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Multiple Loci of *Pseudomonas syringae* pv. syringae Are Involved in Pathogenicity on Bean: Restoration of One Lesion-Deficient Mutant Requires Two tRNA Genes

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A mutational analysis of lesion-forming ability was undertaken in *Pseudomonas syringae* pv. syringae B728a, causal agent of bacterial brown spot disease of bean. Following a screen of 6,401 Tn*5*-containing derivatives of B728a on bean pods, 26 strains that did not form disease lesions were identified. Nine of the mutant strains were defective in the ability to elicit the hypersensitive reaction (HR) and were shown to contain Tn*5* insertions within the *P. syringae* pv. syringae *hrp* region. Ten HR$^+$ mutants were defective in the production of the toxin syringomycin, and a region of the chromosome implicated in the biosynthesis of syringomycin was deleted in a subset of these mutants. The remaining seven lesion-defective mutants retained the ability to produce protease and syringomycin. Marker exchange mutagenesis confirmed that the Tn*5* insertion was causal to the mutant phenotype in several lesion-defective, HR$^+$ strains. KW239, a lesion- and syringomycin-deficient mutant, was characterized at the molecular level. Sequence analysis of the chromosomal region flanking the Tn*5* within KW239 revealed strong similarities to a number of known *Escherichia coli* gene products and DNA sequences: the *nusA* operon, including the complete initiator tRNA^{Met} gene, *metY*; a tRNA^{Leu} gene; the *tpiA* gene product; and the MrsA protein. Removal of sequences containing the two potential tRNA genes prevented restoration of mutant KW239 in *trans*. The Tn*5* insertions within the lesion-deficient strains examined, including KW239, were not closely linked to each other or to the *lemA* or *gacA* genes previously identified as involved in lesion formation by *P. syringae* pv. syringae.

The majority of *Pseudomonas syringae* genes known to be involved in plant disease also control plant interaction phenotypes not directly related to pathogenicity. Mutations in *hrp* genes, for example, disrupt symptom development, growth in association with host plants, and the hypersensitive reaction (HR) on nonhost plants (for a review, see reference 60). The discovery of the *lemA* gene of *P. syringae* pv. syringae B728a, a causal agent of bacterial brown spot disease of bean (*Phaseolus vulgaris*), demonstrated that lesion manifestation can be genetically separated from other interactions with plants. The *lemA* gene is required for disease lesion formation but not for nonhost hypersensitivity (59). Furthermore, the *lemA* gene is essential for production of the toxin syringomycin and an extracellular protease activity (21, 46). Genetic evidence and DNA sequence analysis of the *lemA* gene support a regulatory role for the predicted LemA protein, which is similar to a family of transmembrane histidine protein kinase sensors of two-component regulatory systems (4, 20, 46, 61). More recently, a study of spontaneous mutants of B728a and other *P. syringae* pv. syringae strains led to the identification of the *gacA* gene as the second component, the response regulator (43, 47). Mutants of *gacA* have a phenotype similar to that of *lemA* mutants, being deficient in disease lesion formation and in toxin and protease production.

The pleiotropy of *lemA* and *gacA* mutants and the complex nature of plant-bacterium interactions motivated the search for other loci necessary for lesion formation, with the aim to identify genes more specific to disease progression. This report describes the isolation of novel loci in B728a that are essential for lesion formation but not for the HR. The lesion-deficient mutants that form the basis of this work were examined for pleiotropy; the data presented indicate the existence of genetic loci distinct from *lemA* and *gacA* and from the *hrp* genes that are required for disease manifestation. This analysis establishes the complex, multigenic nature of lesion-forming ability. In particular, non-*hrp* mutations affecting lesion formation but not syringomycin or protease activities have been isolated. These mutations may identify loci that are involved in disease more specifically than the genes presently known to affect lesion formation. In addition, the characterization of nine *hrp* mutants identified in this study shows that *hrp* genes are not required for the expression of known *lemA* and *gacA* phenotypes. A more extensive analysis was undertaken for mutant KW239, defective in lesion formation and syringomycin production but unimpaired in protease activity, elicitation of the HR, and growth in the plant environment (reference 21 and this work). The cloning, localization, and sequence analysis of genomic DNA required to restore lesion formation and toxin production to mutant KW239 are described.

(Preliminary reports of parts of this work have been published elsewhere [43, 44, 49, 61].)

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MATERIALS AND METHODS

Bacteria and plasmids. The sources and genotypes of bacterial isolates, plasmids, and phage are listed in Table 1. Plasmid pME3008 was kindly provided by J. Laville and D. Haas. Bacteria were grown as specified previously (59). The chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was used as previously described (47). Electroporation of plasmids into *Pseudomonas* cells was achieved by using the Bio-Rad Gene Pulser, following the manufacturer's procedures for *Escherichia coli*. Triparental matings with the mobilizing helper plasmid pRK2013 and recombinational mutagenesis were performed as described previously (58, 59).

Tn*5* mutagenesis of *P. syringae* pv. syringae B728a. B728a was mutagenized by mating as follows. A log-phase culture of *P. aeruginosa* PAO11(pMO75) (0.5 ml)

TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
E. coli		
DH5α	F⁻ *recA1 hsdR17 endA1 thi-1 gyrA96 relA1 supE44* f80d*lacZ*Δ*M15* λ⁻	BRL
CC170	CC118 [Δ(*ara leu*)*7697* Δ*lacX4* Δ*phoA20 galE galK thi rpsE rpoB argE*(Am) *recA1*] containing a chromosomal insertion of Tn*lacZ*	35
P. aeruginosa PAO11	*trp-54 nal-19 fon-1*	B. W. Holloway; 56
P. syringae pv. syringae		
B728a	Rif^r, causal agent of brown spot of bean	S. S. Hirano; 59
BUVS1	Rif^r Spc^r Δ(*recA*)*51*::Ω	20
NPS3136	Rif^r Kan^r *lemA1*::Tn*5* derivative of B728a	59
NUVS1	Rif^r Spc^r Kan^r Δ(*recA*)*51*::Ω *lemA1*::Tn*5*	20
KW21	Rif^r Kan^r Lem⁻ Tn*5* mutant	21, 48; this study
KW239	Rif^r Kan^r Lem⁻ Tn*5* mutant, previously designated KW301	21; this study
KW239UVS1	Rif^r Spc^r Kan^r Δ(*recA*)*51*::Ω *lem-239*::Tn*5*	This study
KW626, KW1625, KW1916, KW2163, KW2803, KW3621, KW3777, KW3815, KW3899, KW5949	Rif^r Kan^r Lem⁻ Tn*5* mutants	This study
KW1858, KW1986, KW2652, KW2948, KW3845, KW4777, KW5891, KW6282, KW6358	Rif^r Kan^r Lem⁻ HR⁻ Tn*5* mutants	This study
KW35, KW628, KW1152, KW1163, KW1166, KW1177, KW5279, KW6254	Rif^r Kan^r Tn*5* mutants deleted for pYM101 region	21; this study
KW256, KW1156	Rif^r Kan^r mutants containing Tn*5* insertions linked to *lem-5949*::Tn*5* mutation; KW256 was previously designated KW302	21; this study
BSR	Rif^r, spontaneous mutant of B728a deleted for pYM101 region	This study
KW239XC5, KW239XC11, KW239XC13, KW1625H, KW1916H, KW2803H, KW3815H, KW3899C, KW3899G, KW5949B, KW5949F	Rif^r Kan^r, marker exchange mutants	This study
Plasmids		
R91-5	Crb^r, IncP10 plasmid, derepressed for transfer	6
pBluescript II KS+	Amp^r, cloning vector for producing single-stranded DNA	Stratagene
pCUV8	Tet^r, pLAFR1 clone containing *P. syringae recA* gene	16
pEMH9	Tet^r, Δ(*recA*)*51*::Ω in pLAFR3	20
pHIR11-18	Tn*phoA* mutant of pHIR11, a pLAFR3 clone with a 31-kb *P. syringae* pv. syringae 61 genomic DNA insert, containing *hrp* genes	23
pKMB4	Tet^r, pRK7813-based cosmid containing genomic DNA from *P. syringae* pv. tomato DC3000	3
pKW331, pKW352	Tet^r, pLAFR3 clones containing the *lemA* gene from B728a	59
pLAFR3	Tet^r, cosmid vector	50
pLAFR6	Tet^r, cosmid vector with *trp* transcriptional terminators and pUC18 polylinker	B. Staskawicz
pMO75	Kan^r Crb^r Tn*5* mutant of R91-5	56
pME3008	Tet^r, contains *gacA* and *uvrC* genes from *P. fluorescens* CHA0 in a 19-kb *Hin*dIII fragment	30
pRK415	Tet^r, cosmid vector	26
pRK7813	Tet^r, cosmid vector	25
pRK2013	Kan^r, mobilizing plasmid	11
p5	12-kb *Eco*RI fragment containing *syrA* gene from *P. syringae* pv. syringae B301D, cloned into pBR325	N. Quigley and D. Gross
pYM101	16-kb *Hin*dIII fragment containing *syrBCD* region from *P. syringae* pv. syringae B301D, cloned into pUC19	42
pJJR628-Tn*5*	Tet^r Kan^r, ~13-kb *Eco*RI Tn*5*-containing fragment from KW628, cloned into pLAFR3	This study
pJJR3621-Tn*5*	Tet^r Kan^r, ~15-kb Tn*5*-containing *Eco*RI fragment from KW3621, cloned into pRK415	This study
pJJR21-1, -2, -3, -6, -1′ to -8′, -10′	Tet^r, cosmid clones containing B728a genomic DNA overlapping Tn*5* insertion site of mutant KW21	48; this study
pJJR628-1, -2, -3, -5, -9, -10, -15, -2′, -3′, -6′	Tet^r, cosmid clones containing B728a genomic DNA overlapping mutant KW628 Tn*5* insertion site	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant characteristics[a]	Source or reference
pJJR3621-1, -3, -14, -1′ to -13′	Tet^r, cosmid clones containing B728a genomic DNA overlapping mutant KW3621 Tn*5* insertion site	This study
pJJR2, pJJR3, pJJR6	Tet^r, cosmid clones containing B728a genomic DNA overlapping *lem-239*::Tn*5* in pLAFR3	This study
pJJR1625-Tn*5*H, pJJR1916-Tn*5*H, pJJR2803H, pJJR3815-Tn*5*H, pJJR3899-Tn*5*C and -G, pJJR5949-Tn*5*B and -F	Tet^r Kan^r, pRK7813 containing *Sau*3A1 partial Tn*5*-containing fragments from specified mutants	This study
pJJRTn*5*5, pJJRTn*5*11, pJJRTn*5*13	Tet^r Kan^r, pRK7813 containing *Sau*3A1 partial Tn*5*-containing fragments from mutant KW239	This study
pJJRH	Tet^r, deletion derivatives of pJJR3	This study
pJJRBE, pJJRBam, pJJRΔSH	Tet^r, deletion derivatives of pJJRH	This study
pJJR-AH, -EA, -E13, -Sa, -Sb, -A22, -A36p, -E21a, -E21b, -A12	Tet^r, DNA fragments of pJJR3 in pRK415	This study
pJJRBamΩ	Tet^r Spc^r, designated clone with the Ω fragment blunt-end ligated into the single *Sac*I site	This study
pJJRBAM-3, -13, -35, -37, -40	Tet^r Kan^r, pJJRBam containing specified Tn*lacZ* insertion	This study
pJJRBE95, pJJRBE119	Tet^r Kan^r, pJJRBE containing specified Tn*lacZ* insertion	This study
pJJRB36a, pJJRB36b	Tet^r, 3.6-kb *Bam*HI fragment from pJJRBAM3 in pLAFR6, both orientations	This study
pJJRKS10, pJJRKS12	Amp^r, 3.6-kb *Bam*HI fragment from pJJRBAM3 in pBluescript II KS+, both orientations	This study
pJJRA1BTn*5*, pJJRA2BTn*5*	*Bam*HI fragments spanning each Tn*5*-239 junction in pBluescript II KS+	This study
pJJR-Δ40B, -ΔScB, -Δ37B, -ΔS37, -Δ119S, -ΔX119, -ΔX37, -Δ13X, -Δ13S	Tet^r, deletion derivatives of pJJRBam or Tn*lacZ*-containing clones	This study

[a] Abbreviations not defined in the text: Rif, rifampin; Tet, tetracycline; Spc, spectinomycin; Amp, ampicillin.

and an equal volume of a stationary culture of B728a were washed together and spotted onto a King's medium B (KB medium) (27) agar plate and incubated at 28°C for 1 h. Dilutions were plated onto KB medium containing kanamycin (20 μg/ml) and rifampin (200 μg/ml). Single colonies were picked and patched in a grid (24 or 48 colonies/plate) and grown at 28°C. The colonies were checked for resistance to carbenicillin (500 μg/ml), a marker for pMO75, and nalidixic acid (250 μg/ml), a chromosomal marker for PAO11; carbenicillin-resistant (Crb^r) (ca. 50%) or nalidixic acid-resistant (Nal^r) (0.34%) colonies were discarded. The overall frequency of auxotrophs obtained was 0.52% (0.78% of Crb^s Nal^s colonies); this was determined by transferring colonies onto Turner's minimal agar (55; with 0.4% glycerol substituting for glucose). Auxotrophic mutants were excluded from further analysis.

Screening for lesion-deficient mutants. To screen for mutants altered in lesion-forming ability, single colonies were stabbed laterally under the epidermis of detached bean pods, using sterile toothpicks (up to 12 single colonies per bean pod). The pods were surface sterilized in 10% bleach for 10 to 20 s, thoroughly rinsed with deionized water, and air dried prior to inoculation. The bean pods were stored individually in sterile plastic bags (Nasco Whirl-Pak) for 36 to 48 h at 24°C before scoring. Each mutant showing reduced lesion-forming ability compared to the wild type was restreaked and confirmed to be a Crb^s Nal^s prototroph, and a single colony was isolated for use in all further experiments.

Plant assays. *P. syringae* pv. syringae strains were assayed for lesion-forming ability on bean (*Phaseolus vulgaris* L. cv. Bush Blue Lake 274; Rogers Bros. NK Seeds, Twin Falls, Idaho) by leaf infiltration and injection of detached pods as previously described (58, 59). For spray inoculations, cell suspensions of ca. 10^6 CFU/ml were misted onto 16- to 20-day-old bean plants, and the plants were incubated for 6 to 7 days in a humid chamber (24°C with a 14-h photoperiod) before recording symptoms. The HR was assayed on the nonhost tobacco (*Nicotiana tabacum* cv. Havana 142) as described previously (59). As expected, the necrosis observed within 24 h after inoculation with a high-cell-density solution (10^8 CFU/ml) intensified on the second and third days but did not spread beyond the boundaries of the infiltrated tissue.

Microbial plate bioassays and isolation of mutant BSR. Protease activity was detected as a zone of clearing surrounding a colony grown at 28°C on NYGA agar (53) supplemented with sterile skim milk to 10% (vol/vol). To assay for syringomycin production, colonies were grown at 28°C on SRM-modified (SRM-M) medium (15) for 4 days before spraying or overlaying the plates with a suspension of the sensitive yeast *Rhodotorula pilimanae* ATCC 26423 (64). SRM-M melted top agar (0.9%) was used in the overlay assay. Strain BSR, a spontaneous toxin-deficient derivative of B728a, was isolated from a bioassay plate in which the positive control B728a appeared syringomycin negative. Where noted in the text, the toxin assay medium was supplemented with D-fructose (0.1%, wt/vol) and arbutin (100 μM) from aqueous, filter-sterilized stock solutions. The analysis of syringomycin activity in cell lysates was done as previously described (21).

Determination of bacterial population densities in liquid culture and in association with plants. Bacterial population sizes attained in association with bean plants were assessed by two methods. For measuring total bacterial populations associated with bean leaves, ca. 3-week-old plants were sprayed with a bacterial suspension adjusted to 10^6 CFU/ml and incubated in a mist chamber (24°C, 14-h day). At each time point, 10 trifoliolate leaflets were detached, homogenized individually, and plated as previously described (19). Numbers of bacteria in the intercellular spaces of bean leaves were measured after primary leaf infiltration (5, 43). The growth rate in KB medium (27) was determined by monitoring absorbance at 600 nm and by plating serial dilutions of bacteria.

DNA manipulations. General recombinant DNA techniques were performed as described elsewhere (34). Genomic DNA was prepared as described by Kinscherf et al. (28). Tn*5* target fragments from the mutant genomes were observed by Southern analysis of chromosomal DNAs digested separately with *Eco*RI, *Eco*RV, and *Asp*718, probing with Tn*5* sequences from λ::Tn*5* (λ *c*I857 *rex*::Tn*5*; from D. E. Berg) or pRZ705 (pBR322::Tn*5*; from M. P. Krebs and W. Reznikoff). Sizes were approximated by comparing to size markers (Bethesda Research Laboratories [BRL] 1-kb ladder, λ DNA digested with *Apa*I or *Sal*I) electrophoresed in adjacent lanes. DNA fragments were purified as described previously (48). DNA concentrations were determined fluorometrically (Hoefer TKO 100 Dedicated Mini Fluorometer). Probe DNAs were labeled with ^{32}P by priming with random hexamers (Pharmacia) as described previously (12). The isolation of DNA probes containing sequences flanking Tn*5* insertions by using PCR, including conditions for DNA template preparation, was done as published elsewhere (48). Construction of the B728a genomic library in pLAFR3 was detailed previously (4, 28).

Plasmid constructions. The restoring cosmid pJJR3 was isolated from a B728a genomic library constructed in vector pLAFR3 by colony hybridization using a PCR-generated probe containing chromosomal DNA sequences flanking the *lem-239*::Tn*5* insertion (48). Restoring subclone pJJRH was derived from pJJR3 by digestion with *Hin*dIII and self-ligation of the vector-containing fragment. Restoring plasmids pJJRBE and pJJRBam were constructed from pJJRH by digestion with *Hin*dIII and *Bam*HI followed by ligation of the vector-containing fragment. For pJJRBE, the ends of the 4.5-kb *Hin*dIII/*Bam*HI fragment were made flush with Klenow prior to ligation. For pJJRBam, *Bam*HI linkers (phosphorylated 8-mers; P-L Biochemicals, Milwaukee, Wis.; generously provided by A. Budde) were ligated to the ends of the Klenow enzyme-treated fragment, followed by digestion with *Bam*HI and recircularization of the plasmid. Plasmid pJJRBamΩ was constructed by restriction of the parent plasmids at the unique *Sac*I site with end filling followed by ligation to the Ω fragment (Amersham Life Sciences). pJJRA36p was constructed by partial digestion of pJJR3 with *Asp*718,

followed by religation. The deletion subclones pJJR36a and pJJR36b were obtained by digestion of pJJRBAM3 with *Bam*HI, isolation of the 3.6-kb fragment, and ligation of the fragment into pLAFR6 in both orientations (a and b). For construction of clones pJJR628-Tn*5* and pJJR3621-Tn*5*, mutant genomic *Eco*RI fragments of the appropriate size ranges (ca. 13 and 15 kb, respectively) were isolated and ligated with *Eco*RI-digested, dephosphorylated vector DNA. Tn*5*-containing transformants were selected with kanamycin. A *P. syringae* pv. tomato DC3000 genomic library was constructed in pLAFR3 and screened with the insert from clone pJJRH to obtain pKMB4 (45).

Plasmids pJJRBam and pJJRBE were mutagenized by transposition of the Tn*5* derivative Tn*lacZ* from the chromosome of *E. coli* CC170 (35). CC170 was transformed with the appropriate plasmid, and cells from a single colony were spread onto Luria-Bertani (LB) plates (34) containing tetracycline (15 μg/ml) and kanamycin (300 μg/ml). After incubation for 2 days at 37°C, larger colonies were observed above a background lawn of much smaller colonies. The larger colonies were pooled, and plasmid DNA prepared from these cells was used to transform *E. coli* DH5α, selecting on LB containing tetracycline (15 μg/ml) and kanamycin (50 μg/ml). The resulting Tn*lacZ* insertions were mapped by restriction endonuclease digestion and by sequencing of the junction between the transposon and the *P. syringae* pv. syringae DNA.

Construction of a *recA* deletion derivative of strain 239. The Δ(*recA*)*51*::Ω mutation (conferring resistance to spectinomycin) was introduced into mutant KW239 by recombinational mutagenesis with plasmid pEMH9 as described previously (20) except that pEMH9 was introduced into KW239 via electroporation. Marker exchange strains were identified by Southern blot analysis and UV light sensitivity tests (36), using *P. syringae* pv. syringae *recA* strains BUVS1 and NUVS1 as controls. Exchange strains were checked for lesion formation, toxin and protease production, and HR. Representative *recA* deletion strain KW239UVS1 was selected for further experiments. Resistance to UV light exposure was restored to this strain by the *recA*-containing plasmid pCUV8.

DNA sequencing and analysis. Double-stranded and single-stranded DNAs were sequenced by the dideoxy-chain termination method using 7-deaza nucleotides and [α-^{35}S]dATP with either T7 DNA polymerase (Pharmacia) or Sequenase (U.S. Biochemical). Double-stranded templates were subclones of pJJR3 carried in the vector pRK415, pLAFR3, or pBluescript II KS+ (Stratagene). The sequences of the Tn*5*-*P. syringae* pv. syringae junctions were determined from subclones pJJRA1BTn*5* and pJJRA2BTn*5*, using a Tn*5*-specific primer (bases 77 to 61). Plasmid DNAs for sequencing were prepared according to a standard alkaline lysis miniprep procedure with or without purification on cesium chloride-ethidium bromide equilibrium gradients (34). Single-stranded DNAs of pBluescript II KS+-based subclones pJJRKS10 and pJJRKS12 were isolated from *E. coli* DH5αF′ as described by the manufacturer (Stratagene). Commercial sequencing primers and 17-mers complementary to the sequence generated or to Tn*5* (synthesized by the University of Wisconsin—Madison Biotechnology Center) were used. Sequencing reactions were resolved by electrophoresis on 6% acrylamide–50% urea gels in 0.5× Tris-borate-EDTA buffer or 1× TTE buffer (34). The gels were dried directly onto paper (Whatman 3MM) without fixing. The DNA sequence was analyzed with software from the Genetics Computer Group, Inc., Madison, Wis. (10), and from The DNA Inspector (Textco, Inc., West Lebanon, N.H.).

Nucleotide sequence accession number. The sequence reported has been submitted to the GenBank database and assigned accession number U85643.

RESULTS

Isolation of *P. syringae* pv. syringae mutants that did not form disease lesions on bean. B728a was mutagenized by mating with pMO75, a Tn*5*-containing derivative of plasmid R91-5 from *P. aeruginosa*. A total of 6,401 prototrophic Tn*5* mutants obtained from 25 separate matings were screened on bean pods for altered lesion-forming ability. The defects in lesion formation were confirmed by leaf infiltration and pod injection assays. A spectrum of phenotypes was observed, but only mutants that did not produce a reaction at an inoculum level of 10^6 CFU/ml in these assays were considered lesion manifestation negative (Lem$^-$). Variances among the Lem$^-$ strains were observed in the pod injection assay at 10^8 and 10^7 CFU/ml, but at 10^6 CFU/ml, there is a clear difference between nonpathogenic strains (no reaction) and brown spot pathogens (full reaction at this and lower cell concentrations). Twenty-six Lem$^-$ mutants were isolated (0.41%). Three strains were further characterized by spray inoculation onto bean plants followed by incubation of the plants in a mist chamber. After spray inoculating the wild-type B728a, small necrotic lesions surrounded by an area of slight chlorosis appeared, accompanied by puckering of the surrounding leaf tissue; these symptoms matched those caused by *P. syringae* pv. syringae in nature (17). When mutants KW21, KW239, and KW626 were assayed by spray inoculation, no brown spot symptoms were observed (data not shown).

As a primary verification of lesion deficient strains, chromosomal DNA isolated from each Tn*5* mutant was digested separately with one or more of the enzymes *Eco*RI, *Eco*RV, and *Asp*718. After agarose gel electrophoresis, the chromosomal DNA digestion patterns were compared to that of B728a. In all cases, the digested mutant DNAs matched the B728a profile, ruling out any possibility of contamination of the mutant collection with a different *P. syringae* strain not pathogenic on bean. None of the mutant DNAs contained sequences that hybridized with R91-5, the parent plasmid of pMO75. This finding confirmed that the suicide vector had not integrated into the chromosome, a phenomenon observed in the course of other Tn*5* mutageneses (59). In this and subsequent hybridizations, the *lemA* mutant NPS3136 and 17 Lem$^+$ Tn*5* strains generated in this project were included as controls, comprising 44 Tn*5* mutants altogether (data not shown). Each mutant contained a single Tn*5* insertion, as indicated by Southern blots of restricted mutant genomic DNAs probed with Tn*5* sequences. From the varied sizes of the genomic target fragments, it appeared that Tn*5* inserted randomly into the B728a chromosome.

Southern analysis confirmed that within the examined strains, there were no Tn*5* insertions into the *lemA* gene. The mutant chromosomal DNAs were probed with two cosmid clones (pKW331 and pKW352) that together encompass the *lemA* gene and ca. 40 kb of flanking chromosomal DNA. All bands hybridizing to the wild-type B728a genomic DNA were present and apparently intact within each of the mutant genomes. Thus, no linkage was detected between these mutations and the *lemA* gene. Similarly, no linkage to or disruptions or rearrangements of the *P. syringae* pv. syringae B728a *gacA* gene were revealed by probing the collection of mutant DNAs with the *gacA* gene from *P. fluorescens* CHA0, carried on plasmid pME3008 (Table 1). Nucleotide identity between the CHA0 and B728a *gacA* homologs is 80% (47).

Genetic separation of lesion formation from production of HR, toxin, and protease. The 26 Lem$^-$ strains were infiltrated into tobacco leaves, and 17 of the mutants retained the ability to induce the HR. Since the Lem$^-$ HR$^+$ phenotype is one of the hallmarks of the *lemA* mutant, these 17 strains were tested for the other phenotypes known to be controlled by *lemA*, syringomycin and protease production (Table 2). All strains except KW3621 showed zones of clearing in the protease assay (Table 2). Ten strains did not produce syringomycin (i.e., were (Syr$^-$) on SRM-M medium. Reduced zones of clearing in the skim milk assay or inhibition in the toxin bioassay were observed in some strains, including KW21 (Table 2). In this study, such mutants were still considered syringomycin or protease positive, due to the nonquantitative nature of these assays. Seven Lem$^-$ strains retained the ability to produce both syringomycin and protease. These characterizations suggested that the majority of the new mutants were not disrupted at the *lemA* or *gacA* locus. Furthermore, these data indicated that a loss of lesion formation could occur independently of syringomycin or protease production as measured in bioassays.

All of the Lem$^-$ strains were assayed for toxin production on SRM-M medium supplemented with fructose and arbutin, compounds that stimulate syringomycin production in some strains (38). The production of syringomycin by the parent B728a did not appear to be increased by fructose and arbutin in this nonquantitative plate assay. The *lemA* mutant NPS3136, and all but two of the presently characterized Syr$^-$ strains, did

TABLE 2. Phenotypes of wild-type and lesion-deficient strains

Strain	Lesion formation	HR	Syringomycin production	Protease production	Region[e]	
					pYM101	*lem-5949*::Tn*5*
B728a (wild type)	+	+	+	+	I	I
NPS3136(*lemA*)[a,c]	−	+	−	−	I	I
KW3621[a]	−	+	−	−	I	I
KW21[b]	−	+	+	+	I	I
KW626,[c] KW1625,[c,f,g] KW1916,[c] KW3815,[c,f] KW3899, KW3777	−	+	+	+	I	I
KW239,[c] KW2803,[c] KW5949,[c] KW2163	−	+	−	+	I	I
KW1166[d]	+	+	−	+	Δ	I
KW35,[d] KW1152[d]	+	+	−	+	Δ	Δ
KW628, KW1163, KW1177, KW5279, KW6254	−	+	−	+	Δ	Δ
BSR	−	+	−	+	Δ	Δ

[a] Restored by *lemA*.
[b] Restored by *gacA*. Mutant KW21 exhibited reduced zone sizes in toxin and protease assays.
[c] Tn*5* found to be causal to the mutant phenotype.
[d] Consistently scored intermediate in pathogenicity between B728a and NPS3136 on bean pods.
[e] Δ, region is deleted; I, region is intact.
[f] Indicates mucoid colony morphology on KB medium.
[g] Exhibited reduced zone size in the protease assay.

not produce syringomycin activity in the presence of these compounds. Mutants KW239 and KW21, by contrast, gave increased (comparable to the wild type) zones of inhibition on SRM-M amended with fructose and arbutin (data not shown).

Identification of nine independent insertions into the *P. syringae* pv. syringae *hrp* region. Nine of the 26 Lem$^-$ mutants failed to induce the HR on the nonhost tobacco (0.14% of the total of 6,401). Southern blots indicated that all of the mutants generated in this study contained sequences that hybridized to pHIR11-18, a plasmid containing the *hrp* cluster of genes from *P. syringae* pv. syringae 61 (23). While the hybridizing bands within the Lem$^-$ HR$^+$ mutant DNAs were the same sizes as within B728a, each of the nine HR$^-$ mutants appeared to contain a Tn*5* insertion within one of the *hrp*-specific fragments (Fig. 1). All of the *hrp* insertion mutants produced syringomycin and protease, demonstrating that *lemA*- and *gacA*-regulated phenotypes are expressed in a *hrp* mutant background in *P. syringae* pv. syringae.

Detection of a high-frequency deletion event. Because a large number of the Lem$^-$ mutants did not produce syringomycin, and because concurrent loss of these phenotypes has been observed within other *P. syringae* pv. syringae strains, it was important to determine whether any known loci had been disrupted in the new mutants. The *syrA*, *syrB*, *syrC*, and *syrD* genes, essential for syringomycin production in *P. syringae* pv. syringae B301D, were used as hybridization probes of all the mutant DNAs.

The *syrA* gene, contained on plasmid p5, is required for pathogenicity on cherry fruits as well as syringomycin production (62, 63). Southern blot analysis showed that none of the Tn*5* insertions analyzed were located within or near the *syrA* gene. The 16-kb *Hin*dIII fragment of plasmid pYM101 contains the linked *syrB*, *syrC*, and *syrD* genes required for syringomycin biosynthesis in *P. syringae* pv. syringae B301D. Mutants bearing disruptions of these genes do not produce toxin but show attenuated disease symptoms and have been designated path$^+$ (37, 63). After probing with pYM101, three major hybridizing bands were observed in the wild-type B728a and the majority of the mutant chromosomes (Fig. 2). Surprisingly, these bands were absent from the genome of the non-Tn*5* containing Syr$^-$ mutant BSR and from the genomes of eight Tn*5* mutants, including five Lem$^-$ strains and three strains that were attenuated in the ability to form lesions on bean pods (Table 2; Fig. 2).

Prior to discovery of the *syr* gene deletion event, the Tn*5* mutation was cloned from mutant KW628, generating pJJR628-Tn*5*. A set of 10 overlapping cosmids hybridizing to pJJR628-Tn*5* were isolated from a partial *Sau*3A1 wild-type genomic library (plasmid series beginning with pJJR628-1 in Table 1). Attempts to restore mutant KW628 with these clones were unsuccessful, suggesting that the Lem$^-$ and Syr$^-$ phenotypes of this strain were unlinked to the Tn*5* insertion. The *syr* region deletion, as opposed to the Tn*5* mutation, was likely responsible for the phenotype of mutant KW628 and the other deleted strains.

Mutant restorations mediated by the *lemA* and *gacA* genes. Mutant KW3621 was the only mutant found with the same array of defects as a *lemA* mutant (loss of lesion formation,

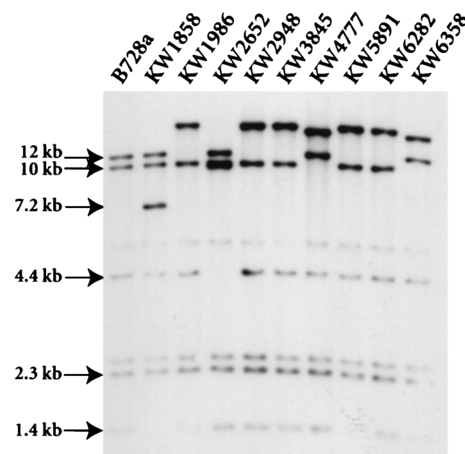

FIG. 1. Southern blot autoradiograph of *P. syringae* pv. syringae chromosomal DNAs digested with *Eco*RI and probed with pHIR11-18, a plasmid containing *P. syringae* pv. syringae *hrp* genes. The wild-type B728a and the nine HR$^-$, lesion-deficient Tn*5* mutants are shown. All of these strains are unimpaired in the production of syringomycin and protease. Approximate sizes labeled were determined by comparison to size markers (BRL 1-kb ladder) electrophoresed in an adjacent well (not shown).

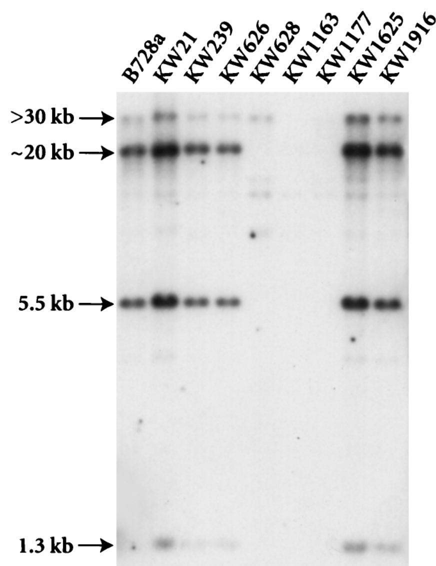

FIG. 2. Southern blot of *P. syringae* pv. syringae chromosomal DNAs digested with *Eco*RI and probed with pYM101 containing the *syrBCD* genes of *P. syringae* pv. syringae B301D. The 1.3-kb band was more clearly visible in longer exposures, but bands were not observed in the KW628, KW1163, and KW1177 lanes even after prolonged exposure times. The sizes of the labeled bands were estimated by comparison to a BRL 1-kb ladder run in an adjacent lane of the gel (not shown).

syringomycin, and protease). Considering the possibility that this strain contains a disruption of a response regulator gene corresponding to *lemA*, the Tn*5* mutation was isolated from mutant KW3621 (in plasmid pJJR3621-Tn*5*) and used to obtain hybridizing clones from a genomic library of wild-type B728a DNA. Restoration of the protease defect was not achieved with 16 independent clones containing DNA sequences flanking the KW3621 Tn*5* insertion (plasmid series beginning with pJJR3621-1 in Table 1). As described above, hybridization with *lemA*-containing clones indicated the Tn*5* mutation within KW3621 was not linked to the *lemA1*::Tn*5* insertion. However, introduction of the *lemA* gene carried on pKW352 restored protease activity to mutant KW3621. By contrast, pKW352 was unable to complement mutants KW21, KW239, KW626, and KW628. Thus, it seemed likely that the *lemA* gene within KW3621 contained a mutation undetectable by Southern blot analysis.

The Lem$^-$ mutant KW21 showed reduced production in both protease and syringomycin assays. Sequences surrounding the Tn*5* insertion of mutant KW21 were isolated by a PCR-based method (48). A total of 13 overlapping clones isolated from a wild-type genomic library based on hybridization to the PCR fragment (plasmid series beginning with pJJR21-1 in Table 1) failed to restore mutant KW21. However, lesion-forming and protease activities were restored to KW21 by the *gacA* gene carried on plasmid pME3008. It was probable, therefore, that a mutation of the *gacA* gene, not detectable by Southern blot hybridization, was responsible for the mutant phenotype of KW21.

Confirmation of causality between the Tn*5* insertions and the Lem$^-$ phenotypes. The exclusion of KW3621, KW21, and the five *syr* deletion strains described above left 10 Lem$^-$ HR$^+$ mutants as candidates for further analysis. Before additional work could be undertaken on these mutants, it was necessary to establish linkage between the Tn*5* insertions and observed defects in lesion-forming ability. Marker exchange mutagenesis was performed to recreate the Tn*5* mutations in B728a, for a fresh assessment of phenotypic alteration.

To obtain clones suitable for introduction of the Tn*5* into the wild-type B728a, a partial *Sau*3A1 genomic library was constructed for each Lem$^-$ HR$^+$ mutant in the vector pRK7813. After packaging and transduction, eight kanamycin-resistant (Kan^r) colonies were isolated from each library. Restriction digestion (*Eco*RI and *Eco*RI-*Hin*dIII) and agarose gel electrophoresis revealed that the eight cosmids from each library contained many common fragments (data not shown). One or more cosmids (Table 1) from each library were introduced by electroporation into B728a for transplacement of the Tn*5*. Analysis by transplacement mutagenesis was completed for eight strains. Marker exchange strains were compared to the original mutants in assays for HR, lesion formation, syringomycin, and protease. As summarized in Table 2, this analysis confirmed that the Tn*5* insertions in strains KW239, KW626, KW1625, KW1916, KW2803, KW3815, and KW5949 were causal to their respective mutant phenotypes. Exchange mutants containing the Tn*5* insertion from mutant KW3899 did not support the causal nature of this insertion. We were not able to successfully exchange the respective Tn*5* insertions from mutant KW2163 or KW3777 into the chromosome of B728a. Thus, in seven of the Lem$^-$ mutants, the Tn*5* insertion was established as responsible for the loss of lesion formation.

Linkage relationships between Lem$^-$ HR$^+$ mutants. The entire collection of mutants was probed separately with cosmids containing genomic DNA flanking several mutations that were determined by marker exchange to cause the loss of lesion phenotype (using probes pJJR3, pJJR6, pJJR1625-Tn*5*H, pJJR1916-Tn*5*H, and pJJR3815-Tn*5*H). The majority of the mutants were also probed with DNA containing the Tn*5* target regions of mutants KW21 (PCR-amplified fragment [48]) and KW628 (pJJR628-2′ and pJJR628-3′); linkage between mutants KW628 and KW3815 was detected by using pJJR628-2′ as a probe. The mutations within KW239, KW1625, KW1916, and KW3815 were not located close to any other Tn*5* insertion examined (data not shown). Therefore, these mutations identified four unlinked loci required for lesion formation on bean.

Strikingly different observations were made with probe pJJR5949-Tn*5*F, containing the Tn*5* insertion cloned from the Lem$^-$ Syr$^-$ mutant KW5949. Southern hybridization of the mutant collection revealed extensive deletions within the *lem-5949*::Tn*5* region in eight Syr$^-$ mutants (Table 2). The deletion events were not site specific; several different patterns of missing *Eco*RI fragments were observed in the mutant genomes, from a total of at least nine fragments of the wild-type B728a that hybridized to the probe (data not shown). The same eight mutant genomes also had the pYM101 region deleted as discussed above. By contrast, mutant KW1166, containing a pYM101 deletion, did not have the pJJR5949-Tn*5*F hybridizing region deleted. Two mutants (KW327 and KW329 in reference 21) that contain Tn*5* insertions into the pYM101 region and retain pathogenicity also had the intact *lem-5949*::Tn*5* region. Plasmids pYM101 and pJJR5949-Tn*5*F defined unstable chromosomal regions that may be in close proximity or overlapping. In addition, the Tn*5* insertions within two mutants with reduced pathogenicity on bean, KW256 and KW1156, were found to be linked to the *lem-5949*::Tn*5* mutation. Strains KW256, KW1156, and KW5949 were isolated from separate mutagenesis matings.

Characterization and restoration of *P. syringae* pv. syringae mutant KW239. To build upon the mutant studies described above, we undertook a more detailed characterization of the Lem$^-$ Syr$^-$ mutant KW239. We measured the population den-

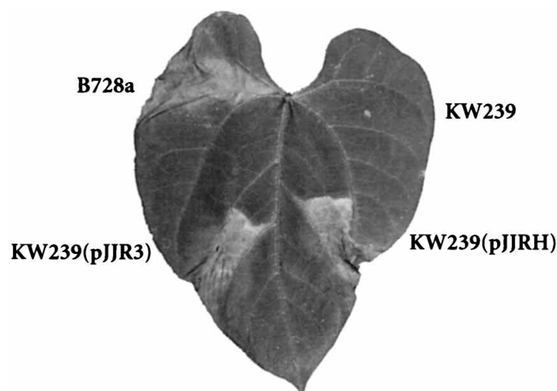

FIG. 3. Bean primary leaf infiltrated with bacterial suspensions at 10^6 CFU/ml of B728a, mutant KW239, and mutant KW239 containing the cosmid clone pJJR3 or the subclone pJJRH. The leaf was inoculated by using a plastic disposable pipette (Sarstedt), and the plant was incubated for 5 days in a 24°C growth chamber with a 14-h photoperiod before detaching and photographing the leaf.

sities achieved by KW239 and the wild-type B728a in association with bean leaves. Plant-associated bacteria were measured after both leaf surface inoculation and infiltration into the intercellular spaces of leaf tissue. Total and intercellular population versus time curves indicated that mutant KW239 attained population levels over time similar to those for B728a. Mutant KW239 grew equivalently to the wild-type strain in liquid KB medium (data not shown).

To assess whether the syringomycin defect observed with mutant KW239 might be due to a secretion failure, KW239 and B728a cell-free lysates and sterile concentrated medium in which the strains had been grown were tested in syringomycin bioassays. Syringomycin activity was detected in both the lysate and the growth medium of B728a, while no toxin activity was observed in either sample of mutant KW239.

A 2.3-kb hybridization probe containing sequences flanking the Tn*5* within KW239 was isolated by using PCR (48). In a screen of 1,200 colonies from a wild-type genomic library, the radiolabeled 2.3-kb probe hybridized to three cosmid clones (data not shown). These clones, pJJR2, pJJR3, and pJJR6, with insert sizes of ca. 25 kb, restored lesion formation and syringomycin production when conjugated into KW239 (Fig. 3). Restoration of a recreated mutant, the marker exchange strain KW239XC5, was also accomplished, thoroughly establishing the causative relationship between the Tn*5* insertion and mutant phenotypes.

Analysis of *P. syringae* pv. syringae subclones and Tn*lacZ* mutants in a *recA* background. A *recA* deletion derivative of mutant KW239 was constructed to facilitate complementation analysis. The *recA* strain KW239UVS1 was sensitive to UV light but otherwise retained the same phenotypes as mutant KW239. KW239UVS1 was restored by pJJR3 and was the recipient in all further restoration experiments. This restoration, therefore, likely occurred in *trans* and was not the result of recombinational rescue.

Restriction mapping and subcloning of pJJR3 were undertaken to identify the boundaries of the region required for restoration. The subclones pJJRH (insert of 9.8 kb) and pJJRBE (insert of 4.5 kb) restored lesion formation and toxin activity to both KW239 and KW239UVS1 (Fig. 3 and data not shown). To further delineate the DNA region required for restoration, subclones pJJRBE and pJJRBam (containing the same insert DNA as pJJRBE with a *Bam*HI site introduced into one end) were mutagenized with the transposon Tn*5* derivative Tn*lacZ* (Fig. 4). None of the Tn*lacZ* clones produced significant β-galactosidase activity in *E. coli* DH5α. However, when introduced into KW239UVS1, two insertions (BAM35 and BE95) with the *'lacZ* gene oriented in the same direction were blue on media containing X-Gal. None of the Tn*lacZ* insertions blocked the ability of the subclone to restore mutant KW239UVS1 in plant or syringomycin assays. Similarly, clone pJJRBamΩ, disrupted in the unique *Sac*I site with the Ω fragment (Fig. 4), also restored KW239UVS1 in both toxin and plant assays.

The Tn*lacZ* mutation within the restoring clone pJJR BAM37 was located near the original Tn*5* insertion responsible for the mutant KW239 phenotypes (27 bp away as determined by sequencing [see below]). This finding raised the question of whether cosmid clones containing *lem-239*::Tn*5* could restore KW239UVS1 in *trans*. To address this, the Tn*5*-containing clone pJJRTn*5*11 isolated from a partial *Sau*3A1 genomic library of mutant KW239 was introduced into the *recA* derivative. This clone did indeed restore lesion formation and toxin production to KW239UVS1. The transconjugants assayed were confirmed to contain the intact Tn*5*-containing clones by Southern blot (data not shown).

The restoring activity was further localized to a 3.6-kb *Bam*HI fragment that was isolated from the Tn*lacZ*-containing clone pJJRBAM3. The fragment (containing 59 bp of Tn*lacZ*) was cloned in both orientations into the vector pLAFR6, yielding pJJRB36a and pJJRB36b (Fig. 4). Restoration of KW239UVS1 was achieved with the 3.6-kb fragment in both orientations, suggesting that the insert DNA contained an active promoter(s).

DNA sequence analysis of the region restoring mutant KW239. The DNA sequence of the 3.6-kb region that restored KW239UVS1 was determined. The junctions between the KW239 Tn*5* insertion, various Tn*5-lacZ* insertions, and the *P. syringae* pv. syringae genomic DNA were also sequenced. A BLASTX (1) search of protein and nucleotide sequence databases at the National Center for Biotechnology Information was conducted with the 3.6-kb DNA sequence including all predicted open reading frames (ORFs). The search revealed that this section of the *P. syringae* pv. syringae chromosome bore strong similarities to a unique and unusual assortment of nucleotide and protein sequences from *E. coli* and other bacteria. Included in this group are the product of the regulatory gene *mrsA* (61% identity; GenBank accession number U01376); the triose phosphate isomerase protein (54% identity; accession number P04790); the *leuU* gene of *E. coli* specifying a leucine tRNA (96% identity; accession number X52801); and the *metY* gene encoding an initiator tRNA gene (92% identity) with the 15-kDa protein (50% identity) from the beginning of the *nusA* operon (accession number X00513). As depicted in Fig. 5, none of these genes or ORFs contained the site of the original Tn*5* insertion. One ORF with similarity to the *E. coli* SecG protein (52% identity; accession number P33582) spans the site of the *lem-239*::Tn*5* insertion (Fig. 5). However, both the BAM37 and *lem-239*::Tn*5* insertions are located within this ORF, and plasmids containing these mutations restore the *lem-239*::Tn*5* mutation in *trans*. In addition, pJJRA36p contains the entire *secG* ORF and does not restore the *lem-239*::Tn*5* mutation (Fig. 4).

Targeted deletions of a cosmid clone restoring mutant KW239. Due to the complex nature of the chromosomal region surrounding the *lem-239*::Tn*5* mutation, a comprehensive deletion analysis of the region required for restoration of mutant KW239UVS1 was undertaken (Fig. 5). Several of these deletions originated from the *Bam*HI site of pJJRBam. Other deletion subclones were

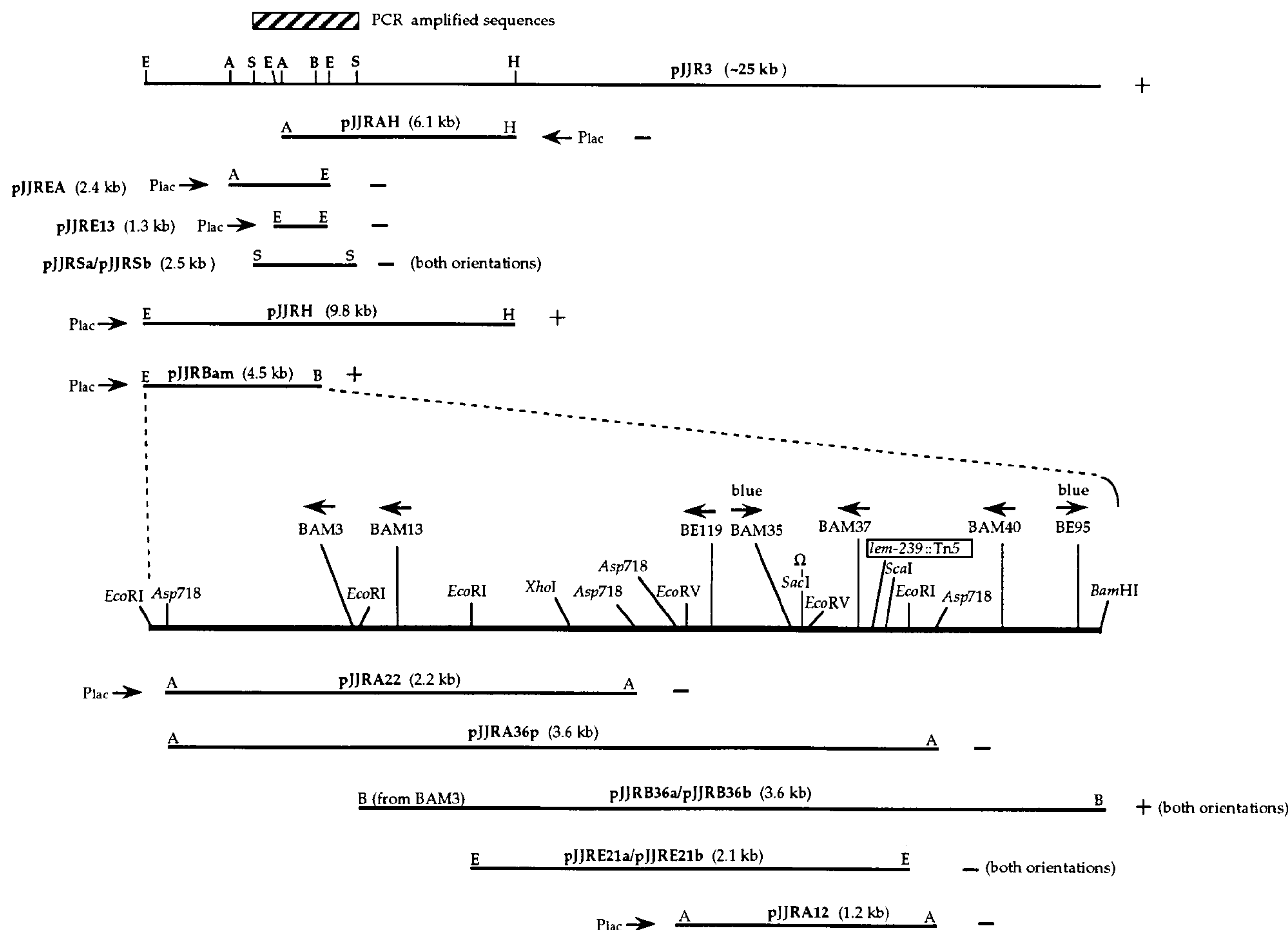

FIG. 4. Restriction map, subcloning, insertional mutagenesis, restoration information, and ORFs detected following DNA sequence analysis of region restoring mutant KW239. The locations of sequences amplified by PCR from mutant KW239 after *Eco*RV digestion and religation are indicated above pJJR3. The name of each clone is given, and size of the insert is noted in parentheses. The orientation of the insert with respect to the *lacZ* promoter is shown for most of the clones. A, *Asp*718; H, *Hin*dIII; E, *Eco*RI; S, *Sac*I; B, *Bam*HI. A + at the right indicates that the clone restored lesion formation and toxin production to mutant KW239UVS1 (see below), while a − indicates lack of restoration. The *lem-239*::Tn*5* mutation, Tn*lacZ* insertions BAM3, BAM13, BE119, BAM35, BAM37, BAM40, and BE95, and the Ω insertion into the *Sac*I site are indicated on the partial restriction map; the restoration phenotype of each of these insertions was +. Insertions BAM35 and BE95 were blue in KW239UVS1, as noted; the remaining Tn*lacZ* insertions were white in KW239UVS1.

constructed from the restoring Tn*lacZ*-containing derivatives of pJJRBE and pJJRBam by using *Sac*I and *Xho*I sites located within the *P. syringae* pv. syringae DNA and within Tn*lacZ*. Since these restriction enzymes do not have sites within the vector sequences of these clones, the deletions were constructed without moving the insert into a different vector.

As indicated in Fig. 5, three separate deletions removing the tRNA genes (pJJRΔScB, pJJRΔ37B, and pJJRΔSH) rendered the deletion subclones incapable of restoring mutant KW239UVS1 in lesion formation and toxin assays. By contrast, deletion of the 15.5-kDa protein ORF (pJJRΔ40B), the *mrsA* ORF (pJJRΔ13X), the *tpiA* ORF (pJJRΔ119S), or the *tpiA* and *mrsA* ORFs together (pJJRΔ13S, pJJRΔX37, and pJJRΔX119) did not prevent the in *trans* restoration of mutant KW239UVS1. In addition, the deletion subclone pJJRΔS37 removed 23% of the amino terminus of the *secG* ORF sequence that spanned the *lem-239*::Tn*5* insertion site but still was able to restore KW239UVS1. These data indicated that of the six regions of similarity sequenced, only clones containing the two tRNA genes were able to restore the *lem-239*::Tn*5* mutation in *trans.*

Conservation of *lem-239*::Tn*5* locus in pseudomonads. Southern blot analysis indicated the physical conservation of the *lem-239*::Tn*5* region in a variety of pseudomonads (data not shown). This assembly comprised numerous plant pathogenic *P. syringae* strains, including *P. syringae* pv. tomato DC3000 and nonpathogenic *P. syringae* Cit7; two *P. solanacearum* strains; and three strains of the human pathogen *P. aeruginosa*, including PAO1. A functional homolog of the *lem-239*::Tn*5* region was isolated from *P. syringae* pv. tomato DC3000, a causal agent of bacterial speck of tomato (*Lycopersicon esculentum*) and a pathogen of *Arabidopsis thaliana*. The cosmid pKMB4, isolated from a DC3000 genomic library, restored both syringomycin production and lesion formation to the *P. syringae* pv. syringae mutant KW239 (data not shown). The isolation of an active allele from DC3000, a strain that does not produce syringomycin, suggests a regulatory function for the gene(s) affected in KW239.

DISCUSSION

The identification of a collection of unlinked loci in *P. syringae* pv. syringae B728a provides insight into the complexity of the genetics of pathogenicity. This research supports the hypothesis that rather than there being a single gene or gene

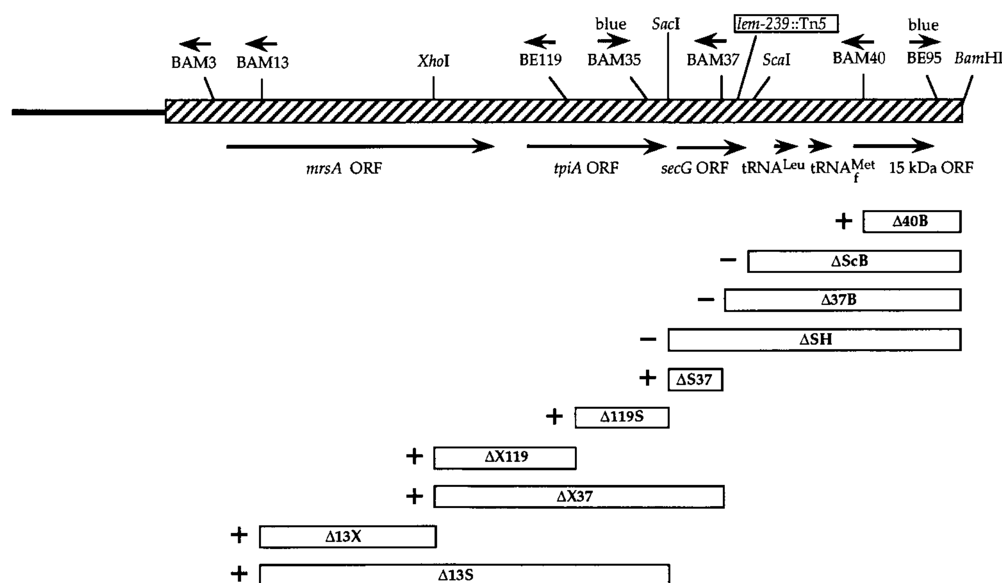

FIG. 5. The ORFs and potential tRNA genes identified in the DNA sequence, as described in the text. The sequenced portion of pJJRBam is indicated by a cross-hatched bar. Also indicated is extent of the deletion generated within the region required for restoration of lesion-forming ability and toxin production to mutant KW239UVS1. The open boxes represent the deleted sequences. A + to the left of the deletion box indicates restoration, while a − specifies lack of restoration. Other features are as described in the legend to Fig. 4.

cluster that distinguishes this organism from nonpathogenic bacteria, lesion formation is a multigenic process, with a number of distinct genes or pathways contributing to the ability to form lesions on bean. The B728a *lemA* and *gacA* genes are required for disease lesion formation on bean but not for the ability to induce the HR on the nonhost tobacco. In this study, we characterized new B728a mutants that still incited the HR on tobacco but did not form disease symptoms on the host, bean. Prior to this research, it was not known whether mutations in genes required for lesion formation but not involved in the HR would define a discrete segment of the genome. By contrast with the *hrp* genes, which are clustered together on the *P. syringae* chromosome, none of the mutations analyzed here were closely linked to the *lemA* or *gacA* genes or to each other.

Our mutational analysis of B728a, based on plant infiltration ssays, strongly suggests that the production of extracellular protease and syringomycin is not a requirement for lesion formation on bean. Syr$^-$ mutants that formed disease lesions have been observed as naturally occurring variants isolated from bean and other hosts (2, 40), during Tn*5* mutagenesis of strains pathogenic on hosts other than bean (62), and within the present 6,401 Tn*5* mutants of B728a (21). Thus, syringomycin production does not appear to play a crucial role in bean pathogenicity by *P. syringae* pv. syringae B728a. In previous work, B728a mutants that had a reduced amount of extracellular protease were unaffected in their ability to form lesions on bean (21). We have also identified a protease-negative mutant (KW5569) from the present screen that is unimpaired in lesion formation on bean (43). Our isolation of seven lesion-defective mutants that retain protease and syringomycin activities demonstrates that the production of these extracellular compounds is not sufficient for lesion formation on bean.

The phenotypes of a significant number of the original mutants appear to be unlinked to the Tn*5* insertion. This result underscores the invalidity of assessing the number of genes involved in lesion formation and other *P. syringae* phenotypes by the number of mutants obtained. Mutant KW3621 was restored by the *lemA* gene, and KW21 was restored by the *gacA* gene, but neither mutant was complemented by sequences flanking the respective Tn*5* insertion. Spontaneous mutation of the *gacA* gene is a relatively frequent event: five derivatives of B728a isolated from a pool of 900 colonies screened for loss of protease activity, and six additional mutants, including KW21, isolated in other experiments have been complemented by the *gacA* gene (43, 47). Mutant KW3621, by contrast, is one of only two spontaneous mutants picked up over the course of our studies that is restored by the *lemA* gene (Table 2 and reference 43).

The >16-kb deletion of the *syrBCD* region is particularly noteworthy. Eight of the 44 Tn*5*-containing strains examined had this region deleted. The *syrBCD* genes have been proposed to be involved in syringomycin biosynthesis, although direct evidence for this has not been reported (37). The deletion event involving the chromosomal region flanking the Tn*5* insertion of mutant KW5949 is a related phenomenon, since all of the strains with the KW5949 region deleted also contain deletions of the *syrBCD* hybridizing region genes. No other deletions or rearrangements were detected within the mutant DNAs with any of the other probes used in this analysis. An interesting parallel is the high-frequency deletion of the tabtoxin biosynthetic cluster from *P. syringae* strain BR2 (28). This finding may imply that under conditions of some stress, such as may be encountered during routine laboratory manipulations, it is advantageous to the survival of the bacterial cell to cease the production of complex secondary metabolites.

The phenotypes observed in this collection of mutants expose fundamental interactions among the new loci and *hrp*, *lemA*, and *gacA* genes, all of which play a role in lesion formation. The HR$^+$ phenotype of the HR$^+$ Lem$^-$ mutants isolated in this study, and also of *lemA* and *gacA* mutants, indicates that *hrp* genes must still be functioning in these Lem$^-$ backgrounds; thus, these new loci, along with *lemA* and *gacA*, do not appear to affect *hrp* gene expression. Our finding that nine B728a *hrp* mutants still produce protease and syringomycin suggests that the known *lemA*- and *gacA*-regulated phenotypes can be expressed in the presence of mutations within the *P. syringae* pv. syringae *hrp* region.

Mutant KW239 was affected in lesion formation but not in other plant interaction phenotypes, namely, the HR and growth in association with bean plants. The ability of mutant KW239 to grow on or in bean leaves argues that the lack of lesion formation of this strain is not a simple result of an incapacity to grow in association with the host plant. The prototrophy of the mutant and its unimpaired growth in KB medium further support the case that mutant KW239 is not defective in a basic housekeeping function, and its deficiency is more specifically related to disease progression.

Restoration of mutant KW239 was accomplished with clones containing wild-type *P. syringae* pv. syringae DNA. Lesion formation on bean leaves and pods and toxin production were also restored to KW239UVS1 and to a marker exchange strain containing the *lem-239*::Tn*5* mutation. These results indicated that the suppression observed was effected in *trans* and was not due to marker rescue. The restoring region was localized to a DNA region smaller than 3.6 kb (pJJRB36a and pJJRB36b) that functioned in both orientations. Nevertheless, an Ω fragment insertion and a collection of Tn*lacZ* mutations into the restoring subclone pJJRBam or pJJRBE did not block suppression of the lesion formation and toxin defects of KW239UVS1. One of the Tn*lacZ* insertions, BAM37, was located only 27 bp from the *lem-239*::Tn*5* mutation. This led to the finding that clones containing the *lem-239*::Tn*5* mutation were also capable of correcting the loss of lesion-forming ability and toxin production. To address this apparent paradox, the subcloning and insertional mutagenesis data were examined in conjunction with the DNA sequence of the 3.6-kb restoring fragment.

The deletion of sequences containing two putative tRNA genes resulted in the loss of restoration activity. By contrast, removal of each of four ORFs identified in the DNA sequence did not prevent restoration of the KW239 defects in *trans*. These results lead to the hypothesis that the *lem-239*::Tn*5* insertion is a *cis*-acting mutation affecting the expression, modification, or processing of one or both of the tRNA genes. In the simplest model, the amounts of the tRNA(s) are decreased by the upstream Tn*5* insertion and are complemented in *trans* to levels sufficient to restore lesion formation and syringomycin production. The lack of syringomycin activity within cell-free lysates of KW239 suggests a defect in synthesis of the toxin rather than in its secretion. Since the *lem-239*::Tn*5* insertion does not reside within a known syringomycin biosynthetic gene, this finding implies that the mutation affects syringomycin gene regulation at some level.

There have been several reports suggesting that $\mathrm{tRNA}_\mathrm{f}^\mathrm{Met}$ is a regulatory factor, in addition to its essential role in translation initiation. The initiator tRNA binds specifically to RNA polymerase, and this binding affects transcription initiation and the pattern of promoter selection in vitro (9, 39, 41). The $\mathrm{tRNA}_\mathrm{f}^\mathrm{Met}$ could act as a signal by which the cell mediates the coupling of transcription and translation. Both the tRNA and the formylmethionine moiety contribute to binding to RNA polymerase and to the stimulation or inhibition of the transcription of different genes (39, 41). In light of this literature, the possibility may be considered that a *P. syringae* pv. syringae initiator tRNA, encoded by the DNA sequence reported here, regulates the transcription of a gene(s) involved in lesion formation and antibiotic production.

There is a precedent for a role for tRNA genes in the regulation of complex phenotypes in bacteria. In *Streptomyces coelicolor*, the *bldA* gene, required for the development of aerial hyphae and spores as well as the production of four different antibiotics, specifies a tRNA molecule that recognizes the leucine codon UUA (31, 33). This codon, which is rare in the G+C-rich *Streptomyces* genome, renders genes containing it dependent on *bldA* (33). It is noteworthy that although *S. coelicolor bldA* mutants are morphologically impaired, they are not affected in vegetative growth. tRNAs have been proposed to function in similar capacities in *E. coli* DNA replication (7) and cell division (8, 52) and are involved in the control of amino acid transport and biosynthesis (13, 57). Furthermore, a variety of functions for tRNAs other than ribosomal protein synthesis have been demonstrated. These include the posttranslational addition of amino acids to the N termini of proteins (32), involvement in the targeting of proteins for degradation (54), porphyrin ring synthesis (24), and peptidoglycan synthesis (51). While it is not known whether there may be a direct parallel with any of these interesting roles for tRNAs in *P. syringae* pv. syringae, these examples show that tRNAs can function in diverse processes that rival lesion formation and antibiotic production in complexity. Finally, there have been reports of tRNAs involved in mediating the interactions of bacteria with eukaryotic organisms. These involve the *miaA* gene of the plant pathogen *Agrobacterium tumefaciens*, encoding a tRNA modification enzyme that affects *vir* gene expression (14), and the partial suppression by tRNA genes of a deletion of the *virR* regulatory gene of the human pathogen *Shigella flexneri* (22). In these cases, the link between the tRNA and the interaction phenotype does not appear to be direct.

This study sheds light on the genome organization of *P. syringae* pv. syringae, about which very little is known. In comparison with *E. coli*, the similarities as well as the differences in the organization of these genes are remarkable. The *metY-nusA* operon, the *leuU* gene, and the operon containing the *mrsA* gene are located in the same region (69 min) on the *E. coli* map, whereas *tpiA* is located elsewhere (88 min). The *lem-239*::Tn*5* region is apparently conserved within *P. syringae*, and the isolation of a restoring cosmid from a pathogen of tomato and *Arabidopsis* demonstrates that a functionally homologous region is contained within *P. syringae* pv. tomato DC3000. Southern hybridization suggests there may be an analogous genetic organization within other pseudomonads (data not shown).

In summary, our analysis of 6,401 Kan^r transconjugants of B728a identified seven lesion-defective mutants that contain causal Tn*5* insertions. It is clear that the *lemA* and *gacA* genes must function at some level in each of the seven mutants, since these mutants still produce the protease activity regulated by the *lemA* and *gacA* genes. This mutant screen has laid the groundwork for our continuing genetic analysis of lesion formation by *P. syringae* pv. syringae on bean. Analysis of the mutated locus in KW3815 revealed that the Tn*5* insertion occurred within the *P. syringae* pv. syringae *ftsK* homolog, a gene that is required for septum formation in *E. coli* (18). This result, together with the finding that tRNA expression may be be affected in KW239, is consistent with a role for bacterial translation machinery in the regulation of lesion formation by *P. syringae* pv. syringae. Perhaps not unexpectedly, it is becoming obvious that the regulation of lesion formation within *P. syringae* is a genetically complex process.

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