from pCUV808) that eliminated the ability of this cosmid to restore UV^r to HB102 (Hickman *et al.* 1987; E. M. Hrabak and D. K. Willis, unpublished data). Both of these insertions occurred in a 0.5-kb region near the middle of the 13.4-kb *Eco*RI fragment. This region was subcloned from pEMH4 as part of the central 5.1-kb *Bgl*II fragment (Fig. 1B) to produce pEMH5. This plasmid restores a UV^r phenotype to strain DH5α (*recA1*) as shown by replica plate test and apparently contains the functional *P. syringae recA* gene.

Construction of Cit7 (*recA4*::Tn*5*). We chose to mutagenize Cit7, the source strain for the *P. syringae recA* gene (Hickman *et al.* 1987), using a two-step process involving a *cis*-merodiploid intermediate. This method was successful in the construction of deletion mutants of Cit7 lacking ice nucleation activity (Orser *et al.* 1984). We constructed the host dependent plasmid pKW11 containing the *recA4*::Tn*5* mutation on a 19.2-kb *Eco*RI fragment for this exchange. As can be seen in Figure 2, the tet^r transconjugants (designated as Cit7 [pKW11]) contained, in addition to the wild-type 13.4-kb fragment present in Cit7, a new fragment that corresponds to the 19.2-kb *P. sryingae recA4*::Tn*5* fragment of pKW11. Because pKW11 is nonreplicative in *P. syringae*, this is consistent with these transconjugants being *cis*-merodiploids in which pKW11 has integrated into the *P. syringae recA* gene by a single crossover event. Supporting this finding is the lack of tet^r transconjugants in test crosses using the pLVC18 vector (data not shown), which suggests that the stable inheritance of pKW11 was homology dependent. We screened 2,000 survivors of the exchange process and found five (0.25%) kan^r tet^s colonies. All five of these isolates (designated as Cit7 *recA4*::Tn*5* numbers 3, 4, 5, 7, and 10) were UV^s by plate test and were therefore likely to be exchange mutants that contain the *recA4*::Tn*5* mutation. Southern blot analysis confirmed this hypothesis and all of these strains now contain the 19.2-kb *recA4*::Tn*5* fragment in place of the wild-type 13.4-kb fragment (Fig. 2).

To ascertain the phenotype of a *P. syringae recA* mutant in more detail, one of the exchange mutants (Cit7 *recA4*::Tn*5* number 4) was analyzed for quantitative UV survival. As can be seen in Figure 3A, a Cit7 derivative containing the *recA4*::Tn*5* mutation is very sensitive to UV irradiation at dosages that have little effect on the parental strain. That this sensitivity is due to a lesion in the *P. syringae recA* gene is shown by the ability of pCUV8 to complement Cit7 *recA4*::Tn*5* to wild-type levels of UV survival (Fig. 3A).

Construction of B728a (*recA4*::Tn*5*). The 13.4-kb *Eco*RI fragment containing the *recA* gene is apparently conserved between *P. syringae* strain Cit7 and *P. syringae* pv. *syringae* strain B728a (Fig. 2). Unlike Cit7, B728a is pathogenic and causes bacterial brown spot disease of bean. Due to the apparent homology, we attempted to perform a gene replacement of the wild-type *recA* gene in B728a with the *recA4*::Tn*5* mutation in pEMH1 using the transplacement technique (Gutterson *et al.* 1986). Approximately 50% of the kan^r colonies plated after the repeated cycling without selection proved to be tet^s by replica plate test. Forty-five of the tet^s colonies were tested by the replica plate method and were found to be UV^s. Five of these potential exchanges were analyzed by Southern transfer using the 13.4-kb *recA* fragment as a probe. In all five isolates the wild-type 13.4-kb fragment had been replaced by a fragment migrating at 19.2 kb, which indicated that they had inherited the 19.2-kb *recA4*::Tn*5* mutation from pEMH1 (data not shown).

One of the exchange mutants, designated as B728a (*recA4*::Tn*5*), was further assayed for quantitative UV survival. Figure 3B shows that the presence of the *recA4*::Tn*5* mutation in B728a causes a reduction in UV survivors that is comparable to Cit7 (*recA4*::Tn*5*). Again, B728a (*recA4*::Tn*5*) shows a marked UV sensitivity at dosages that have little effect on B728a.

Effect of the *recA4*::Tn*5* mutation on the *in planta* growth and pathogenicity of B728a. In order for a *recA* mutation to be useful as a genetic tool for the analysis of phytopathogenic *P. syringae*, it is necessary to establish that the presence of a *recA* mutation does not significantly affect the pathogenicity or virulence of the bacteria. Other than the effect on UV survival, the *recA4*::Tn*5* mutation did not appear to significantly affect the growth parameters that we assayed. Figure 4a shows the results of an *in planta* growth assay conducted in a high humidity growth chamber environment. The presence of the *recA4*::Tn*5* allele had no significant effect on the rate of growth or final mean colony-forming units per leaflet of B728a *in planta*. In addition, we did not find a significant difference between the growth rates of B728a or B728a (*recA4*::Tn*5*) when grown in KB broth with aeration at 28° C (Fig. 4B).

We assayed both B728a and B728a (*recA4*::Tn*5*) for pathogenicity by end point dilution on both pods and leaves of bean. On pods, a typical pathogenic isolate caused the infiltrated area to appear water-soaked at a concentration of 1×10^4 cfu/ml and above. Using our leaf assay, a pathogenic response was indicated by the appearance of a spreading necrotic lesion around the site of inoculation at cell densities of 1×10^5 cfu/ml and above (data not shown). We found no visible difference in pathogenicity between these two strains at any of the dilutions tested.

DISCUSSION

We have localized the *P. syringae recA* gene to a 5.1-kb *Bgl*II fragment by subcloning from the cosmid pCUV8. We mapped two Tn*5* insertions that eliminate the ability of pCUV8 to restore UV^r to a *recA*$^-$ *E. coli* strain. They were located in a 0.5-kb region near the middle of the 5.1-kb fragment.

We used two methods of gene replacement for the site-directed mutagenesis of *P. syringae* isolates. Previously, we successfully used the creation and resolution of a *cis*-merodiploid intermediate using a nonreplicative plasmid to construct ice deletion mutants of *P. syringae* (Orser *et al.*

1984). Therefore, this was the initial method we employed, using pKW11, in the construction of the Cit7 (*recA4*::Tn*5*) mutant. However, cycloserine enrichment was required to facilitate the identification of tet^s segregants that arose at a frequency of 0.25%. Despite this low frequency of exchange, the construction of a *cis*-merodiploid intermediate is useful for exchanges involving point or deletion mutations that do not contain a directly selectable marker (Orser *et al.* 1984). In constructing the B728a (*recA4*::Tn*5*) mutant, we investigated the usefulness of transplacement (Gutterson *et al.* 1986), using the replicative but unstable plasmid pEMH1. In contrast to the *cis*-merodiploid method, transplacement resulted in the isolation of approximately 50% tet^s segregants. In both experiments, the majority of the tet^s colonies contained the desired gene replacement and had acquired the *recA4*::Tn*5* mutation. Clearly, transplacement is the method of choice in those cases where the desired mutation contains a selectable marker. We have recently used the transplacement technique to exchange mutations between molecularly distinct isolates of *P.s.* pv. *syringae* that are pathogenic on bean (Willis *et al.* 1987).

A *recA* mutation in *P. syringae* displays at least one of the phenotypes, UV sensitivity, of an *E. coli recA* mutant. It is interesting to note that, in our hands, the level of UV survival of a *P. syringae recA* mutant is not significantly different from that of an *E. coli recA* mutant (Hickman *et al.* 1987; D. K. Willis, unpublished data). There seem to be no significant additional *recA* independent DNA repair pathways that function in this leaf epiphyte.

The lack of an efficient and well-defined genetic transfer system limits our ability to determine the extent to which the *recA4*::Tn*5* mutation affects recombination in *P. syringae*. In *E. coli*, either Hfr-mediated bacterial matings or genetic transduction via the bacteriophage P1 are used for the determination of quantitative recombination frequencies. It should be noted that the Cit7 *recA* gene was able to restore recombination proficiency to a *recA* deletion mutant of *E. coli* as assayed by quantitative Hfr mating (Hickman *et al.* 1987). We attempted to use the formation of *cis*-merodiploids with a nonreplicative plasmid containing homology to the *P. syringae* chromosome to determine the recombination ability of the Cit7 (*recA4*::Tn*5*) strain, but the frequency of transconjugants in the Rec$^+$ background was too low to reach a definitive conclusion (D. K. Willis, unpublished data). At present, we cannot rule out the existence of an additional recombinational pathway in *P. syringae*. However, this activity could not be fully analogous to the *recA* gene of *E. coli* since a *P. syringae recA4*::Tn*5* mutant is UV-sensitive.

Although the 13.4-kb *recA* gene-containing *Eco*RI

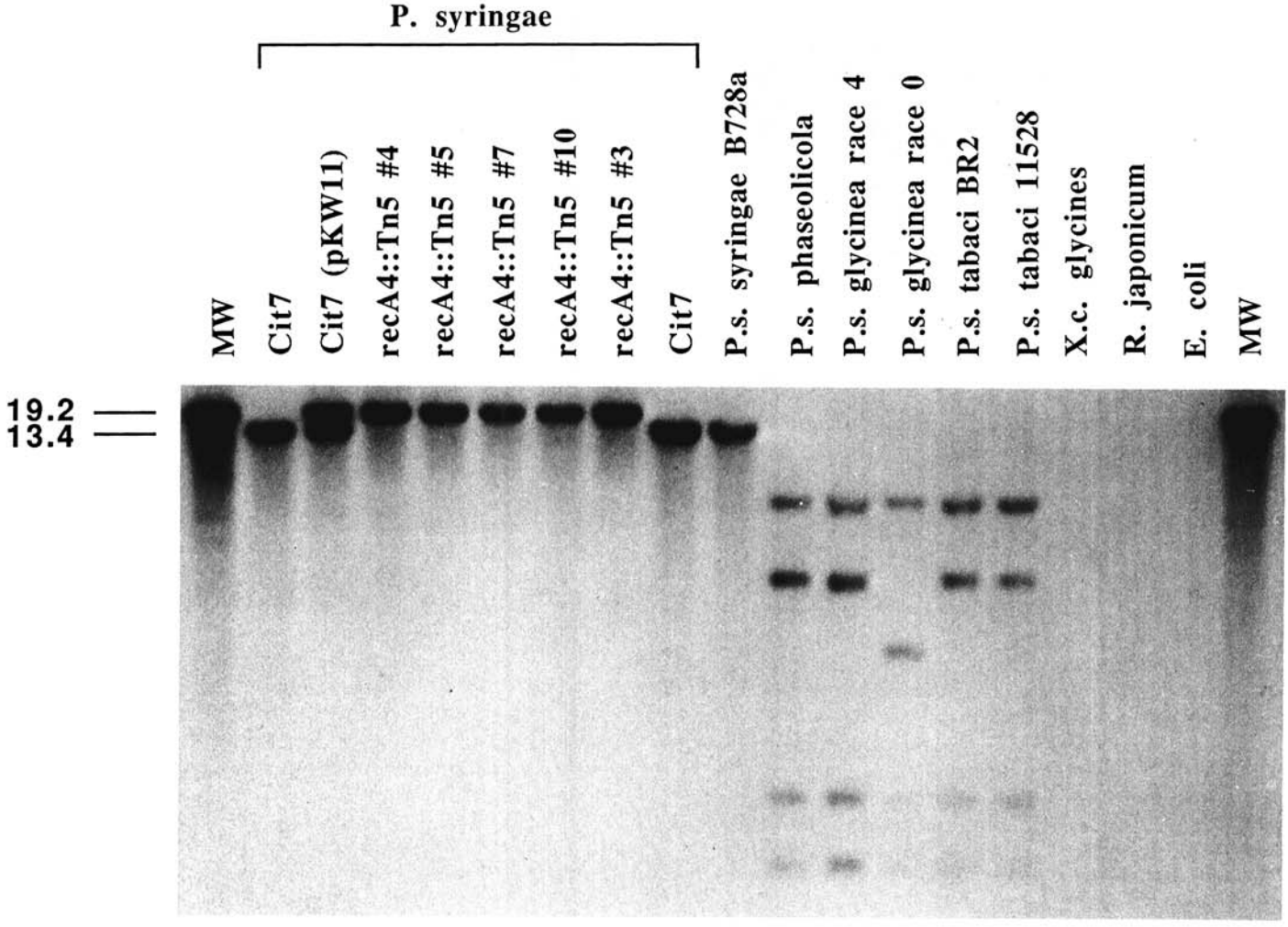

Fig. 2. Southern blot analysis of total bacterial genomic DNA restricted with *Eco*RI and resolved by gel electrophoresis in 0.7% agarose. Normal "high" strigency hybridization and wash conditions were used. The ^{32}P-labeled probe utilized was the *recA4*::Tn*5*-containing 19.2-kb *Eco*RI fragment isolated from pKW11. The bacterial strains analyzed and the expected position of the 13.4 and 19.2 kb *Eco*RI fragments are indicated. The size of restriction fragments was determined by comparison to the 19.2-kb fragment of pKW11 (designated as MW) and to the *Hind*III cut lambda DNA (not shown).

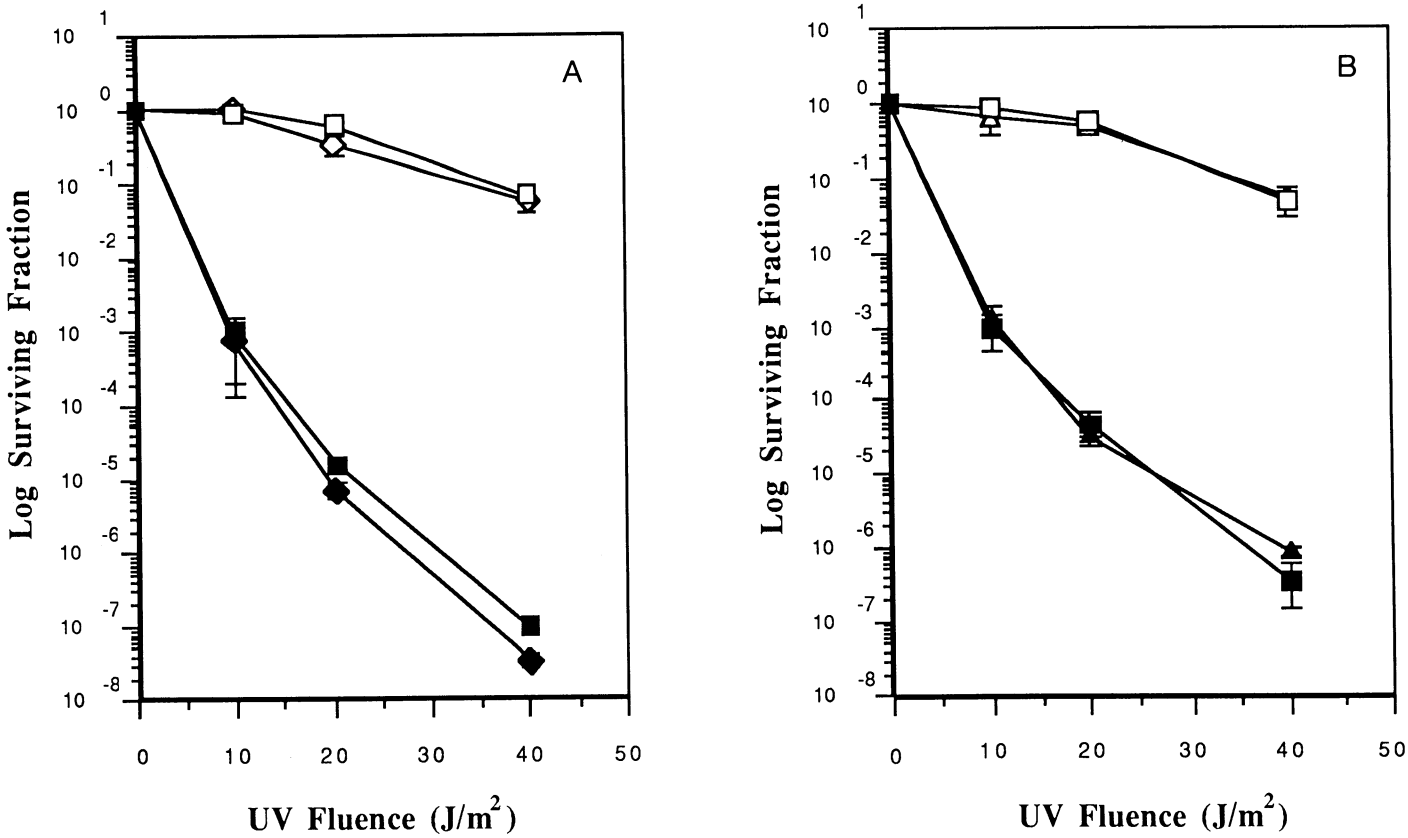

Fig. 3. Quantitative survival of *Pseudomonas syringae* derivatives following exposure to UV irradiation. Viable counts were determined from duplicated dilution series prepared immediately after irradiation. Each data point represents the mean of two separate irradiation trials and bars indicate standard error. **A,** Effect of *recA4*::Tn*5* mutation in strain Cit7, Cit7 (*recA4*::Tn*5*), and Cit7 (*recA4*::Tn*5*) containing either pCUV8 or pLAFR1. **B,** Comparison of the effect of the *recA4*::Tn*5* mutation in strains Cit7 and B728a. Strains were assayed simultaneously to minimize variation due to time of irradiation. □ = Cit7, ■ = Cit7 (*recA4*::Tn*5*), ◇ = pCUV8/Cit7 (*recA4*::Tn*5*), ◆ = pLAFR1/Cit7 (*recA4*::Tn*5*), Δ = B728a, ▲ = B728a (*recA4*::Tn*5*).

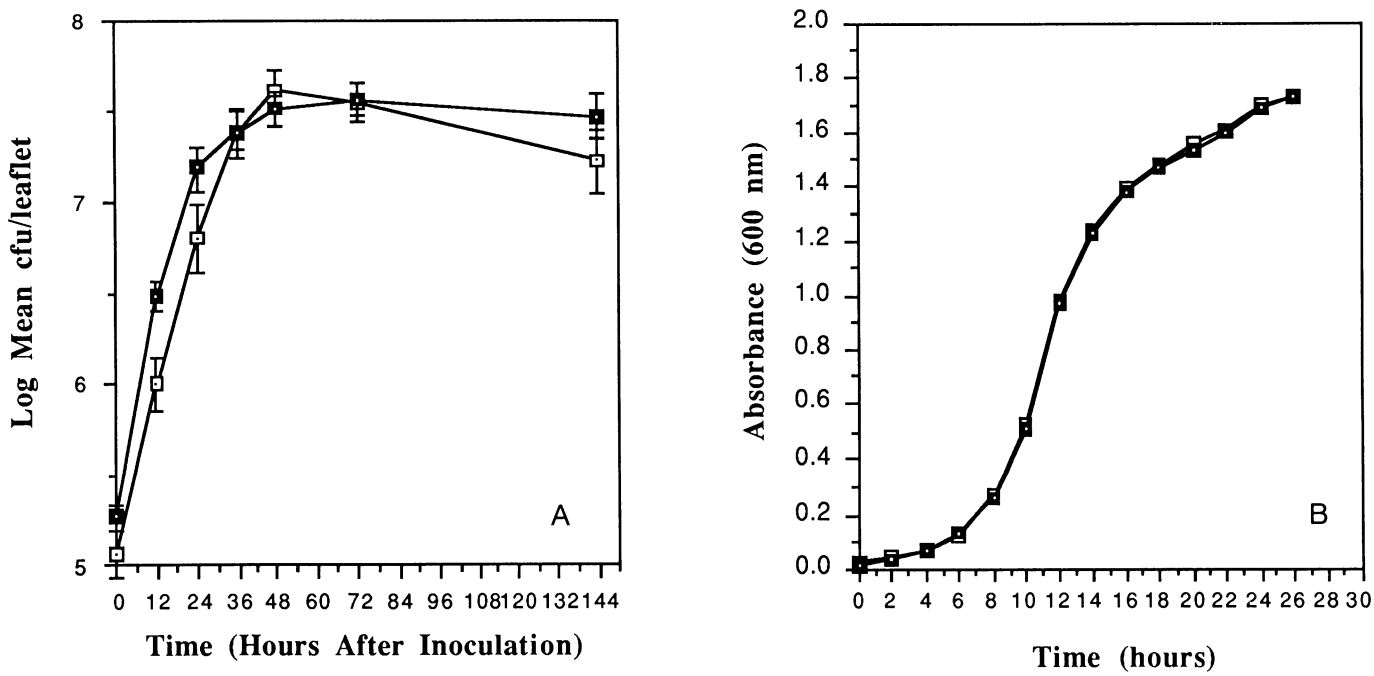

Fig. 4. Effect of the *recA4*::Tn*5* mutation on the growth of B728a. **A,** *In planta* growth of B728a and B728a (*recA4*::Tn*5*). Each data point represents the mean of the bacterial populations on 15 bean leaves. Standard error bars are shown. **B,** Growth of B728a and B728a (*recA4*::Tn*5*) in broth culture. ⊡ = B728a, ■ = B728a (*recA4*::Tn*5*).

fragment is apparently conserved within *P.s.* pv. *syringae* isolates (Hickman *et al.* 1987), there is a considerable amount of restriction site polymorphism within the hybridization patterns of other pathovars of *P. syringae* (Fig. 2). Figure 2 also shows that there is no hybridization under normal stringency conditions between the *P. syringae recA* gene fragment and *Xanthomonas campestris* pv. *glycines*, *Bradyrhizobium japonicum* or *E. coli*. The successful site-directed mutagenesis of the pathogenic strain B728a using DNA originally isolated from strain Cit7 suggests that sufficient homology exists among *P.s.* pv. *syringae* isolates to make pEMH1 generally useful in the construction of *recA4*::Tn*5* mutant strains. Whether *recA* transplacement will be successful in the mutagenesis of *P. syringae* strains more distantly related to pv. *syringae* such as *P.s.* pv. *phaseolicola*, where there exists extensive polymorphism in the hybridization pattern (Fig. 2), remains to be seen.

With isogenic *E. coli* K-12 strains, the presence of the *recA56* mutation causes a 25% increase in doubling time in complete media as measured by monitoring optical density (Capaldo-Kimball and Barbour 1971). Capaldo-Kimball and Barbour proposed two models to explain the effect of the *recA* gene in *E. coli* K-12: 1) that recombination proficiency is required for normal growth or 2) that the *recA* protein regulates genes that are required for normal growth rates. The lack of an observable effect of the *recA4*::Tn*5* mutation on the growth rate of B728a in broth culture (Fig. 4B) is in contrast to the effect of a *recA* mutation in *E. coli* K-12. At present we cannot explain this discrepancy, but one possibility is a potential synergistic effect due to the presence of multiple auxotrophic lesions in *E. coli* K-12 in addition to the *recA56* mutation. We are unaware of any studies on the effect of a *recA* mutation in an otherwise wild-type *E. coli*. A second possibility is the difference in the basic physiology of *E. coli* and *P. s.* pv. *syringae*. The latter bacterium is well adapted for growth in the plant environment and may possess additional *recA*-independent pathways that aid in survival under field conditions.

Zink *et al.* (1985) reported that the *recA* gene is required for the induction of pectin lyase, and the bacteriocin, carotovoricin, in *Erwinia carotovora* subsp. *carotovora*. In addition, *recA*$^-$ *E. c.* pv. *carotovora* did not differ from wild type in its ability to macerate potato tuber tissue. Thus it seems that in *E. c.* pv. *carotovora*, as well as *P.s.* pv. *syringae*, a *recA* mutation has no observable effect on pathogenicity. Our results indicate that *recA*-deficient strains will be useful for the analysis of pathogenicity genes in *P. s.* pv. *sryingae* by definitive complementation analysis in the homologous system. Finally, the *recA*$^-$ strains should simplify the study of repetitive genes, such as the ice nucleation genes previously cloned from strain Cit7 (Orser *et al.* 1984), in the parental genetic background.

ACKNOWLEDGMENTS

We thank T. Barta, S. Hirano, T. Kinscherf, J. Kolmer, and D. Mathews for assistance with the *in planta* growth experiment, and J. Handelsman and S. Leong for their helpful suggestions on the manuscript. This work was supported in part by NSF Grants PCM-8409723 and PCM-8313052.

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