MOLECULAR PLANT-MICROBE INTERACTIONS
Vol. 3, No. 3, pp. 149-156, 1990

Isolation and Characterization of a *Pseudomonas syringae* pv. *syringae* Mutant Deficient in Lesion Formation on Bean

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Received 21 April 1989. Accepted 30 November 1989.

The lesion-forming ability of *Pseudomonas syringae* pv. *syringae* was found to be genetically separable from other *in planta* phenotypes. We identified a Tn*5* mutant, derived from the pathogenic strain B728a, that had lost the ability to form lesions on either the pods or leaves of bean (*Phaseolus vulgaris*). The mutant, designated as NPS3136, was prototrophic, retained the ability to elicit a hypersensitive reaction on the nonhost tobacco, and attained levels of growth similar to the parental strain on bean. NPS3136 was shown to contain a single Tn*5* insertion within a 6.1-kb *Eco*RI fragment. We showed that the transposon insertion was causal to the mutant phenotype by marker exchange mutagenesis of B728a using a clone containing the Tn*5*-disrupted *Eco*RI fragment from NPS3136. We have designated the DNA region affected by the Tn*5* insertion in NPS3136 as the *lemA* locus. Eight overlapping cosmid clones that restored lesion formation to NPS3136 were isolated from a B728a genomic library. By restriction analysis of these cosmids and the original Tn*5* insertion, we have localized the *lemA* locus within a 6.7-kb region of the B728a chromosome.

Additional keywords: epiphytic growth, pathogenicity genes.

The interactions between *Pseudomonas syringae* van Hall pathovars and their plant hosts fall into two general categories: compatible, leading to intercellular bacterial growth and symptom development in the host, or incompatible, resulting in the absence of observable disease symptoms and a reduction in bacterial growth. Among pathovars of *P. syringae* that cause leaf spot disease, symptomatology on susceptible host plants is manifested as the formation of persistent or transient water-soaked lesions (Fahy and Lloyd 1983; Schroth *et al.* 1981). Bacterial populations in such a compatible interaction increase dramatically, with final population densities increasing 10^5- to 10^7-fold over initial inoculum levels.

On nonhost plants, the incompatible interaction is correlated with the elicitation of the hypersensitive reaction (HR) when bacteria are introduced into leaf tissue at artificially high (greater than 10^6 colony forming units [cfu] per milliliter) inoculum levels (Klement 1982). Bacterial growth within the intercellular spaces of resistant leaf tissue is limited, with bacterial populations increasing only 10- to 100-fold in the first 12 to 24 hr and usually decreasing thereafter. Below the 10^6 cfu/ml threshold, a macroscopic plant reaction is not normally seen in an incompatible interaction.

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No *in planta* response or growth (that is, a null reaction) is detected upon introduction of nonpathogenic bacteria, such as *P. fluorescens* (Trevisan) Migula or *Escherichia coli* (Migula) Castellani and Chalmers, into plant tissues at any inoculum level. It is important to note that the above *in planta* responses and growth effects are the result of the introduction of the bacteria into the intercellular spaces of the host plant.

As epiphytes, many *P. syringae* pathovars and *P. fluorescens* isolates are able to colonize extensively the surfaces of both host and nonhost plants. For example, bean pathogenic isolates of *P. s.* pv. *syringae* van Hall are often found on field-grown host plants that do not show disease symptoms (Lindemann *et al.* 1984). In addition, *P. s.* pv. *syringae* isolates that are pathogenic on bean (*Phaseolus vulgaris* L.) may attain population densities greater than 10^4 cfu per gram of leaf tissue on the nonhost corn (*Zea mays* L.) (Lindemann *et al.* 1984).

Recent studies have begun to elucidate the genetic control of the biological processes within the pathogen that contribute to these complex bacteria-plant interactions. Mutational analysis of *P. s.* pv. *phaseolicola* (Burkholder) Young *et al.* has led to the identification and cloning of a gene cluster required for several plant reaction phenotypes (Lindgren *et al.* 1986). Tn*5* insertions in these *hrp* genes eliminate the ability of the bacterium to elicit the HR on nonhost plants such as tobacco (*Nicotiana tabacum* L.), block its ability to form water-soaked lesions on bean, and attenuate growth *in planta*. Work in other laboratories has also led to the isolation of *hrp*-like mutants within *P. s.* pv. *phaseolicola* (Deasey and Matthysse 1988; Somlyai *et al.* 1986), *P. s.* pv. *syringae* (Niepold *et al.* 1985), *P. s.* pv. *tomato* (Okabe) Young *et al.* (Cuppels 1986), and *P.*

s. pv. *pisi* (Sackett) Young *et al.* (Malik *et al.* 1987). Recently, a cosmid clone containing a *hrp* region from *P. s.* pv. *syringae* was shown to enable both *E. coli* and *P. fluorescens* to elicit an HR-like response on tobacco (Huang *et al.* 1988). However, as in the analysis of other *hrp* loci, mutations in this *hrp* cluster abolish the HR caused by *P. s.* pv. *syringae* on tobacco but do not lead to a pathogenic response on this nonhost plant.

While the analysis of *hrp* genes shows that pathogenicity and the elicitation of the HR are functionally linked at some level, several groups have identified mutations that influence virulence without having an effect on the nonhost reaction. Tn*5*-generated nonpathogenic mutants of *P. s.* pv. *pisi* have been identified that elicit an HR on both host (pea, *Pisum sativum* L.) and nonhost (tobacco) plants (Malik *et al.* 1987). A similar mutant of *P. s.* pv. *tomato* has been isolated that is still able to cause an HR on tobacco (Cuppels 1986). The cloning and analysis of avirulence (*avr*) genes from the soybean (*Glycine max* (L.) Merr.) pathogen *P. s.* pv. *glycinea* (Coerper) Young *et al.* clearly show that loss of the ability to elicit the HR does not affect pathogenicity in this race-cultivar system. Mutations within the *avrA* gene eliminate the HR and result in lesion formation on normally resistant soybean cultivars, while the compatible interaction with susceptible cultivars is not altered (Staskawicz *et al.* 1987; Staskawicz *et al.* 1984).

In the hope of simplifying the analysis of the genetic control of pathogenicity and identifying those genes and gene products required for lesion formation, we have sought and obtained bacterial mutants that have lost the ability to cause disease symptoms but have retained their other *in planta* phenotypes. In this study, we describe the isolation and characterization of one such mutant of *P. s.* pv. *syringae* isolate B728a, a causal agent of brown spot disease of bean. This mutant, designated as NPS3136, lost the ability to cause a pathogenic response on the pods or leaves of bean, but retained the ability to elicit the HR on the nonhost plant tobacco and to attain wild-type levels of growth within or on the leaves of bean. We report the isolation of cosmid clones that restore pathogenicity to NPS3136 and the localization of the locus affected by Tn*5* in this strain, designated as *lemA*, to a 6.7-kilobase (kb) region of the B728a chromosome. (Preliminary accounts of this work have been published elsewhere [Willis and Panopoulos 1984; Willis *et al.* 1985].)

MATERIALS AND METHODS

Bacterial strains and plasmids. The sources and relevant genotypes of bacterial isolates and plasmids are listed in Table 1.

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 ambient temperature. Antibiotics were used at the following concentrations for *E. coli*: ampicillin (Ap), 50 μg/ml; tetracycline (Tc), 15 μg/ml; and kanamycin (Km), 30 μg/ml. For selection of resistant *P. syringae*, the antibiotic concentrations were as follows: Tc, 10 μg/ml; Km, 10 μg/ml; and rifampicin (Rif), 100 μg/ml. Stocks of Rif and Tc were made in 100% methanol at 10 mg/ml and stored at 4° C. Km and Ap were dissolved at 30 mg/ml and 50 mg/ml, respectively, in water, filter-sterilized, and stored at 4° C. Washing buffer consisted of KH_2PO_4 (6.25 g/L), $K_2HPO_4 \cdot 3H_2O$ (11.8 g/L), and Difco (Detroit, MI) Bacto peptone (1 g/L).

DNA manipulations. The procedures for isolation of plasmid DNA, restriction endonuclease digestion, ligation, isolation of restriction fragments from agarose gel by electroelution, transfer of DNA fragments to nitrocellulose or nylon membranes, labeling of DNA with ^{32}P by nick translation, and hybridization were conducted essentially as described by Maniatis *et al.* (1982) or according to manufacturers' recommendations. Isolation of total bacterial DNA from *E. coli* and phytobacteria as well as the construction of cosmid libraries in pLAFR3 have been described previously (Peet *et al.* 1986). Colony hybridizations were performed by lifting fresh colonies onto Whatman 541 filter paper and hybridizing with a ^{32}P-labeled probe by the method of Hanahan and Meselson (1980).

Mutagenesis and identification of transposon insertion mutants. The procedure we followed for suicide plasmid mutagenesis of *P. syringae* pathovars was as described by

Table 1. Bacterial strains, plasmids, and phage used in this study

Designation	Relevant genotype or phenotype[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
JM83	*φ80δlacZΔM15*	Messing *et al.* 1981
Pseudomonas syringae		
Cit7	Rif^r, isolate from citrus, incompatible on bean	Orser *et al.* 1985
P. s. pv. *syringae*		
B728a	Rif^r, bean pathogenic isolate	S. S. Hirano, Univ. of Wisconsin, Madison
NPS3136	Rif^r Km^r *lemA1*::Tn*5*	This study
Plasmids and phage		
pCUV8	*recA*$^+$ Tc^r	Hickman *et al.* 1987
pKW3	*lemA1*::Tn*5* Km^r Ap^r	This study
pKW25	*lemA1*::Tn*5* Km^r Tc^r	This study
pKW – 112, 231, 321, 331, 332[b], 333[b], 351[b], 352[b]	*lemA*$^+$ Tc^r	This study
pLAFR3	Tc^r	Staskawicz *et al.* 1987
pUC8	Ap^r	Bethesda Research Laboratories, Gaithersburg, MD
pUW964	Tn*903*::Tn*7* Tn*5*	Weiss *et al.* 1983
λ::Tn*5*	λ*cI857 rex*::Tn*5*	D. E. Berg, Washington Univ. School of Medicine, St. Louis, MO

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

[b] Four of the cosmids identified, pKW332/pKW333 and pKW351/pKW352, make up two probable sibling pairs.

Peet *et al.* (1986). We used the suicide plasmid pUW964 (Weiss *et al.* 1983) for the generation of Tn*5* mutants of *P. s.* pv. *syringae* isolate B728a. This plasmid contains a ColE1 replicon, the *tra* region of RK2, and the transposons Tn*7* and Tn*5*. pUW964 is self-transmissible but cannot replicate in *P. s.* pv. *syringae*, permitting the direct selection of Tn*5* transposition. Presumptive Tn*5* insertion mutants (Km-resistant [Km^r] transconjugants) were selected on KB plates containing Rif and Km and screened for the ability to cause water-soaked lesions on pods of *P. vulgaris* cv. Bush Blue Lake 274 (Northrup King Co., Minneapolis, MN) as described below. The frequency of auxotrophs was established by replica plating Km^r transconjugants on minimal medium. To ensure the elimination of contaminants, Km^r transconjugants of interest were probed with the *P. s.* pv. *syringae recA*-containing cosmid pCUV8 by Southern blot analysis to establish that B728a-specific fragments (Willis *et al.* 1988) were present.

Construction of pKW3 and pKW25. Plasmid pKW3 was isolated from an *Eco*RI genomic library of NPS3136 ligated into the plasmid pUC8 by selection for Ap^r Km^r colonies after transformation into JM83. The insert consisted of an 11.9-kb *Eco*RI fragment containing the *lemA1*::Tn*5* insertion from NPS3136. Plasmid pKW25 was constructed by subcloning this 11.9-kb *Eco*RI fragment from pKW3 into pLAFR3.

Marker exchange. Recombinational exchange mutagenesis of *P. s.* pv. *syringae* isolates was performed using the transplacement technique (Gutterson *et al.* 1986) as previously described (Willis *et al.* 1988).

Pathogenicity assays and determination of *in planta* growth rates. Preliminary pathogenicity assays of Km^r transconjugants of B728a were performed by inoculation of pods of *P. vulgaris* cv. Bush Blue Lake 274. Plants were grown in the greenhouse (approximately 24° C day and 18° C night temperatures with a 16-hr light cycle). Pods between 8 and 12 cm in length were harvested for bacterial inoculation. Bacterial colonies were picked with the pointed end of a sterile flat toothpick and stabbed under the epidermis of the pods. Inoculated pods were incubated individually in Whirl-Pak bags (Nasco, Chelsea, MA) in a growth chamber with a 14-hr light cycle at 24° C. The pods were examined for the presence or absence of symptoms after 2 to 3 days. Mutants showing an altered plant response were rechecked by injection of serially diluted inoculum into the pods and leaves of bean as previously described (Willis *et al.* 1988). It should be noted that the persistent sunken and water-soaked lesion shown by pathogenic strains of *P. s.* pv. *syringae* in our pod assay (Fig. 1) is not a typical field symptom of brown spot disease of bean. However, we judged this assay to be valid in assessing pathogenicity since it has been used successfully to breed *Phaseolus vulgaris* for field resistance to brown spot (Daub and Hagedorn 1979). This pathogenic response is easily distinguished from the necrosis of the inoculated area (HR) within 18 to 24 hr that results from the inoculation of a nonpathogenic *P. syringae* strain such as Cit7 (data not shown). The ability of isolates to induce the HR on the nonhost tobacco cultivar Havana 142 (seed provided by K. K. Knoche and R. D. Durbin, University of Wisconsin, Madison) was determined by the rapid inoculation technique of Staskawicz *et al.* (1984).

We confirmed the plant response phenotype of wild-type and mutant strains using two different plant inoculation methods. The first was localized infiltration of primary bean leaves as described in the legend of Figure 2. In the second method, bacterial suspensions adjusted to 10^6 cfu/ml were spray-inoculated onto the leaves of bean, and the plants were incubated under high humidity conditions as previously described for the measurement of epiphytic growth of bacteria on bean (Willis *et al.* 1988). With the latter method, the plants were observed for the presence or absence of lesions after 7 days of incubation.

Two methods were used to assess bacterial growth in association with bean plants. First, we determined the epiphytic growth rate as previously described (Willis *et al.* 1988) with the following modifications. For sampling, 60 trifoliate leaflets were picked and divided into three groups of 20 leaflets. Bacteria were dislodged from the leaf surfaces by sonication of each group in 100 ml of washing buffer for 7 min before dilution plating on KB agar medium containing Rif (B728a) or Rif plus Km (NPS3136).

The second method, used to measure *in planta* growth, was modified from Bertoni and Mills (1987). Bacterial cultures were grown to an $A_{600\ nm}$ of 0.3 in KB medium

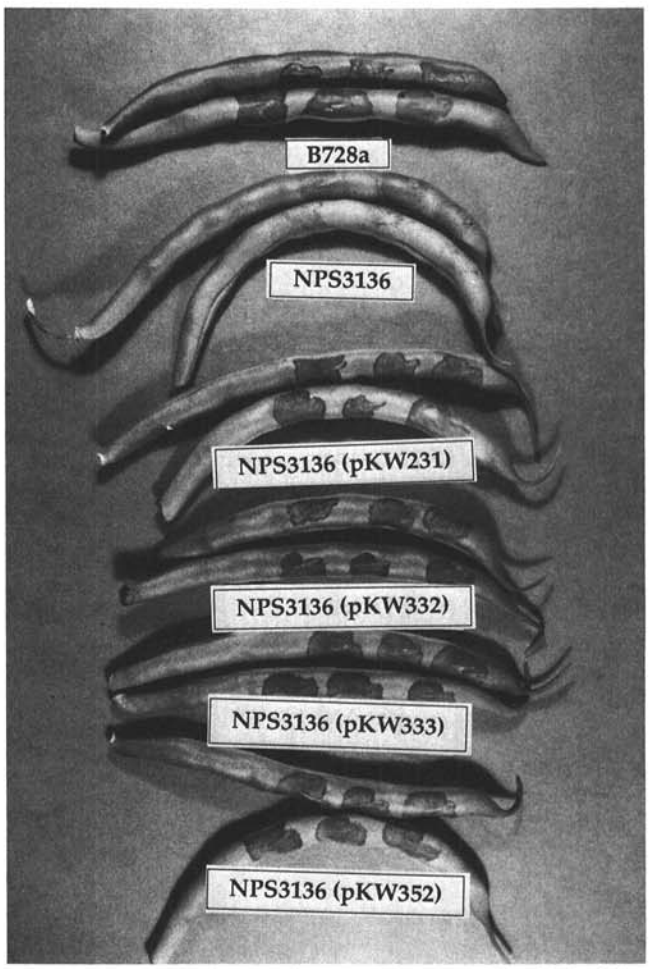

Fig. 1. Response of the pods of bean cultivar Bush Blue Lake 274 to inoculation with B728a(*lemA*$^+$), NPS3136(*lemA1*::Tn*5*), or NPS3136 containing various cosmid clones. The pods were surface-sterilized in 10% bleach for 30 sec and injected just below the epidermis with a bacterial suspension in water containing approximately 10^8 colony forming units per milliliter. A 25-gauge needle attached to a 1-ml disposable syringe was used. Pods were incubated in individual Whirl-Pak bags as described in the text. The photograph was taken 2 days after inoculation.

at 24° C, and then 1.0 ml of the bacterial cells was harvested by centrifugation and resuspended in 1.0 ml of K buffer (0.01 M K_2HPO_4/KH_2PO_4, pH 7.0). The cells were diluted 100-fold in K buffer and infiltrated into primary leaves of 13-day-old bean plants using a disposable transfer pipette (catalog no. 86.1171, Sarstedt, Inc., Princeton, NJ). The edge of the pipette was used to wound the top epidermis of the leaf slightly to facilitate the infiltration. For each time point, five samples were taken from five different plants using a cork borer with a diameter (8 mm) smaller than the area of infiltration. The leaf disks were ground in 300 μl of K buffer in a 1.5-ml microcentrifuge tube using a disposable pellet pestle (Kontes Glass Co., Vineland, NJ), and viable counts were determined by serial dilution on KB agar medium containing Rif (B728a) or Rif plus Km (NPS3136).

RESULTS

Isolation of mutants deficient in lesion formation on bean. To identify genes required for the pathogenicity of *P. s.* pv. *syringae* on bean, we generated transposon Tn*5* mutants of the wild-type isolate B728a. This strain was originally recovered from a Wisconsin bean field and is able to cause typical brown spot lesions on bean. As a transposon vector, we used the suicide plasmid pUW964 that has been shown to efficiently deliver Tn*5* into *P. syringae* pathovars (Lindgren *et al.* 1986; Peet *et al.* 1986). Mutagenesis of strain B728a yielded mutants with random Tn*5* insertion sites as ascertained by Southern blot analysis using λ::Tn*5* as a probe and by the frequency of auxotrophic strains with different nutritional requirements (approximately 1% of all Km^r isolates). A total of 1,008 presumptive Tn*5*-containing transconjugants were screened for alteration of their pathogenic response on the pods of bean. Of these, one prototrophic mutant, designated as NPS3136, was identified that had lost the ability to cause a disease reaction in the initial pod assay.

***In planta* phenotypes of NPS3136.** When injected into bean pods, NPS3136 caused a slow necrosis in the inoculated area. This reaction was markedly different from the sunken, water-soaked lesion caused by the pathogenic strain B728a (Fig. 1) and from the HR caused by the nonpathogenic *P. syringae* strain Cit7 (data not shown). Inoculation of 10^7 to 10^8 cfu/ml of the mutant strain into bean leaves resulted in necrosis of the inoculated area that was difficult to distinguish from the necrosis typical of B728a. At lower inoculum concentrations (10^6 cfu/ml or

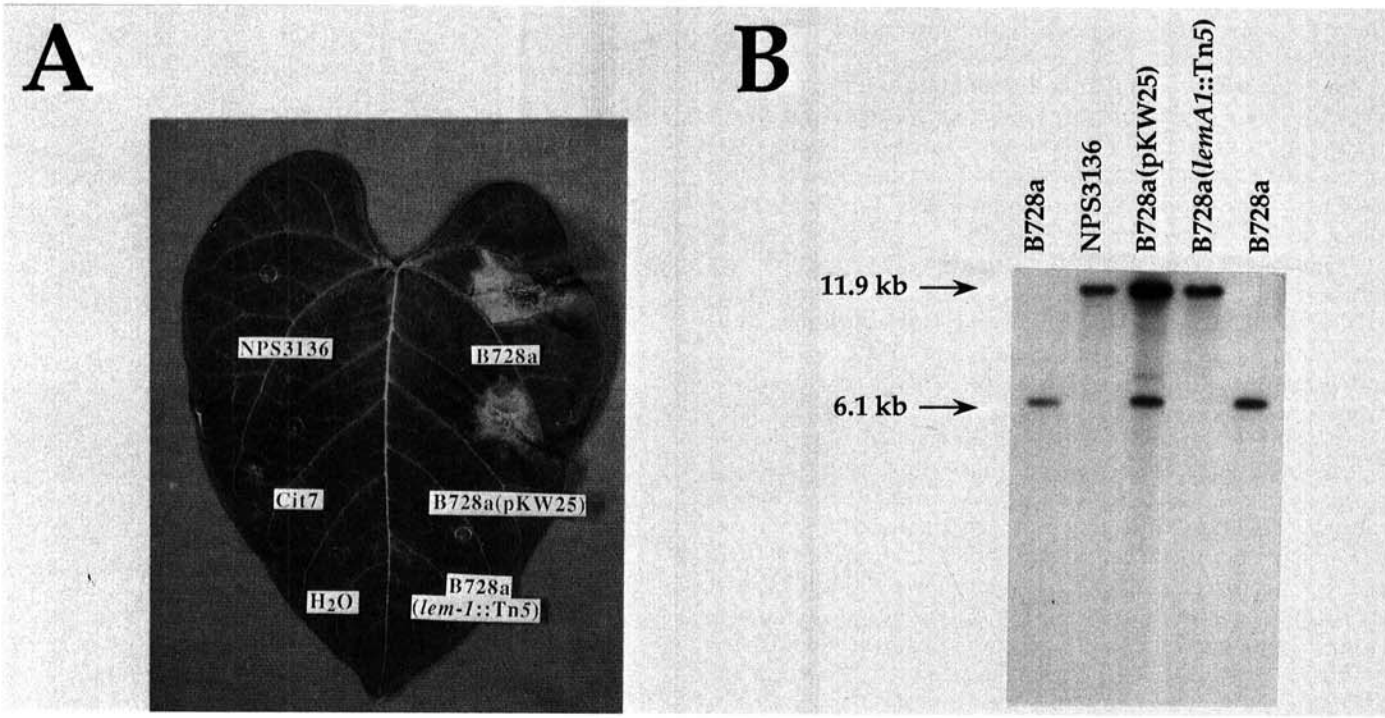

Fig. 2. A, Reaction on a primary leaf of *Phaseolus vulgaris* cv. Bush Blue Lake 274 after bacterial infiltration. Shown are phenotypes of B728a(*lemA*$^+$), NPS3136(*lemA1*::Tn*5*), the exchange intermediate merodiploid B728a(pKW25), and the exchange mutant B728a(*lem-1*::Tn*5*). It should be noted that *lem-1*::Tn*5* was the provisional designation for the *lemA1*::Tn*5* mutation. Also inoculated were the nonpathogenic strain Cit7 and a sterile water control. Bacterial suspensions in water adjusted to 10^6 colony forming units per milliliter were infiltrated into the primary leaves (midrib length 8 to 10 cm) of intact bean plants (approximately 6 days postemergence). A disposable plastic transfer pipette was used to infiltrate inoculum through a slight nick in the upper leaf surface made with a sterile razor blade. The circular marks seen above several of the labels (see the H_2O control as an example) resulted from pressure on the leaf by the pipette during inoculation. The plants were incubated in growth chambers with a 14-hr light cycle at 24° C and reactions were scored after 4 to 5 days. The photograph was taken 5 days after inoculation. **B**, Southern blot analysis of a representative recombinational exchange of the *lemA1*::Tn*5* mutation. The lanes contain *Eco*RI-digested DNA from B728a, NPS3136, B728a(pKW25), or B728a(*lemA1*::Tn*5*). The restricted DNA was resolved by gel electrophoresis in 0.7% agarose. The probe used was pKW3 labeled with ^{32}P by nick translation as described by Maniatis *et al.* (1982). The sizes in kilobases (kb) of *Eco*RI fragments homologous to the 6.1-kb *Eco*RI fragment within pKW3 are indicated at the left. Sizes of the pKW3 homologous fragments were determined by comparison to *Hin*dIII cut λ DNA and a 1-kb ladder (Bethesda Research Laboratories, Gaithersburg, MD) in adjacent lanes of the gel (not shown).

lower), no symptoms were seen with NPS3136, while typical spreading lesions developed when 10^6 cfu/ml of the parental strain was used (Fig. 2A). When bean plants were spray-inoculated with either B728a or NPS3136, brown spot lesions were always observed with the parental strain but never with NPS3136 (data not shown). Inoculation of NPS3136 on tobacco showed that this mutant retained the ability to induce an HR on this nonhost plant (Fig. 3). We detected no difference in the time course of HR induction by NPS3136 when compared to parental strain B728a or in the maximum dilution at which the HR was elicited on tobacco.

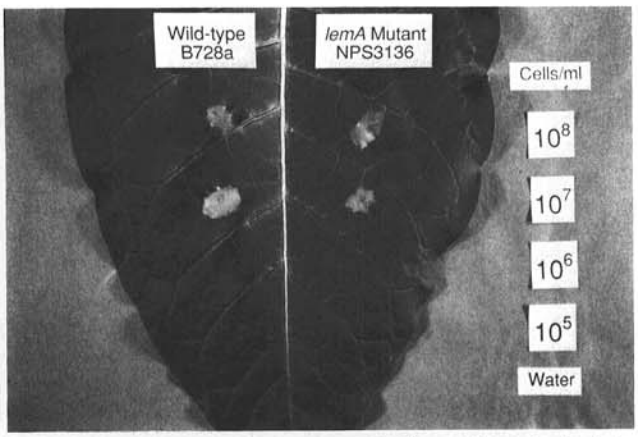

Fig. 3. Response of B728a and NPS3136 infiltrated into the leaf tissue of the nonhost tobacco. Bacterial suspensions in water at the cell densities indicated at the right were infiltrated into an almost fully expanded leaf of 8- to 10-wk-old tobacco cultivar Havana 142 using a disposable transfer pipette. The plants were incubated at ambient temperature for 48 hr before being photographed.

NPS3136 was not altered in its ability to colonize the leaves of bean. There was no significant difference in the growth rates or the final population levels of this mutant when compared with B728a under greenhouse conditions (Fig. 4A). In addition, we tested the ability of NPS3136 to grow *in planta* by infiltration inoculation (Fig. 4B). Using this protocol, NPS3136 not only attained population densities equivalent to B728a by 2 days after inoculation but was able to maintain a high cell number more effectively than its parental strain over an 11-day period. Based on the above phenotypic characteristics, the mutation in NPS3136 appeared to affect lesion production on bean but not other plant interaction phenotypes.

Cloning and characterization of the Tn*5* insertion and flanking DNA from NPS3136. Southern blot analysis of total genomic DNA from NPS3136 using λ::Tn*5* as a probe revealed a single *Eco*RI fragment containing Tn*5* homology with an apparent length of 11.9 kb. After subtracting 5.8 kb, representing the length of Tn*5* (Berg 1989), the predicted length of the *Eco*RI fragment within NPS3136 that suffered the insertion of Tn*5* was 6.1 kb. We cloned the 11.9-kb Tn*5*-containing *Eco*RI fragment from NPS3136 into pUC8 and used this plasmid, designated as pKW3, as a probe in the Southern hybridization analysis of *Eco*RI-digested chromosomal DNA. Figure 2B shows a single fragment with a length of 6.1 kb in the DNA of the parental strain B728a, representing the target *Eco*RI fragment. This frag-

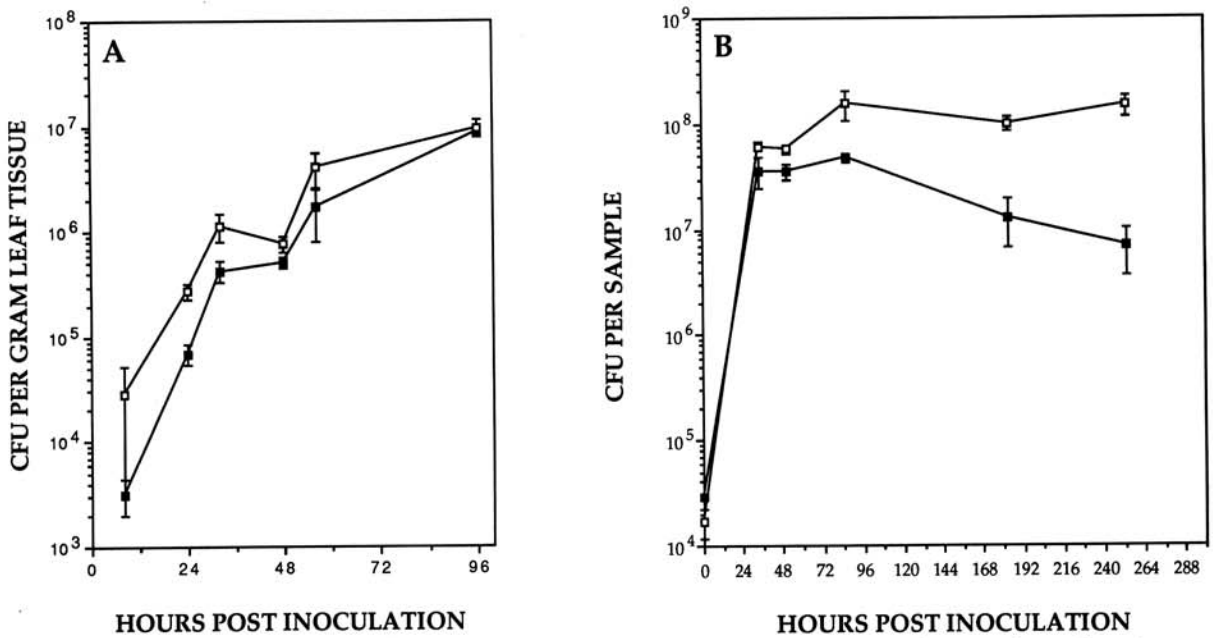

Fig. 4. A, Epiphytic growth of B728a (■) and NPS3136 (□) on the leaves of *Phaseolus vulgaris* cv. Eagle. Bacterial suspensions, adjusted to approximately 10^8 colony forming units (cfu) per milliliter in sterile water, were sprayed onto leaves until runoff of the liquid occurred. Plants were incubated under greenhouse conditions (approximately 24° C day and 18° C night temperatures with a 16-hr light cycle) in mist tents at high humidity. The vertical lines represent the standard error of the determination of the mean of three samples collected as described in the text. **B,** Growth of B728a (■) and NPS3136 (□) after infiltration inoculation into the leaves of *P. vulgaris* cv. Bush Blue Lake 274. Bacterial suspensions, adjusted to approximately 10^6 cfu/ml in sterile water, were infiltrated into the leaves of 13-day-old bean plants. Plants were incubated in growth chambers at 24° C with a 12-hr light cycle. The vertical lines represent the standard error of the determination of the mean of five leaf disks collected as described in the text.

ment was not present in the DNA of NPS3136 and was replaced by the expected 11.9-kb Tn*5*-containing fragment.

To confirm that the Tn*5* insertion was causal to the mutant phenotype of NPS3136, we subcloned the 11.9-kb *Eco*RI fragment from pKW3 into the wide host range cosmid pLAFR3 and used the resulting plasmid, designated pKW25, to perform site-directed mutagenesis of B728a by transplacement. Figure 2B shows the result of the Southern blot analysis of a representative exchange in which the 6.1-kb wild-type fragment in B728a has been replaced by the 11.9-kb Tn*5*-containing *Eco*RI fragment from pKW25. When assayed in bean leaves, B728a and the exchange intermediate merodiploid B728a(pKW25) show the spreading necrotic lesion typical of a pathogenic response in this assay, while an exchange mutant, B728a(*lemA1*::Tn*5*), shows no lesion-forming ability and is indistinguishable from NPS3136 (Fig. 2A). Fifteen additional exchange mutants were tested for their ability to form lesions on the pods or leaves of bean, and all 15 lost their lesion-forming ability. Due to the genetic linkage between the Tn*5* insertion and the loss of lesion-forming ability, we have designated the locus affected by Tn*5* in NPS3136 as *lemA* (*le*sion *m*anifestation) and the specific mutation as *lemA1*::Tn*5* in accordance with the guidelines for bacterial nomenclature (Demerec *et al.* 1966).

Cloning and localization of the *lemA* locus. Using pKW3 as a probe, we performed colony hybridizations of a 1,100-member genomic library of B728a. The library consisted of size-fractionated, *Sau*3A partially digested B728a chromosomal DNA ligated into the *Bam*HI site of pLAFR3, packaged *in vitro* into λ phage particles, and transduced into strain HB101. We identified eight positive clones in the initial screen, and seven of these were found to contain an intact 6.1-kb *Eco*RI target fragment (Fig. 5).

All eight of the above cosmids were able to complement a *lemA1*::Tn*5* mutant to the Lem$^+$ phenotype. Figure 1 shows the results obtained by introducing four of these cosmids into NPS3136 by triparental mating (Willis *et al.* 1988). All four cosmids restored lesion-forming ability to NPS3136 inoculated into bean pods. A transconjugant containing pKW352 was assayed by spray inoculation on the leaves of bean, and typical brown spot lesions developed that were indistinguishable from those formed by B728a. We have restriction mapped all eight cosmids and have found them to consist of an overlapping set encompassing approximately 35 kb of the B728a chromosome (Fig. 5). The region common to all eight cosmids is 6.7 kb and presumably contains the *lemA* locus or at least a large enough portion of the locus to complement the *lemA1*::Tn*5* mutation. This common region includes the insertion site of Tn*5* within the *lemA1* mutation.

DISCUSSION

Following Tn*5* mutagenesis, we identified a mutant of a pathogenic isolate of *P. s.* pv. *syringae* that had lost its ability to produce lesions on either the pods or leaves of bean. This nonpathogenic mutant, NPS3136, retained the ability to elicit the HR on the nonhost tobacco and to epiphytically colonize the leaves of bean. When infiltrated into bean leaves, NPS3136 manifested a 10-fold higher number of viable cells than did B728a at the end of the 11-day growth period. While this seems significant, we believe that this difference reflects an artifact of the experimental procedure. The area infiltrated by B728a collapsed and became increasingly necrotic during the time course of this experiment, while the leaf tissue in the area inoculated with NPS3136 remained healthy in appearance. The difference between these two strains, as shown by the

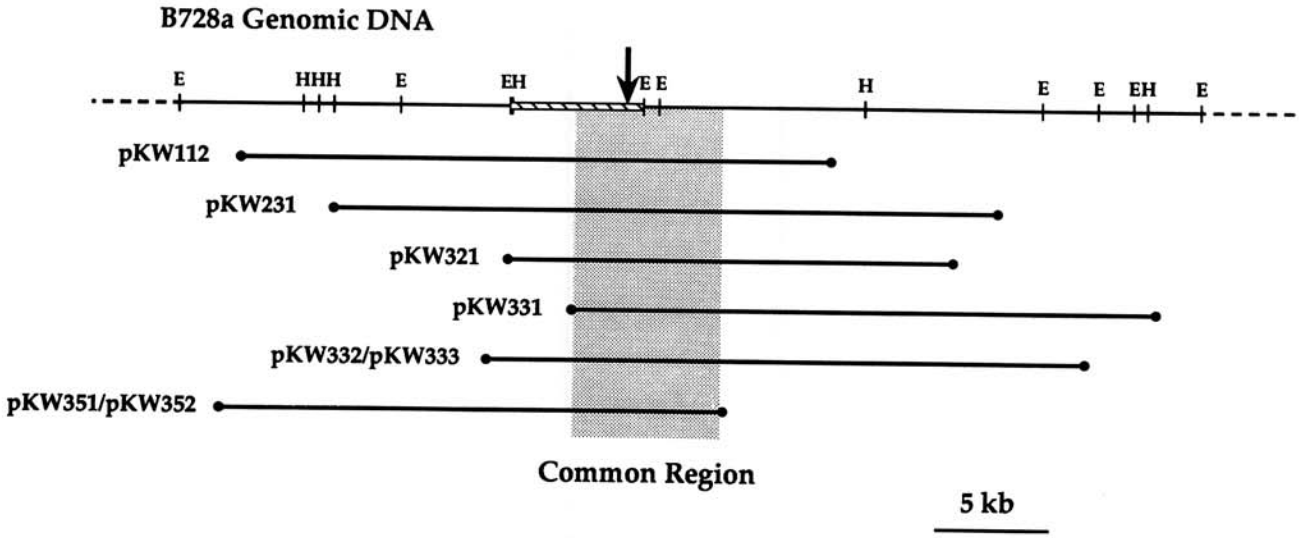

Fig. 5. Restriction map of the chromosomal inserts within eight cosmid clones that restore lesion formation to a *lemA1*::Tn*5* mutant. The designation of individual cosmids is given at the left of each cosmid insert. The probable sibling pairs pKW332/pKW333 and pKW351/pKW352 are indicated. The 35-kilobase (kb) region of the B728a chromosome encompassed by this overlapping cosmid set is shown at the top of the figure. The approximate positions of *Hin*dIII (H) and *Eco*RI (E) restriction sites are indicated. The 6.1-kb *Eco*RI target fragment is represented by a crosshatched box, and the site of the Tn*5* insertion causal to the *lemA1* mutation is shown by an arrow. The shaded box illustrates the 6.7-kb common DNA region shared by all eight restoring cosmids and includes the site of the *lemA1*::Tn*5* mutation.

leaf inoculation procedure, is most likely due to the difference in the physiological state of the sampled leaf area.

Our data show that disease symptom formation can be genetically separated from other *in planta* reaction phenotypes. This work contrasts with the analysis of *hrp* mutations that shows a definite linkage of pathogenicity, the elicitation of the HR, and growth *in planta* in several pathovars of *P. syringae*, including *P. s.* pv. *syringae* (Cuppels 1986; Huang *et al.* 1988; Lindgren *et al.* 1986; Malik *et al.* 1987; Niepold *et al.* 1985). Unlike the *hrp* clone isolated by Huang *et al.* (1988), the *lemA* locus is apparently not expressed in *E. coli* at a phenotypic level since the presence of pKW352 did not alter the typical null response of *E. coli* when infiltrated into bean or tobacco. It is interesting that we did not find a Hrp^- mutant in our screen of more than 1,000 Km^r transconjugants. We did identify a second mutant with the Lem^- phenotype, designated as NPS3139, that was also unaffected in its ability to grow epiphytically on the leaves of bean. Unfortunately, the inheritance of Tn*5* in NPS3139 was the result of a complex molecular event apparently involving the duplication of chromosomal DNA sequences and the insertion of all or part of the suicide vector pUW964. We have not been able to isolate a clone that restores lesion formation to this mutant (T. M. Barta and D. K. Willis, unpublished data).

We are continuing the screening of B728a Tn*5* mutants by pod inoculation and have provisionally identified several other mutants that have lost their lesion-forming ability but remain HR^+ on tobacco. The isolation of additional mutants with a Lem^- phenotype further strengthens our finding that lesion formation is not dependent on *in planta* growth or the ability to induce the HR on nonhost plants.

Lindgren *et al.* (1986) did not find a $Path^- HR^+$ mutant in their screen of 796 Km^r transconjugants using leaf assays on red kidney bean. However, potential Lem^- mutants have been described in *P. s.* pv. *pisi* (Malik *et al.* 1987) and *P. s.* pv. *tomato* (Cuppels 1986) using leaf assays. Currently, we do not fully understand the reason for the difference in distribution of the phenotypes of mutants isolated by various researchers analyzing the genetics of pathogenicity. However, the symptoms produced by the inoculation of the two Lem^- mutants NPS3136 and NPS3139 into the leaves of bean would have made the detection of these mutants difficult, if not impossible, had we chosen leaf inoculation for our initial screen.

The necrosis shown by both of these strains is difficult to distinguish from the response shown by B728a when the bacteria are introduced at the high inoculum levels typical of mutagenesis screening on leaves. The differential response on bean leaf tissue only becomes dramatically apparent when the cultures are diluted to approximately 10^6 cfu/ml (Fig. 2A). Although artifactual, the persistent water-soaked appearance of the pods inoculated with Lem^+ strains was easily distinguished from the slow necrosis shown by the Lem^- mutants in our initial screen. It is likely, therefore, that the method of plant inoculation chosen for the initial screening of mutagenized phytopathogenic bacteria may influence the predominant type of altered *in planta* phenotype found.

It is clear from the exchange mutagenesis of B728a using pKW25 that the *lemA1*::Tn*5* mutation is genetically linked to the Lem^- phenotype of NPS3136. The restoration of lesion-forming ability to NPS3136 by six distinct cosmids containing a common DNA region suggests that the *lemA* locus is actively transcribed from these cosmids. The capacity of several of these cosmids to complement the *lemA1*::Tn*5* mutation *in trans* has recently been confirmed by the ability of these plasmids to restore a Lem^+ phenotype to a *recA* deletion derivative of NPS3136 (E. M. Hrabak and D. K. Willis, unpublished data). We have localized the *lemA* locus to a 6.7-kb region of the B728a chromosome that encompasses the *lemA1*::Tn*5* insertion.

ACKNOWLEDGMENTS

We thank J. Handelsman and S. S. Hirano for their helpful suggestions on the manuscript. We also thank the Northrup King Co. and Rogers Brothers Seed Co. (Twin Falls, ID) for providing bean seed.

This work was supported by USDA Grant Award 88-37263-3856 and in part by National Science Foundation grants PCM-8409723 and PCM-8313052.

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