The Use of Transposon Mutagenesis in the Isolation of Nutritional and Virulence Mutants in Two Pathovars of *Pseudomonas syringae*

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ABSTRACT

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Transposon Tn*5*, which confers kanamycin resistance, was introduced into the genomes of *Pseudomonas syringae* pv. *syringae* PS9020 and *P. syringae* pv. *phaseolicola* PP7010 by conjugation with *Escherichia coli* SM10 containing the suicide plasmid vector pSUP1011. Approximately 0.5–0.6% of over 4,000 kanamycin-resistant (Km^r) colonies that were analyzed mutated to auxotrophy. Most auxotrophs reverted at low frequencies ($<10^{-8}$), and most prototrophic revertants were kanamycin-sensitive. Of approximately 2,400 Km^r colonies assayed on leaves of *Phaseolus vulgaris*, 0.3–0.4% had partially or completely lost the ability to induce wild-type virulence symptoms. Blot hybridization analysis of *Eco*RI-digested total DNA from these strains revealed insertion of Tn*5* at unique sites. The identification of avirulent mutants that resulted from transposition of Tn*5* provides a mechanism for cloning and characterizing virulence determinants in these strains.

Modern molecular genetics provides tools for the isolation and characterization of the disease-causing determinants of plant pathogenic bacteria. The elucidation of molecular mechanisms of pathogenesis can eventually provide the basis for developing effective disease control strategies. The successful application of molecular genetics in the characterization of phytopathogenic bacteria is best exemplified by recent advances in the study of the genetics of *Agrobacterium tumefaciens* (22). For the majority of bacterial plant pathogens, however, limited fundamental genetic information is available (18) and modern molecular genetic analysis has not been pursued. Consequently, there is a great need for the generation and characterization of a variety of mutants in these pathogens.

Transposon mutagenesis, which results in inactivation of a gene by insertion of a drug-resistance transposable element (17), provides a method for the generation of selectable, single-site mutations in bacteria. Furthermore, mutants obtained by transposon mutagenesis are ideal for subsequent molecular manipulation (17). This kind of mutational scheme has been exploited in the genetic analysis of plant pathogenic bacteria such as *Agrobacterium* spp. (12,16,28), *Erwinia* spp. (6,29), and *Pseudomonas* spp. (5,7,26).

Pseudomonas syringae pv. *syringae* Van Hall, causal agent of bacterial brown spot, and *P. syringae* pv. *phaseolicola* (Burkholder) Young et al, causal agent of halo blight in beans, are two phytopathogens whose genetics have not been well developed (13,19). We sought to isolate genetic mutants with selectable phenotypes in strains of these pathovars by using transposon mutagenesis. Reported here is the transposition of transposon Tn*5* (3) into the genomes of two strains, and the isolation and characterization of Tn*5*-induced nutritional and virulence mutants. A preliminary report of a portion of this work has been presented (2).

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MATERIALS AND METHODS

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

Media. MaNY medium (9) and LB medium (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) were used to culture bacteria. MaS minimal medium, used for the screening of auxotrophs, has been described (9). Media were supplemented, when appropriate, with kanamycin (Km) (50 μg/ml), rifampicin (Rif) and streptomycin (Sm) (100 μg/ml each), chloramphenicol (Cm) (25 μg/ml), or combinations of these. MaNY and LB media were solidified with 1.5% agar. Noble agar (Difco) was used to solidify MaS medium.

Genetic techniques. Bacterial conjugations between *E. coli* SM10 (pSUP1011) and *Pseudomonas* recipient strains were performed on 3% agar (LB) plates by mixing loopfuls of approximately 10^{10} cells from 3-day-old plates in a 1 : 2, donor : recipient ratio. Following incubation at room temperature (22 C) for 4 hr, cells were suspended in LB medium containing 0.01% Tween-80, serially diluted, and plated onto MaNY plates containing appropriate antibiotics to select for Tn*5*-containing transconjugants and to counterselect against donor cells. Dilutions were also plated onto LB agar plates containing appropriate antibiotics and incubated at either 37 C overnight to determine viable donor cell count, or at 22 C for 3 days to determine recipient cell count. Following incubation at room temperature for 3–4 days, Km^r colonies were then transferred to MaNY and MaS plates for further characterization, and to chloramphenicol-containing MaNY plates to test for the presence of pSUP1011.

Kanamycin-resistant derivatives of *Pseudomonas* that failed to grow on unsupplemented MaS medium were characterized for specific nutritional requirements by the method of Davis et al (10) on supplemented MaS media. Reversion frequencies of putative auxotrophs were determined by growing single-cell isolates in LB medium to stationary phase, washing with MaS medium, concentrating 100-fold, and plating about 2×10^{11} cells onto MaS minimal medium. Prototrophs were subsequently tested for growth on medium containing kanamycin. Dilutions were prepared and plated onto LB agar to determine the viable cell count.

Bioassay. A leaf bioassay of Tn*5*-containing colonies on *Phaseolus vulgaris* L. 'Red Mexican' UI-36 was used to screen for mutants with altered virulence. Fresh 2- to 3-day-old MaNY-grown colonies were swabbed with a toothpick onto needle puncture wounds on young, expanding primary leaves. Wounds were wetted with a drop of 0.01 M phosphate buffer (pH 7.2) containing 0.2% Tween-80 prior to application of mutant cells. Plants were incubated at 17 C under a 12-hr light/12-hr dark cycle. At day 10, symptoms were compared with those induced by wild-type virulent strains. Following initial screening with the needle puncture assay, putative mutants were tested using more sensitive assays to distinguish relative differences in virulence. Loopfuls of cells of strains of *P. syringae* pv. *syringae* grown 2 days on LB medium were suspended in 0.01 M potassium phosphate (pH 7.2), serially diluted, and forcibly injected (in 0.05–0.1 ml aliquots) with a hypodermic needle into the undersides of 1- to 2-wk-old primary leaves of cultivar Red Mexican until temporary watersoaking was observed. Plants were incubated as described above, and necrotic symptoms were recorded as a function of inoculum density after incubation for 3–5 days. Cells of mutant strains of *P. syringae* pv. *phaseolicola* were suspended in 0.01 M potassium phosphate buffer (pH 7.2), and cell densities were adjusted to approximately 10^9 cells per milliliter by using standard turbidometric and dilution plating techniques. Cotton swabs saturated with bacterial suspensions were rubbed over Carborundum-dusted, expanding primary leaves (24) of *Phaseolus lunatus* L. PI 199791 (W-6 Regional Plant Introduction, Pullman, WA). This cultivar was determined to be more susceptible to PP7010 than Red Mexican, and developed a more uniform lesion distribution (*unpublished*). Plants were incubated at 100% RH in the dark at 17 C for 24 hr prior to subsequent incubation as described above.

DNA isolations. Total DNA was isolated from strains of *Pseudomonas* by a procedure of Comai and Kosuge (8), as modified by Szabo and Mills (27). Plasmid DNA was isolated from *E. coli* following amplification with spectinomycin or chloramphenicol by the cleared lysate technique (15). DNA was further purified by equilibrium centrifugation in cesium chloride-ethidium bromide (27). DNA was precipitated following removal of ethidium bromide (10) and stored at 4 C. DNA fragments from agarose gels were isolated and purified by electroelution, as described by Maniatas et al (20).

Restriction endonuclease digestion. Restriction endonuclease *Eco*RI was purified as previously described (27) and used as described by Davis et al (10). Conditions for restriction endonuclease digestion with *Bgl*II and *Xho*I (PL Biochemicals, Milwaukee, WI) were those specified by the manufacturer.

Agarose gel electrophoresis and DNA:DNA hybridization. Agarose gel electrophoresis was performed as described by Szabo and Mills (27). DNA fragments were transferred from agarose gels to diazobenzyloxymethyl (DBM) paper following the procedure of Alwine et al (1). Radioactively labeled DNA fragments were prepared by nick translation (23) and hybridized to DBM blots at 42 C in 50% formamide buffer (1) for 18–36 hr. Autoradiographs were prepared using Kodak X-Omat X-ray film. Between hybridizations, hybridized DNA was washed from the blots with 0.4 N NaOH then neutralized with several distilled water washes and 0.1 M sodium phosphate, pH 7.0.

TABLE 1. Bacterial strains and plasmids

Strains	Characters[a]	Source or reference
Pseudomonas syringae pv. *syringae*		
R32	wild type, prototrophic	K. Rudolph
PS9020	same as R32; Sm^r	spontaneous in R32
pv. *phaseolicola*		
LR700	wild type, prototrophic	27
PP7010	same as LR700; $Sm^r Rif^r$	spontaneous in LR700
Escherichia coli		
SM10	chr RP4.2.Tc::Mu	25
Plasmids		
pSUP101	Cm^r(pACYC184.Mob)	25
pSUP1011	$Cm^r Km^r$(pACYC184.Mob::Tn5)	25

[a] Cm, chloramphenicol; Km, kanamycin, Rif, rifampicin; Sm, streptomycin; ^{r}, resistant; ^{s}, sensitive.

RESULTS

Transposition of Tn*5*. *Pseudomonas syringae* pv. *syringae* PS9020 transconjugants with resistance to kanamycin and streptomycin were recovered at frequencies ranging from 0.7 to 3.8 $\times 10^{-5}$ per donor cell, whereas *P. syringae* pv. *phaseolicola* PP7010 transconjugants (Km^r, Sm^r, and Rif^r) were detected at frequencies ranging from 0.03 to 2.1 $\times 10^{-7}$ per donor cell, in separate conjugation experiments. None of the kanamycin-resistant transconjugants of *Pseudomonas* was resistant to chloramphenicol, indicating that Tn*5* transposition had occurred, rather than maintenance of the Tn*5* donor plasmid, pSUP1011.

Auxotroph characterization. Auxotrophic mutants were obtained from several conjugation experiments (Table 2). The auxotrophs characterized were: adenine$^-$, arginine$^-$, cysteine$^-$, glutamine$^-$, isoleucine-valine$^-$, leucine$^-$, lysine$^-$, methionine$^-$, serine$^-$, thiamine$^-$, and tryptophan$^-$. Tn*5* insertions resulting in auxotrophy in *P. syringae* pv. *syringae* PS9020 and *P. syringae* pv. *phaseolicola* PP7010 were detected at frequencies of 0.5% and 0.6%, respectively. Nutritional requirements of three auxotrophs could not be determined by using the procedure of Davis et al (10). All auxotrophs except one (PP7012, methionine$^-$) retained symptom-producing capabilities when bioassayed on bean leaves.

Reversion to prototrophy was tested to determine the stability of Tn*5* insertion, as well as the linkage of Km^r (conferred by Tn*5*) to mutant phenotypes (Table 2). A majority of the auxotrophs had low reversion rates ($<10^{-8}$), indicating stability of Tn*5* insertion; and, except for two strains, all of the prototrophic revertants from each strain were kanamycin-sensitive (Km^s), indicating that Tn*5* insertion was the cause of mutation to auxotrophy. Two auxotrophs (PS9513 and PP7512) yielded revertants that remained Km^r. Reversion to prototrophy in PP7012 (met$^-$ path$^-$ [reduced pathogenicity]) was accompanied by reversion to wild type symptom-producing capabilities.

TABLE 2. Reversion characteristics of Tn5-induced auxotrophs

Strain	Mutant phenotype[a]	Reversion frequency	Revertant phenotype
PS9501	$cys^- Km^r$	10^{-10}	$cys^+ Km^s$
PS9502	$met^- Km^r$	$<10^{-10}$	$met^+ Km^s$
PS9503	$gln^- Km^r$	10^{-9}	$gln^+ Km^s$
PS9504	$arg^- Km^r$	10^{-9}	$arg^+ Km^s$
PS9505	$ade^- Km^r$	10^{-9}	$ade^+ Km^s$
PS9507	ND, Km^r	10^{-10}	prototrophic, Km^s
PS9509	$lys^- Km^r$	$<10^{-10}$	$lys^+ Km^s$
PS9510	$trp^- Km^r$	10^{-10}	$trp^+ Km^s$
PS9511	$gln^- Km^r$	10^{-6}	$gln^+ Km^s$
PS9513	$ser^- Km^r$	10^{-7}	$ser^+ Km^r$
PS9514	$arg^- Km^r$	10^{-9}	$arg^+ Km^s$
PP7504	ND, Km^r	10^{-9}	prototrophic, Km^s
PP7507	$leu^- Km^r$	10^{-9}	$leu^+ Km^s$
PP7508	$ade^- Km^r$	10^{-9}	$ade^+ Km^s$
PP7012	$met^- vir^- Km^r$	10^{-8}	$met^+ vir^+ Km^s$
PP7510	$arg^- Km^r$	10^{-9}	$arg^+ Km^s$
PP7511	$ilv^- Km^r$	10^{-10}	$ilv^+ Km^s$
PP7512	$arg^- Km^r$	10^{-7}	$arg^+ Km^r$
PP7514	$leu^- Km^r$	10^{-10}	$leu^+ Km^s$

[a] ade, adenine; arg, arginine; cys, cysteine; gln, glutamine; ilv, isoleucine-valine; leu, leucine; lys, lysine; met, methionine; ser, serine; thi, thiamine, trp, tryptophan; Km, kanamycin; ^{r}, resistant; ^{s}, sensitive.
[b] ND, nutritional requirement(s) could not be determined.

Characterization of virulence mutants. Several mutants were isolated that failed to induce wild-type symptoms when bioassayed on wounded bean leaves. Mutants varied from those with almost complete inability to induce symptoms (strains PS9021 and PP7011, Fig. 1) to those that induced mild symptoms. To further characterize virulence levels in these mutants, a leaf injection bioassay was utilized because it yielded reproducible results when *P. syringae* pv. *syringae* PS9020 was injected into leaves of bean cultivar Red Mexican. Mutant strains required 20- to 2,000-fold greater inoculum densities than strain PS9020 to induce necrosis (Fig. 2).

Symptom expression induced by mutants of *P. syringae* pv. *phaseolicola* was determined using the highly susceptible lima bean (*Phaseolus lunatus*) cultivar, PI 199791. When primary leaves of lima bean were swabbed with cell suspensions of the various strains, a uniform distribution of watersoaked lesions appeared on the leaves. The mutant strains induced only 1 to 50% as many lesions as the wild-type strain (Fig. 3).

Physical analysis of Tn*5* insertions. To determine if a single, random insertion had occurred, total genomic DNA from several Km^r Cm^s strains expressing virulence and nutritional mutations was digested to completion with restriction endonuclease *Eco*RI, electrophoresed, transferred to DBM paper, and hybridized to nick-translated pSUP1011 (Tn*5*) (Fig. 4). Only one band in each lane was expected to hybridize to the probe, since Tn*5* does not contain an *Eco*RI recognition site (14). All of the isolates tested contained a single band that hybridized strongly to the Tn*5* probe; this band was in a different location in each lane. In some DNA digests, a second band also hybridized weakly to the Tn*5* probe (Fig. 4, lanes A,F,H,I). Hybridization of the blot to nick-translated pSUP101, which has homology with pSUP1011 but does not contain Tn*5* (Table 1), showed no hybridization to DNA in any of the lanes except for the control lane containing pSUP1011 (*unpublished*). This indicated that the weak bands seen in the first hybridization were due to secondary transposition of all or a part of Tn*5*.

In other Tn*5* transposition experiments in *E. coli* (4) and *P. solanacearum* (26), IS50 from the terminal-inverted-repeat region of Tn*5* (14) was reported to transpose independently. To determine if this had occurred in those strains with secondary hybridization bands, the DBM blot was hybridized to the internal, non-IS50 portion of Tn*5*. A fragment with this region of Tn*5* was isolated by electroelution following digestion of pSUP1011 with *Bgl*II and labeled by nick translation. Only the bands that strongly hybridized to pSUP1011 (Fig. 4) hybridized to this internal Tn*5* probe (*unpublished*).

DISCUSSION

Conjugation of the suicide vector pSUP1011 (25) from *E. coli* donor strain SM10, into *Pseudomonas syringae* pv. *syringae* PS9020 and *P. syringae* pv. *phaseolicola* PP7010, resulted in Tn*5* transposition into the genomes of these strains. Genetic and physical evidence confirmed that random Tn*5* transposition, and not maintenance of the donor plasmid, pSUP1011, had occurred. Screening of Tn*5*-containing isolates on appropriate media revealed that 0.5–0.6% of these isolates had mutations resulting in auxotrophy. This frequency is consistent with physical evidence for mutation rates in Tn*5* mutagenesis experiments with other organisms (5,11,21). Analysis of these auxotrophs showed a wide range of nutritional deficiencies which were consistent with random insertion of Tn*5* into the genomes of these bacteria. Loss of kanamycin resistance, concomitant with reversion to prototrophy in 17 of 19 mutants (Table 2), showed linkage of mutant phenotypes to the Km^r determinant of Tn*5*. In addition to auxotrophy,

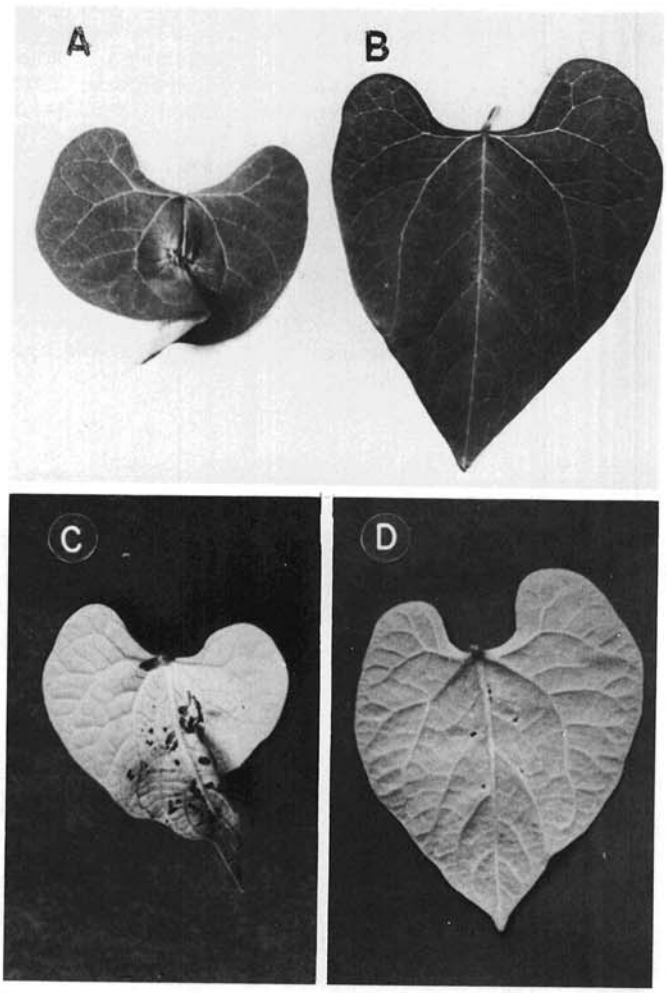

Fig. 1. Symptoms induced by wild-type and Tn*5*-induced mutant strains of *Pseudomonas syringae* pv. *syringae* and pv. *phaseolicola* on *Phaseolus vulgaris* 'Red Mexican.' **A and B,** Symptoms induced by strains PS9020 (wild-type) and PS9021 (mutant), respectively; **C and D,** symptoms induced by strains PP7010 (wild-type) and PP7011 (mutant), respectively.

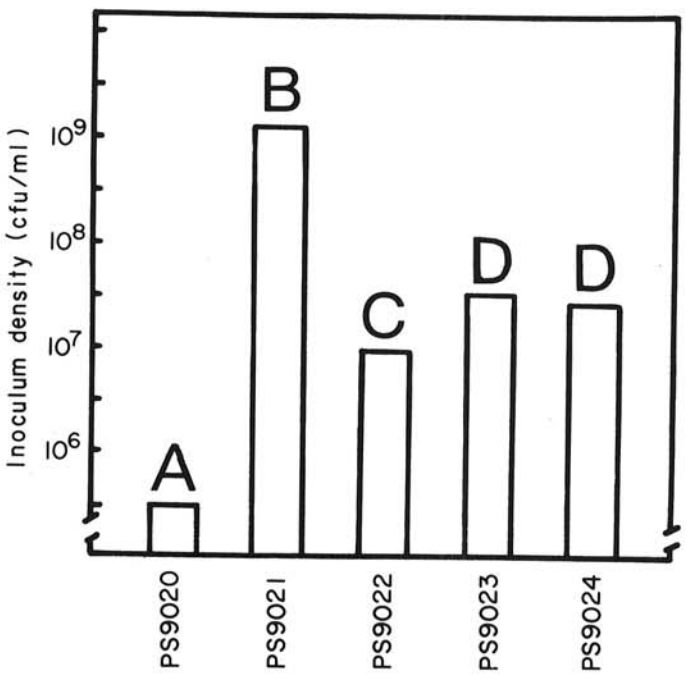

Fig. 2. Inoculum densities (colony-forming units per milliliter) of wild-type (PS9020) and Tn*5*-induced mutant strains of *P. syringae* pv. *syringae* required to induce symptoms when injected into leaves of *Phaseolus vulgaris* 'Red Mexican.' Bars with the same letter are not significantly different, according to the Student's *t* test ($P = 0.05$). Each bar represents the average of three experiments, each with five replicates .

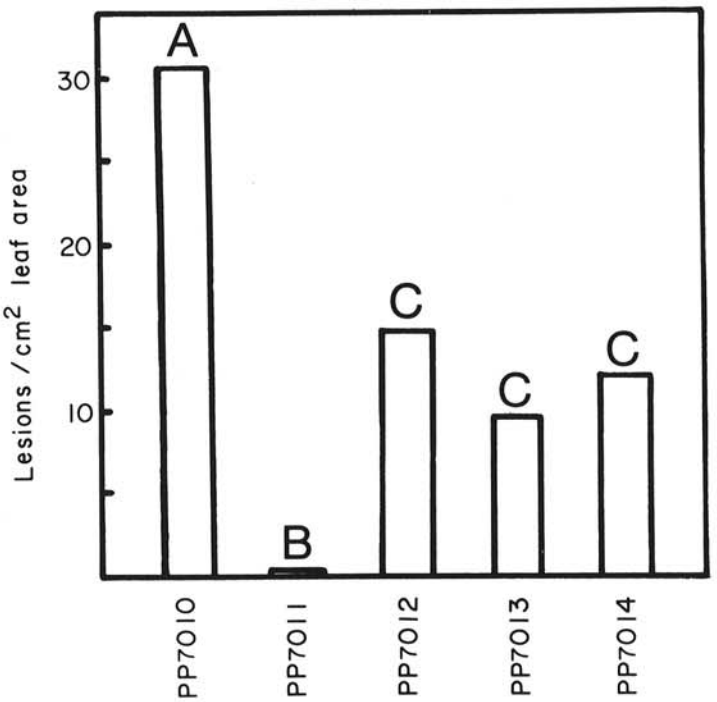

Fig. 3. Number of watersoaked lesions per square centimeter of leaf area of *Phaseolus lunatus* PI 199791 after swab-inoculation (approximately 10^9 colony-forming units per milliliter) with wild-type (PP7010) and Tn*5*-induced mutants of *P. syringae* pv. *phaseolicola*. Bars with the same letter are not significantly different according to Student's *t* test ($P = 0.05$). Each bar represents two experiments, each with two replicates.

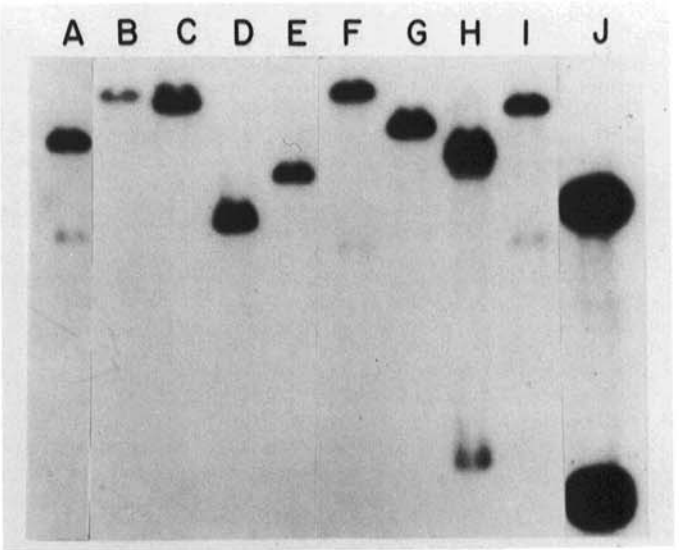

Fig. 4. *Eco*RI-digested total genomic DNA from Tn*5* containing auxotrophic and virulence mutants of *Pseudomonas syringae* pv. *syringae* and pv. *phaseolicola*, hybridized to ^{32}P-labeled pSUP1011 (Tn*5*) probe. **A,** PS9021, **B,** PS9023, **C,** PS9024, **D,** PS9510, **E,** PS9513, **F,** PP7011, **G,** PP7012, **H,** PP7013, **I,** PP7014, and **J,** *Xho*I-digested pSUP1011 DNA.

0.3–0.4% of Tn*5* insertion mutants assayed on leaves of *Phaseolus vulgaris* showed either reduced ability or complete inability to incite disease symptoms (Figs. 1 to 3).

We infer from the hybridization data (see Fig. 4 and Results) that transposition of IS50 from Tn*5* occurred in some of the Tn*5*-containing isolates, since neither pSUP101 nor the internal, non-IS50 portion of Tn*5* have homology with bands that weakly hybridize to pSUP1011. A potential problem posed by this phenomenon is that mutations due to IS50 insertion would not be linked to kanamycin resistance, and would not be readily selectable. IS50 transposition may have been the cause of auxotrophy in strains PS9513 ser$^-$ and PP7512 arg$^-$ whose revertants remained Km^r. Tn*5* insertion was also the cause of both auxotrophy and lowered symptom-inducing capabilities in PP7012, as evidenced by kanamycin sensitivity of revertants that were both prototrophic and able to induce wild-type levels of disease symptoms. There was no direct method for testing reversion in the mutants with phenotypes other than auxotrophy. Since it is clear that IS50 transposition has occurred in some of the isolates, its involvement in experimental anomalies cannot be ruled out in Tn*5* mutational studies. Anomolous results also reported during Tn*5* mutagenesis of *Rhizobium meliloti* (21) and *Erwinia carotovora* (29) could be explained by secondary transposition of IS50. Indeed, it appears that secondary bands of DNA of *E. carotovora* hybridized weakly to Tn*5* (29), as in our study (Fig. 4). Complementation of modified virulence mutants with cloned genomic sequences from a clone library should elucidate the cause (Tn*5* or IS50) of mutation in these strains.

The isolation and characterization of Tn*5*-induced mutations with selectable phenotypes in *P. syringae* pv. *syringae* and *P. syringae* pv. *phaseolicola* provides enhanced opportunities for the study of the genetics of these organisms. Tn*5* transposition to many regions of the genome is evidenced by the variety of mutants obtained (Table 2, Figs. 2 and 3), and by the different location of *Eco*RI fragments from various mutants hybridizing to Tn5 in blot hybridization analysis (Fig. 4). Although mutants exhibiting a common nutritional requirement (eg, leu$^-$ and arg$^-$ [Table 2]) might suggest nonrandom insertion of Tn*5* into the genome, the different reversion frequencies of these mutants (Table 2) suggest that different gene loci have been affected. The occurrence of several Tn5-induced virulence mutants with insertions into different *Eco*RI fragments, as well as the different responses of these mutants on bean leaves (Fig. 2 and 3), suggest that different genes involved in the virulence of these organisms have been identified. The nature of the virulence mutations in these strains is unknown; further characterization of the nature of pathogenicity of these mutants, and the genetic sequences identified, is needed. Our results suggest that pSUP1011 may be useful in transposon mutagenesis of other pathovars of *P. syringae*. Detailed mutational analysis of the diverse *P. syringae* group of plant pathogens should lead to an enhanced understanding of the genetics and molecular nature of pathogenicity of these bacteria.

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