Isolation and Characterization of Pathogenicity Genes of *Pseudomonas syringae* pv. *tabaci*

YANGKYO P. SALCH AND PAUL D. SHAW*

Department of Plant Pathology, University of Illinois at Urbana-Champaign, 1102 South Goodwin Avenue, Urbana, Illinois 61801

Received 14 September 1987/Accepted 29 February 1988

Pseudomonas syringae pv. *tabaci* BR2 produces tabtoxin and causes wildfire disease on tobacco and bean plants. Approximately 2,700 Tn5 insertion mutants of a plasmid-free strain, PTBR 2.024, were generated by using suicide plasmid pGS9. Of these Tn5 mutants, 8 were no longer pathogenic on tobacco plants and 10 showed reduced symptoms. All of the eight nonpathogenic mutants caused typical wildfire disease symptoms on bean plants. Two of the nonpathogenic mutants failed to produce tabtoxin. The eight nonpathogenic mutants have Tn5 insertions into different *Eco*RI and *Sal*I restriction fragments. The *Eco*RI fragments containing Tn5 from the eight nonpathogenic mutants were cloned into vector pTZ18R or pLAFR3. A genomic library of the parent strain was constructed in the broad-host-range cosmid pLAFR3. Three different cosmid clones that hybridized to the cloned Tn5-containing fragment from one of the nonpathogenic mutants, PTBR 4.000, were isolated from the genomic library. These clones contained six contiguous *Eco*RI fragments (a total of 57 kilobases [kb]). A 7.2-kb *Eco*RI fragment common to all three restored pathogenicity to mutant PTBR 4.000. None of the six *Eco*RI fragments hybridized to Tn5-containing fragments from the other seven mutants. The 7.2-kb fragment was conserved in *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata*, but not in other pathovars or strains. Because the mutants retained pathogenicity on bean plants and because of the conservation of the 7.2-kb *Eco*RI fragment only in pathovars of tobacco, we suggest that genes on the fragment might be related to host specificity.

Pseudomonas syringae pv. *tabaci* BR2 causes wildfire disease on green bean and tobacco plants (29, 33). It produces tabtoxin, a dipeptide phytotoxin containing tabtoxinine- β -lactam linked to either threonine or serine (40, 41). The toxin causes chlorotic halos on infected plants (43). Strain BR2 harbors one detectable plasmid, pBPW1 (31; B. J. Staskawicz, M. Sato, and N. J. Panopoulos, *Phytopathology* 71:257, 1981), which is not directly involved either in tabtoxin production or in other aspects of pathogenicity in *P. syringae* pv. *tabaci* (29, 31).

The development of Tn5 mutagenesis and recombinant DNA procedures has provided tools for the isolation and characterization of large numbers of mutant derivatives of phytopathogenic bacteria, defective in various aspects of the pathogenic process (6, 28). A number of genes involved in the plant-pathogen interactions have been cloned; examples are pathogenicity genes from *P. syringae* pv. *phaseolicola* (25), avirulence genes of *P. syringae* pv. *glycinea* (38), and toxin-production genes of *P. syringae* pv. *phaseolicola* (30).

Little is known about the molecular genetics of pathogenicity mechanisms of *P. syringae* pv. *tabaci*, even though the structure of tabtoxin is well established and its inhibition mechanism is well understood (41). Turner and Taha (44) suggested that tabtoxin production is significant in pathogenicity, since their spontaneous nontoxigenic mutants of *P. syringae* pv. *tabaci* 11528 were nonpathogenic on tobacco leaves of *Nicotiana tabacum* cv. White Burley. However, other *P. syringae* pv. *tabaci* strains incapable of producing tabtoxin were still pathogenic on tobacco plants (11). Also, nontoxigenic strains of *P. syringae* pv. *phaseolicola* were still pathogenic on host plants (30).

In this study, a plasmid-free BR2 strain was selected for

the investigation of pathogenicity traits by using Tn5 mutagenesis and recombinant DNA techniques.

(A preliminary report of this study was presented at the Third International Symposium on the Molecular Genetics of Plant-Microbe Interactions, Montreal, Quebec, Canada, 27 to 31 July 1986.)

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used are listed in Table 1. Bacteria were grown in Luria-Bertani broth (LB) (24), King B medium (KB) (23), Vogel-Bonner minimal medium (VB) (49), or M9 medium (5) at 26 or 37°C. Filter-sterilized antibiotics (Sigma Chemical Co.) were added to media at the following concentrations (in micrograms per milliliter) unless otherwise stated: ampicillin, 300; kanamycin, 100; streptomycin, 100; nalidixic acid, 200; and tetracycline, 15.

Tn5 mutagenesis. Bacterial conjugations with *Escherichia coli* WA803 (containing the suicide plasmid pGS9::Tn5) as the donor and *P. syringae* pv. *tabaci* BR2 (strain PTBR 2.024) as the recipient strain were carried out by liquid mating. Both strains were grown overnight in 5 ml of LB supplemented with appropriate antibiotics. A 1-ml portion of each culture was transferred into 50 ml of fresh LB without antibiotics and grown to mid-log phase ($A_{660} = 0.5$). The donor and recipient cells (1 ml each) were mixed and incubated at 30°C with constant shaking overnight (about 16 h). The cells were plated on minimal medium (VB) containing kanamycin to select for Tn5-containing transconjugants. For further characterization, the transconjugants were replica plated on KB containing nalidixic acid and streptomycin (50 μ g/ml). They were also replica plated on media containing chloramphenicol (300 μ g/ml) to test for the presence of plasmid vector pGS9.

Pathogenicity, tabtoxin production, and resistance assays.

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype ^a	Source or reference
<i>P. syringae</i> pv. <i>tabaci</i>		
PTBR 2.024	Nal ^r Tox ⁺ Pat ⁺ Flu ⁺ Tox ^r	29
PTBR 3.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁻	This study
PTBR 4.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 5.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 6.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 7.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁻	This study
PTBR 8.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 9.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 10.000	PTBR 2.024::Tn5 Pat ⁻ Tox ⁺	This study
PTBR 4.005	PTBR 4.000::pQS409 Pat ⁺ Tox ⁺	This study
<i>S. typhimurium</i>		
TA 1975	<i>his</i> Rif ^r	J. B. Johnston
<i>E. coli</i>		
HB101	<i>hsd-20</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>recA13</i> Str ^r <i>arg pro ara gal F</i> ⁻	4
WA803	<i>met thi</i>	51
TB-1	<i>r_K</i> ⁻ <i>m_K</i> ⁻ <i>ara thi lacZ</i> Str ^r	48, 50
NM522	<i>thi pro lacI lacZ</i>	14
Plasmids		
pGS9	30.5 kb, Tra ⁺ (N-type), p15A replicon, Cm ^r Kan ^r (Tn5)	35
pTZ18R	2.86 kb, Amp ^r	27
pWS6	12.0 kb, Amp ^r Kan ^r Str ^r	50
pLAFR3	23.0 kb, Tet ^r <i>cos rlx</i>	B. J. Staskawicz
pTZ184.1	pTZ18R::12.9-kb <i>EcoRI</i> fragment::Tn5 from PTBR 4.000	This study
pQS405	pTZ18R::7.2-kb <i>EcoRI</i> fragment from PTBR 2.024	This study
pQS409	pWS6::7.2-kb <i>EcoRI</i> fragment from PTBR 2.024	This study

^a Abbreviations: Nal^r, Str^r, Kan^r, Rif^r, Amp^r, Tet^r, and Cm^r, resistant to nalidixic acid, streptomycin, kanamycin, rifampin, ampicillin, tetracycline, and chloramphenicol, respectively; Tox⁻, no toxin production by in vitro assay; Tox^r, resistant to tabtoxin; Pat⁻, nonpathogenic for tobacco plants; Flu⁺, fluorescent pigment on KB.

Pathogenicity tests were performed on detached tobacco (*N. tabacum* cv. Havana 38) or bean (*Phaseolus vulgaris* cv. Top Crop) leaves. Fully expanded leaves were removed from plants and washed with distilled water, and excess water was removed by air drying at room temperature. The leaves were placed in petri dishes containing Whatman no. 1 filter paper moistened with sterile distilled water. Bacterial inocula were prepared by growing cells in 5 ml of VB at 26°C overnight and washing them with 0.9% sterile saline solution. Inoculations were performed by gently pin-pricking leaves with a sterilized needle through droplets of inoculum (20 µl, containing approximately 4 × 10⁷ viable cells). Inoculated leaves were incubated at 26°C and illuminated continuously with fluorescent lights. Populations of bacteria in leaves at times after inoculation were estimated by the method of Mills et al. (D. Mills, G. Bertoni, and P. Mukopadhyay, Abstr. 3rd. Int. Symp. Mol. Genet. Plant-Microbe Interactions, abstr. no. 34, 1986). Tabtoxin production and tabtoxin resistance were assayed as described previously (31).

DNA isolations. Minipreparations of plasmid DNAs were carried out by the sodium dodecyl sulfate-alkaline lysis method (34). Large-scale isolation of plasmid DNA was performed by the procedure of Johnston and Gunsalus (21). Total DNA was isolated by the procedure of Chesney et al. (7).

Recombinant DNA techniques. Restriction digestion of DNA, agarose gel electrophoresis, and DNA-DNA hybridization were performed as described by Maniatis et al. (26). Restriction endonucleases obtained from Bethesda Research

Laboratories, Inc., or Anglian Biotechnology Ltd. were used as recommended by the suppliers. Bacterial alkaline phosphatase (BAP) was obtained from Bethesda Research Laboratories and used as recommended by the manufacturer. T4 DNA ligase was purchased from Promega Biotec, and ligation was performed under conditions specified by the manufacturer. DNAs used as probes were nick translated with [α -³²P]dCTP (800 µCi/µmol) by using a nick translation kit (from Bethesda Research Laboratories) to a specific activity of more than 10⁶ cpm/µg of DNA by following the protocol outlined in the kit. The DNA probe was separated from unincorporated nucleotides by Sephadex G-50 (Sigma) column chromatography and denatured by boiling for 10 min prior to use. DNA was transferred onto GeneScreen membranes (New England Nuclear Corp.) under conditions described by the manufacturer. Multiple hybridizations were performed as described by Anderson and Young (1). Colony hybridization was performed as described previously (16, 17).

Transformation. Transformation was carried out by a modification of the procedure of Gross and Vidaver (15). Cells grown in 50 ml of LB were harvested at mid-log phase for *E. coli* (*A*₆₆₀ = 0.5) and at late log phase for *P. syringae* pv. *tabaci* BR2 (*A*₆₆₀ = 1.0) by centrifugation at 6,000 rpm in a Sorvall SS34 rotor for 10 min. Cells were suspended in 20 ml of transformation buffer containing 150 mM CaCl₂ and 15% glycerol. The suspension was incubated on ice for 20 min and then centrifuged as described above. The cells were resuspended in 2.5 ml of the transformation buffer. Small portions (0.2 ml) of the suspensions were dispensed into

FIG. 1. Southern blot analysis of Tn5 insertions present in nonpathogenic mutants. DNA samples digested with *Eco*RI (a), *Eco*RI and *Sal*I (b), or *Sal*I (c) were separated by agarose gel electrophoresis, transferred onto a GeneScreen membrane, and probed with pGS9. Lanes: 1, lambda DNA *Hind*III digest (fragment sizes are given in kilobases); 2, pGS9; 3, total DNA from *E. coli* WA803::pGS9; 4, total DNA from PTBR 2.024 plus one equivalent of pGS9; 5, total DNA from PTBR 2.024; 6, PTBR 3.000; 7, PTBR 4.000; 8, PTBR 5.000; 9, PTBR 6.000; 10, PTBR 7.000; 11, PTBR 8.000; 12, PTBR 9.000; 13, PTBR 10.000.

plastic tubes and stored at -80°C . For transformation, 0.2 ml of the cell suspensions thawed on ice prior to use was mixed with 0.1 ml of DNA (1 μg) and incubated on ice for 30 min (for *E. coli*) or for 60 min (for BR2 strains). The cell suspensions were heat shocked either at 42°C (for *E. coli*) or at 45°C (for BR2) for 2 min and then incubated at room temperature for 10 min. After 1 to 2 ml of fresh LB was added to the suspensions, the *E. coli* mixtures were incubated at 37°C for 30 to 60 min and the BR2 mixtures were incubated at 26°C for 2 h with shaking to allow time for expression of resistance to the antibiotics used for selection. The cells were plated on media containing appropriate antibiotics.

Construction of genomic library. Total DNA from PTBR 2.024 was partially digested with *Eco*RI (25 U/ml for 100 μg of DNA), and DNA fragments in the range of 20 to 30 kilobase pairs (kb) were isolated by fractionation of the digests on 1.25 to 5 M sodium chloride gradients (8). These fragments were ligated into cosmid vector pLAFR3 which had been digested to completion with *Eco*RI and dephosphorylated with BAP. The ligated DNA was packaged in vitro by using packaging extracts (Promega Biotec) as recommended by the supplier. The packaged DNA was transduced into *E. coli* HB101, and the recombinant cosmids were selected on a medium containing tetracycline. The packaging efficiency was 7.5×10^4 CFU/ μg of DNA. The cosmid clones were transferred onto sterilized nitrocellulose filters (HATF; Millipore Corp.) which were sandwiched between two sheets of sterile Whatman 3MM filter paper. These were placed on tetracycline medium containing 0.8 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.2 g of KH_2PO_4 , 0.25 g of MgSO_4 , 0.6 g of trisodium citrate, and 44 ml of glycerol per 100 ml and stored at -80°C .

RESULTS

Tn5 insertion mutants. The Tn5 insertion mutants of *P. syringae* pv. *tabaci* BR2.024 (strain PTBR 2.024), isolated on minimal medium, produced fluorescent pigment on KB, were resistant to kanamycin, streptomycin, and nalidixic acid, and were sensitive to chloramphenicol. The phenotypes of the Tn5 insertion mutants indicate that these are derived from the parent strain PTBR 2.024. A streptomycin resistance gene is encoded by Tn5 and is expressed in *Pseudomonas* strains, but not in *E. coli* (36). The sensitivity of transconjugants to chloramphenicol indicates the absence of pGS9 in the cells and indicates that Tn5 transpositions had

occurred. DNA was isolated from 10 randomly chosen Tn5 mutants and digested to completion with *Eco*RI. Agarose gel electrophoresis and Southern hybridization with pGS9 as the probe showed that Tn5 had inserted into a range of different *Eco*RI restriction fragments (data not shown).

Tn5 mutants were tested for pathogenicity on detached tobacco leaves. Of the 2,686 mutants generated, 8 (0.3%) showed no symptoms and 10 exhibited reduced symptoms (limited chlorosis without necrotic lesions) on tobacco leaves. However, all 18 still produced wildfire symptoms on detached bean leaves. When the eight nonpathogenic mutants (designated as PTBR 3.000, PTBR 4.000, PTBR 5.000, PTBR 6.000, PTBR 7.000, PTBR 8.000, PTBR 9.000, and PTBR 10.000) were assayed for tabtoxin production and resistance in vitro, two mutants (PTBR 3.000 and PTBR 7.000) produced no tabtoxin and all eight were resistant to tabtoxin.

Physical analysis of Tn5 insertions of the nonpathogenic mutants. Total genomic DNA was isolated from the eight nonpathogenic mutants and digested to completion with *Eco*RI or *Sal*I, or both. Tn5 does not contain *Eco*RI recognition sites, but does contain one *Sal*I site in the middle of the transposon (22). Tn5 insertions were detected by agarose gel electrophoresis followed by Southern hybridization analysis with pGS9 as a probe. Similar results (data not shown) were obtained by using the internal *Hind*III fragment of Tn5 as a probe. Mutants PTBR 3.000, PTBR 4.000, PTBR 6.000, PTBR 7.000, PTBR 8.000, and PTBR 10.000 (lanes 6, 7, 9, 10, 11, and 13) contain one *Eco*RI fragment (Fig. 1a) and two *Sal*I fragments (Fig. 1b and c) that hybridize to Tn5 (the film in Fig. 1 was deliberately overexposed to reveal the faint bands; e.g., panel a, lane 9). This indicates that only one copy of Tn5 was present per mutant genome in these strains. Mutant PTBR 5.000, however, has additional *Eco*RI and *Sal*I fragments that hybridize to Tn5 (Fig. 1a and c, lanes 8). The *Eco*RI-*Sal*I double digest of PTBR 5.000 also shows one additional band hybridizing to pGS9 (Fig. 1b, lane 8). PTBR 9.000 shows two *Eco*RI bands (Fig. 1a, lane 12) and four *Sal*I bands (Fig. 1c, lane 12) or *Eco*RI-*Sal*I bands (Fig. 1b, lane 12) hybridizing to Tn5. Tn5 has a single internal *Sal*I site but no *Eco*RI sites. These results therefore suggested that the PTBR 5.000 chromosome contained IS50 as well as Tn5 and that the PTBR 9.000 chromosome contained two copies of Tn5.

Bacterial growth of the mutant PTBR 4.000 in planta. Strain PTBR 4.000 was investigated further to determine the

FIG. 2. Bacterial growth curves of PTBR 2.024 and PTBR 4.000 in tobacco leaves. The number of viable cells in 100 mg of leaf tissues was counted daily after inoculation by plating plant cell extracts on KB. Samples are PTBR 2.024 (■) and PTBR 4.000 (+).

possible reasons for its failure to cause symptoms on tobacco leaves. The growth of PTBR 4.000 and its parent strain, PTBR 2.024, was monitored for 5 days after inoculation (Fig. 2). Parent PTBR 2.024 and mutant PTBR 4.000 populations reached a maximum at 24 h after inoculation on tobacco leaves. PTBR 2.024 began to produce symptoms 2 days after inoculation, but symptoms were never observed with PTBR 4.000. The PTBR 2.024 population remained relatively constant at 5×10^6 to 3×10^7 CFU/100 mg of tissue from 2 days after inoculation. However, the number of cells of PTBR 4.000 had decreased essentially to zero by 5 days after inoculation. Three repetitions of this experiment gave essentially the same results, but the cell populations varied by an order of magnitude. In all three experiments, the parent and mutant populations reached approximately the same levels, but the mutant populations declined, whereas the parent populations remained nearly constant.

Cloning of *EcoRI* fragments containing Tn5 from the nonpathogenic mutants. The *EcoRI* fragments containing Tn5 from seven nonpathogenic mutants were cloned into pLAFR3 (pQS301, pQS501, pQS601, pQS701, pQS801, pQS901, and pQS101, respectively, from mutant strains PTBR 3.000, PTBR 5.000, PTBR 6.000, PTBR 7.000, PTBR 8.000, PTBR 9.000, and PTBR 10.000), and the fragment from mutant PTBR 4.000 was cloned into pTZ18R (pTZ184.1). To verify that the clones contained Tn5, we digested them with *EcoRI* or *Bam*HI and analyzed them by agarose gel electrophoresis and Southern hybridization with pGS9 as a probe. Tn5 has a single *Bam*HI cleavage site (22). All the digests showed one *EcoRI* and two *Bam*HI signals when probed with pGS9 (data not shown). The identity of the cloned fragments was further confirmed by digestion with *Hind*III followed by the same analysis as described above. Transposon Tn5 has two *Hind*III sites just inside the proximal ends of the terminal inverted repeat sequence IS50 (22). All clones have the internal *Hind*III sequence (3.4 kb) of Tn5 (Fig. 3, arrow), as well as chromosomal DNA sequences that flank Tn5. pGS9 hybridized only to the internal *Hind*III fragment of Tn5 and to the two regions of chromosomal DNA immediately adjacent to Tn5 (data not shown). The sizes of the *EcoRI* fragments containing Tn5 were estimated to be as follows: pQS301, 24 kb; pQS501, 12 kb; pQS601, 18 kb; pQS701, 28 kb; pQS801, 25 kb; pQS901, 15 kb; pQS101, 15 kb; and pTZ184.1, 12.9 kb.

FIG. 3. *Hind*III digests of the cloned *EcoRI* fragments containing Tn5 from the nonpathogenic mutants. DNA samples were digested with *Hind*III and analyzed by agarose gel electrophoresis. Lanes: 1, lambda DNA *Hind*III digest (fragment sizes are given in kilobases); 2, pQS301; 3, pTZ184.1; 4, pQS501; 5, pQS601; 6, pQS701; 7, pQS801; 8, pQS901; 9, pQS101; 10, pTZ18R; 11, pWS6; 12, pLAFR3. The designations of the clones are explained in the text.

Construction of genomic library. A genomic library of the parent strain, PTBR 2.024, was constructed in pLAFR3. The length of the inserts in the clones was determined to be 19 to 34 kb (average, 26.5 kb) by *EcoRI* digestion of 15 randomly selected colonies. This is consistent with the size selection imposed by the lambda packaging system and is similar to results obtained with *Rhizobium meliloti* (12) and *Xanthomonas campestris* pv. *campestris* (8). The probability that this library of 1,750 cosmid clones contains any DNA sequence is 99% (26), assuming that the *Pseudomonas* genomic DNA contains 7.5×10^6 base pairs (19).

Isolation of parental pathogenicity genes. The initial attempt to isolate pathogenicity genes was made by using mutant PTBR 4.000. PTBR 4.000 has only one Tn5 insert in a 12.9-kb *EcoRI* fragment (Fig. 1a, lane 7) and is completely avirulent. To isolate the intact region complementary to the mutated sequence in PTBR 4.000, we hybridized cosmid colonies from the wild-type genomic library to 32 P-labeled pTZ184.1. Five recombinant cosmids that hybridized to the probe were identified and designated pQS1212, pQS1315, pQS2822, pQS2821, and pQS3220. Restriction endonuclease analysis of DNA from the five clones (Fig. 4A) showed that three of them (pQS2822, pQS2821, and pQS3220) contained an identical insert, having (in addition to the 23-kb pLAFR3 band) four *EcoRI* bands of 3, 7.0, 7.2, and 16 kb. pQS1212 has three *EcoRI* bands, of 4, 7.2, and 19 kb, and pQS1315 has four *EcoRI* bands, of 4, 7.0, 7.2, and 16 kb. The faint bands are fragments resulting from partial digestion. Each of the five cosmid clones was subjected to repeated partial digestion by *EcoRI* to determine the order of the fragments. *EcoRI* restriction maps of the cosmid clones are shown in Fig. 4C. Southern hybridization analysis with 32 P-labeled pTZ184.1 indicated that the 12.9-kb *EcoRI* fragment hybridized to the 7.2-kb *EcoRI* fragment that is common to all of three cosmid clones, but hybridized to none of the other *EcoRI* fragments (Fig. 4B). The 7.2-kb *EcoRI* fragment was subcloned into plasmid pTZ18R, and this recombinant plasmid was designated pQS405. Restriction maps of the 7.2-kb fragment from the parent strain and the Tn5-containing 12.9-kb *EcoRI* fragment from mutant PTBR 4.000 (Fig. 5) confirm that the 12.9-kb *EcoRI* fragment contains the chromosomal 7.2-kb *EcoRI* fragment plus the Tn5 insertion. Tn5 is located at approximately 300 base pairs from the right

FIG. 4. Physical analysis and *EcoRI* restriction maps of five cosmid clones. DNA samples were digested with *EcoRI* and analyzed in a 0.8% agarose gel. DNA fragments were transferred onto GeneScreen membrane and hybridized with the ^{32}P -labeled 12.9-kb *EcoRI* fragment. (A) Agarose gel electrophoresis; (B) Southern hybridization; (C) *EcoRI* restriction map. Lanes: 1, lambda DNA *HindIII* digest; 2, pQS1212; 3, pQS2821; 4, pQS1315; 5, pQS2822; 6, pQS3220; 7, plasmid pTZ18R; 8, cosmid pLAFR3. The fragment sizes are given in kilobases.

border of the second-largest *PstI* fragment (3.0 kb). They also show that the transcription of the neomycin phosphoryl-transferase gene residing in Tn5 is from left to right.

Relationships between the parental DNA clones and the mutant clones. The cloned sequences containing Tn5 plus the flanking sequences of chromosomal DNA from the nonpathogenic mutants were cleaved with *HindIII* and hybridized with pQS405, pQS1212, or pQS2822 to detect possible overlaps between the parental DNA sequences and the cloned fragments from the other seven nonpathogenic mutants. All three hybridized only to clone pTZ184.1 and sequences of plasmids pTZ18R, pWS6, and cosmid pLAFR3. None of the other clones hybridized to these probes (data not shown). This result demonstrates that the 7.2-kb *EcoRI* fragment is located in at least 23 kb from the other seven fragments.

Complementation tests. The 7.2-kb *EcoRI* fragment from pQS405 was subcloned into a shuttle vector, pWS6, which can replicate in *E. coli* as well as in *Pseudomonas* strains. The orientation of the insertion fragment was not determined. This clone, called pQS409, and plasmid vector pWS6 were transformed into mutant PTBR 4.000 as well as into the other seven mutant strains. All of the eight mutants containing either pQS409 or pWS6 were tested for pathogenicity. PTBR 4.005, the merodiploid construct from mutant PTBR 4.000, caused wildfire symptoms on tobacco leaves (Fig. 6). None of the other seven nonpathogenic mutants into which pQS409 was transformed caused symptoms, and none of the

FIG. 5. Restriction maps of (A) the 7.2-kb *EcoRI* fragment from the parent strain and (B) the 12.9-kb *EcoRI* fragment from the mutant PTBR 4.000. Panel B shows that the 12.9-kb *EcoRI* fragment contains the 7.2-kb *EcoRI* fragment plus the Tn5 insertion. The fragment sizes are given in kilobases.

eight mutants into which pWS6 was transformed caused symptoms.

Detection of sequences homologous to the 7.2-kb *EcoRI* fragment in other pseudomonads. Southern hybridization

FIG. 6. Tobacco leaves inoculated with PTBR 2.024 (WT), saline solution (Saline B), PTBR 4.000, and PTBR 4.005. Clockwise from the upper left: leaf infected with parent strain PTBR 2.024; leaf treated with saline solution as a control; leaf infected with mutant PTBR 4.000; and leaf infected with merodiploid strain PTBR 4.005 (mutant PTBR 4.000 containing clone pQS409).

TABLE 2. *Pseudomonas* strains used in homology test

Lane in Fig. 7	Strain	Identification
2	<i>P. syringae</i> pv. <i>tabaci</i>	PT15
3	<i>P. syringae</i> pv. <i>angulata</i>	PA130
4	<i>P. syringae</i> pv. <i>angulata</i>	BF1a
5	<i>P. syringae</i> pv. <i>angulata</i>	PA108
6	<i>P. syringae</i> pv. <i>angulata</i>	PA52
7	<i>P. syringae</i> pv. <i>angulata</i>	PA3
8	<i>P. syringae</i> pv. <i>angulata</i>	PA150
9	<i>P. syringae</i> pv. <i>angulata</i>	PA149
10	<i>P. syringae</i> pv. <i>angulata</i>	PA13453
11	<i>P. syringae</i> pv. <i>angulata</i>	PA45
12	<i>P. syringae</i> pv. <i>angulata</i>	PA11
13	<i>P. fluorescens</i>	PF014
14	<i>P. syringae</i> pv. <i>tabaci</i>	WFvir79
15	<i>P. syringae</i> pv. <i>tabaci</i>	PT 14.000
16	<i>P. syringae</i> pv. <i>tabaci</i>	PT15373
17	<i>P. syringae</i> pv. <i>tabaci</i>	PT27881
18	<i>P. syringae</i> pv. <i>tabaci</i>	PT5
19	<i>P. syringae</i> pv. <i>tabaci</i>	PT 12.000
20	<i>P. syringae</i> pv. <i>tabaci</i>	PT13
21	<i>P. syringae</i> pv. <i>tabaci</i>	PT 11528
22	<i>P. syringae</i> pv. <i>tabaci</i>	PT4
23	<i>P. syringae</i> pv. <i>tabaci</i>	PT130
24	<i>P. syringae</i> pv. <i>tabaci</i>	PT 11527
25	<i>P. syringae</i> pv. <i>tabaci</i>	PT107
26	<i>P. syringae</i> pv. <i>mellea</i>	PM1.000
27	<i>P. syringae</i> pv. <i>syringae</i>	BSA.002
28	<i>P. syringae</i> pv. <i>syringae</i>	BSA.001
29	<i>P. cepacia</i>	PCE716
30	<i>P. putida</i>	PPG277
31	<i>P. syringae</i> pv. <i>coronafaciens</i>	PC27
32	<i>P. syringae</i> pv. <i>glycinea</i>	PG59
33	<i>P. syringae</i> pv. <i>tabaci</i>	PT124(42)
34	<i>P. syringae</i> pv. <i>tabaci</i>	PT 24.000
35	<i>P. syringae</i> pv. <i>tabaci</i>	PT3
36	<i>P. syringae</i> pv. <i>tabaci</i>	WFvir78
37	<i>P. syringae</i> pv. <i>tabaci</i>	WF77
38	<i>P. syringae</i> pv. <i>tabaci</i>	PT 11.000
39	<i>P. syringae</i> pv. <i>tabaci</i>	PTBR 2.024

analysis with various strains (Table 2) was done with pQS405 as a probe of digests of total DNA from other *P. syringae* pathovars and *Pseudomonas* species (Fig. 7). pQS405 hybridized to most of the *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata* strains, except strains PT 14.000, PT 12.000, PT 24.000, and PT 11.000. Strains PT 14.000 and PT 12.000 are nonpathogenic on tobacco, but strains PT 24.000

and PT 11.000 are pathogenic on tobacco. Strains PT 11528 and PT 11527 have sequences which are homologous to pQS405 but which are contained in *Eco*RI fragments larger than the 7.2-kb *Eco*RI fragment of strain BR2. There are small fragments (about 4.0 kb) that hybridize weakly to the 7.2-kb fragment in all strains of *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata*. Clone pQS405 hybridized to none of the other *Pseudomonas* pathovars or species.

DISCUSSION

The properties of the mutants (Nal^r Kan^r Str^r Cm^s), as well as the production of a fluorescent pigment on KB, demonstrated that the eight mutants were the result of Tn5 transposition from pGS9 onto the PTBR 2.024 chromosome. Of the eight nonpathogenic mutants, six contained a single Tn5 insertion. In one mutant, PTBR 5.000, an independent transposition of IS50 had occurred, and mutant PTBR 9.000 contained a second copy of Tn5. The evidence for this is the presence of three bands that hybridize to Tn5 in *Sal*I digests of PTBR 5.000 and four bands in *Sal*I digests of PTBR 9.000. The independent transposition of IS50 has been described (2, 3, 39, 45). Multiple insertions of Tn5 into the *E. coli* genome have also been observed (10, 18). For these two mutants, it is not known which of the two insertions caused the loss of pathogenicity on tobacco.

Only two (PTBR 3.000 and PTBR 7.000) of the eight mutants failed to produce tabtoxin or tabtoxinine- β -lactam when assayed against *Salmonella typhimurium*, although the characteristic chlorotic halos were observed on bean plants inoculated with all eight mutants. The lack of toxin production by two mutants was confirmed by R. D. Durbin, University of Wisconsin-Madison (Durbin, personal communication). One possible explanation for these results is that the toxins are not produced by these two mutants in VB or tobacco plants, but that they are produced in bean plants. Alternatively, chlorosis in bean plants might be caused by a mechanism not involving tabtoxin or tabtoxinine- β -lactam, such as a different toxin that does not inhibit *S. typhimurium*. Since we did not attempt to isolate toxins from infected bean plants, we do not know which, if either, of these explanations is correct. Other work (11), indicating that Tox⁻ mutants of *P. syringae* pv. *tabaci* retain pathogenicity, suggests that toxin production does not appear to be essential for pathogenicity. Similar results have also been observed with other *P. syringae* pathovars. For example, mutants of *P. syringae* pv. *phaseolicola* that fail to produce phaseolotoxin are still pathogenic for bean plants (20, 30,

FIG. 7. Detection of sequences homologous to the 7.2-kb *Eco*RI fragment in various *Pseudomonas* species. Total DNA was isolated, digested with *Eco*RI, and analyzed by Southern hybridization with ³²P-labeled pQS405 as described in Materials and Methods. The strains are listed in Table 2 by lane number. Lane 1 is a lambda DNA *Hind*III digest (fragment sizes are given in kilobases).

32). There are conflicting results (44), however, which suggest that *Tox⁻* mutants of *P. syringae* pv. *tabaci* are still pathogenic. Our results indicate that toxin production by that organism does not necessarily lead to the production of symptoms. An alternative to the direct role of toxins in disease is the suggestion that toxins are produced by phytopathogenic bacteria to prevent coinfection of a plant by related bacteria (37, 47).

PTBR 4.000 penetrates and multiplies in intercellular spaces of tobacco leaf tissue to approximately the same level as PTBR 2.024 does, but in contrast to the parent, it does not survive (Fig. 5). One possible explanation is that an initial source of nutrients is available to the mutant, but that this is eventually exhausted, and other nutrient sources that can be accessed by the parent are not available to the mutant, whose population therefore declines. An alternative explanation is that the mutants are unable to protect themselves from plant defense mechanisms. Our results suggest that the production of disease symptoms is not dependent solely on the size of the bacterial population in the leaves, but might require that the population be maintained for some period. A decline in the number of viable cells of the nonpathogenic mutants of *P. syringae* pv. *syringae* (Mills et al., Abstr. 3rd Int. Symp. Mol. Genet. Plant-Microbe Interactions) in host tissues has also been observed. However, there are other examples (25, 42, 45) of nonpathogenic mutants of *P. syringae* pathovars that appear to be unable to reach the population levels of their pathogenic parents in plant tissues, and so in these instances, the population level might be a factor in symptom production.

Lack of hybridization of the six *Eco*RI fragments in clones pQS405, pQS1212, and pQS2822 to the Tn5-containing fragments from the other seven mutants demonstrates that the 7.2-kb *Eco*RI fragment is located at least 23 kb in one direction and 26 kb in the other direction from the other seven mutants. Since sequences complementary to the other seven mutant sequences have not been isolated from the parental genomic library, we cannot comment on the organization of the other Tn5-containing sequences. Thus, unlike the pathogenicity genes from *P. syringae* pv. *phaseolicola* (25) and from *X. campestris* pv. *campestris* (46), which are clustered, at least one of the genes in PTBR 2.024 is removed from the others. pQS409, which contains the parental 7.2-kb fragment, restored the pathogenicity of mutant PTBR 4.000 on tobacco by complementation tests, but did not complement the other seven mutants. This supports the structural evidence indicating that none of the other mutations map in close proximity to the 7.2-kb *Eco*RI fragment. The isolation of parental DNA clones complementary to the other seven mutants and cross-hybridization among those clones will reveal the organization of those genes.

Hybridization indicated that only strains of *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata* contain sequences homologous to the 7.2-kb *Eco*RI fragment from PTBR 2.024. In most strains, hybridization occurred to fragments of the same size, but in two strains, hybridization took place with larger *Eco*RI fragments (about 17 kb). This indicates that sequences on the 7.2-kb *Eco*RI fragment are conserved in strains of *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata*, but there are sequence polymorphisms among the strains. The weak hybridization to the small fragment (4.0 kb) suggests the possibility of repeated sequences in the 7.2-kb *Eco*RI fragment. Two of the *P. syringae* pv. *tabaci* strains that do not contain sequences homologous to the 7.2-kb *Eco*RI fragment were nonpathogenic on tobacco. However, two other strains lacking the homology retained

pathogenicity on tobacco. There are no obvious explanations for the lack of hybridization, but it might suggest that the gene(s) on the 7.2-kb *Eco*RI fragment are not essential for pathogenesis in certain strains or that those strains have alternative mechanisms for overcoming the absence of the gene(s). Since most of the isolates of *P. syringae* pv. *tabaci* and *P. syringae* pv. *angulata* we tested were isolated from tobacco plants in the field, the 7.2-kb *Eco*RI fragment may encode host specificity functions (13) on tobacco. In addition, the observation that all of the eight mutants caused wildfire symptoms indistinguishable from those caused by the parent on bean plants supports the suggestion that all of the eight mutated genes are involved in host specificity on tobacco plants. It is likely that the mechanism of host specificity is a complex process and involves many genes including host range and avirulence genes (13). For example, Daniels et al. (9) failed to isolate a pathovar specificity gene(s) from *X. campestris* pathovars by cross-transferring cosmid clones from genomic libraries.

The results obtained from the present study suggest that the genes inactivated by Tn5 insertion of eight mutants are specifically involved only in the pathogenicity on tobacco. This hypothesis must be supported by the isolation of a set of genes involved in host specificity on bean plants that is different from those involved in host specificity on tobacco plants when Tn5 insertion mutants are initially screened on bean leaves.

ACKNOWLEDGMENTS

We thank V. N. Iyer, B. J. Staskawicz, B. W. Kemper, and M. A. Schuler for generous gifts of plasmid vectors and strains. We are also indebted to R. D. Durbin for the tabtoxin assay of the toxin mutants. We acknowledge A. Hepburn and S. Farrand for their comments and suggestions in preparing the manuscript.

This work was funded in part by Biomedical Research Support Grant PHS RR-7030 and by U.S. Department of Agriculture Competitive Grant 86-CRCR-1-2237.

LITERATURE CITED

1. Anderson, M. L. M., and B. D. Young. 1985. Quantitative filter hybridization, p. 73-111. In B. D. Hames and S. J. Higgins (ed.), *Nucleic acid hybridization: a practical approach*. IRL Press, Oxford.
2. Berg, D. E., L. Johnsrud, L. McDivitt, R. Ramabhadran, and B. J. Hirschel. 1982. Inverted repeats of Tn5 are transposable elements. *Proc. Natl. Acad. Sci. USA* 79:2632-2635.
3. Boucher, C., P. Barberis, A. Trigalet, and D. Demery. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: isolation of Tn5-induced avirulent mutants. *J. Gen. Microbiol.* 131:2449-2457.
4. Boyer, H. W., and D. Rouland-Dussoix. 1969. A complementation analysis for the restriction and modification of DNA in *Escherichia coli*. *J. Mol. Biol.* 41:459-472.
5. Chang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J. Bacteriol.* 134:1141-1156.
6. Chatterjee, A. K., and A. K. Vidaver. 1986. Genetics of pathogenicity factors: application to phytopathogenic bacteria. Academic Press, Inc., New York.
7. Chesney, R. H., J. R. Scott, and D. Vapnek. 1979. Integration of the plasmid prophages P1 and P7 into the chromosome of *Escherichia coli*. *J. Mol. Biol.* 130:161-173.
8. Daniels, M. J., C. E. Barber, P. C. Turner, M. K. Sawczyk, R. J. W. Byrde, and A. H. Fielding. 1984. Cloning of genes involved in pathogenicity of *Xanthomonas campestris* pv. *campestris* using the broad-host range cosmid pLAFR1. *EMBO J.* 3:3323-3328.
9. Daniels, M. J., P. C. Turner, C. E. Barber, M. K. Sawczyk, and

- F. Dums. 1985. Molecular pathology of *Xanthomonas*, p. 125–133. In John Innes Institute 73rd report covering the 2 years 1983–1984. The John Innes Institute, Norfolk, England.
10. De Bruijn, F. J., and J. R. Lupski. 1984. The use of transposon Tn5 mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids — a review. *Gene* 27:131–149.
11. Durbin, R. D. 1971. Chlorosis-inducing pseudomonad toxins: their mechanisms of action and structure, p. 369–385. In S. Akai and S. Ouchi (ed.), *Morphological and biochemical events in plant-parasite interactions*. Mochizuki Publishing Co., Tokyo, Japan.
12. Friedman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. M. Ausubel. 1982. Construction of a broad host range cosmid vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* 18:289–296.
13. Gabriel, D. W. 1986. Specificity and gene function in plant-pathogen interactions. *ASM News* 52:19–25.
14. Gough, J. A., and N. E. Murray. 1983. Sequence diversity among related genes for recognition of targets in DNA molecules. *J. Mol. Biol.* 166:1–19.
15. Gross, D. C., and A. K. Vidaver. 1981. Transformation of *Pseudomonas syringae* with nonconjugative R plasmids. *Can. J. Microbiol.* 27:759–765.
16. Grunstein, M., and D. S. Hogness. 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. USA* 72:3961–3965.
17. Hanahan, D., and M. Meselson. 1980. Plasmid screening at high colony density. *Gene* 10:63–67.
18. Harayama, S., E. T. Palva, and G. L. Hazelbauer. 1979. Transposon insertion mutants of *Escherichia coli* K-12 defective in a component common to galactose and ribose chemotaxis. *Mol. Gen. Genet.* 171:193–203.
19. Holloway, B. W. 1974. Genetic organization of *Pseudomonas*, p. 133–161. In P. H. Clarke and M. H. Richmond (ed.), *Genetics and biochemistry of Pseudomonas*. John Wiley & Sons, Inc., New York.
20. Jamieson, A. F., R. L. Bielecki, and R. E. Mitchell. 1981. Plasmids and phaseolotoxin production in *Pseudomonas syringae* pv. *phaseolicola*. *J. Gen. Microbiol.* 122:161–165.
21. Johnston, J. B., and I. C. Gunsalus. 1977. Isolation of metabolic plasmid DNA from *Pseudomonas putida*. *Biochem. Biophys. Res. Commun.* 75:13–17.
22. Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff. 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. *Mol. Gen. Genet.* 177:65–72.
23. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyrocyanin and fluorescein. *J. Lab. Clin. Med.* 44:301–313.
24. Lennox, E. S. 1955. Transduction of linked genetic characters of the host bacteriophage P1. *Virology* 1:190–206.
25. Lindgren, P. B., R. C. Peet, and N. J. Panopoulos. 1986. Gene cluster of *Pseudomonas syringae* pv. “*phaseolicola*” controls pathogenicity of bean plants and hypersensitivity on nonhost plants. *J. Bacteriol.* 168:512–522.
26. Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. Mead, D. A., E. Szczesna-Skorupa, and B. Kemper. 1986. Single-stranded DNA “blue” T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering. *Protein Eng.* 1:67–74.
28. Mills, D. 1985. Transposon mutagenesis and its potential for studying virulence genes in plant pathogens. *Annu. Rev. Phytopathol.* 23:297–320.
29. Obukowicz, M., and P. D. Shaw. 1985. Construction of Tn3-containing plasmids from plant-pathogenic pseudomonads and an examination of their biological properties. *Appl. Environ. Microbiol.* 49:468–473.
30. Peet, R. C., P. B. Lindgren, D. K. Willis, and N. J. Panopoulos. 1986. Identification and cloning of genes involved in phaseolotoxin production by *Pseudomonas syringae* pv. “*phaseolicola*.” *J. Bacteriol.* 166:1096–1105.
31. Piwowarski, J. M., and P. D. Shaw. 1982. Characterization of plasmids from plant pathogenic pseudomonads. *Plasmid* 7:85–94.
32. Quigley, N. B., D. Lane, and P. L. Bergquist. 1985. Genes for phaseolotoxin synthesis are located on the chromosome of *Pseudomonas syringae* pv. *phaseolicola*. *Curr. Microbiol.* 12:295–300.
33. Ribeiro, R. de L. D., D. J. Hagedorn, R. D. Durbin, and T. F. Uchytel. 1979. Characterization of the bacterium inciting bean wildfire in Brazil. *Phytopathology* 69:208–212.
34. Rodriguez, R. L., and R. C. Tait. 1983. Recombinant DNA techniques: an introduction, p. 50–51. The Benjamin/Cummings Publishing Company, Inc., Reading, Mass.
35. Selvaraj, G., and V. N. Iyer. 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. *J. Bacteriol.* 156:1292–1300.
36. Selvaraj, G., and V. N. Iyer. 1984. Transposon Tn5 specifies streptomycin resistance in *Rhizobium* spp. *J. Bacteriol.* 158:580–589.
37. Smidt, M. L., and A. K. Vidaver. 1982. Bacteriocin production by *Pseudomonas syringae* PsW-1 in plant tissue. *Can. J. Microbiol.* 28:600–604.
38. Staskawicz, B. J., D. Dahlbeck, and N. T. Keen. 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max* (L.) Merr. *Proc. Natl. Acad. Sci. USA* 81:6024–6028.
39. Staskawicz, B. J., D. Dahlbeck, J. Miller, and D. Damm. 1983. Molecular analysis of virulence genes in *Pseudomonas solanacearum*, p. 345–350. In A. Puhler (ed.), *Molecular genetics of the bacteria-plant interaction*. Springer-Verlag KG, Berlin.
40. Stewart, W. W. 1971. Isolation and proof of structure of wildfire toxin. *Nature (London)* 229:174–178.
41. Taylor, P. A., H. K. Schnoes, and R. D. Durbin. 1972. Characterization of chlorosis-inducing toxins from *Pseudomonas* sp. *Biochim. Biophys. Acta* 286:107–117.
42. Trigalet, A., and D. Demery. 1986. Invasiveness in tomato plants of Tn5-induced avirulent mutants of *Pseudomonas solanacearum*. *Physiol. Mol. Plant Pathol.* 28:423–430.
43. Turner, J. G. 1981. Tabtoxin, produced by *Pseudomonas tabaci*, decreases *Nicotiana tabacum* glutamine synthetase and causes accumulation of ammonia. *Physiol. Plant Pathol.* 19:57–67.
44. Turner, J. G., and R. R. Taha. 1984. Contribution of tabtoxin to the pathogenicity of *Pseudomonas syringae* pv. *tabaci*. *Physiol. Plant Pathol.* 25:55–69.
45. Turner, P., C. Barber, and M. Daniels. 1984. Behavior of the transposons Tn5 and Tn7 in *Xanthomonas campestris* pv. *campestris*. *Mol. Gen. Genet.* 195:101–107.
46. Turner, P., C. Barber, and M. Daniels. 1985. Evidence for clustered pathogenicity genes in *Xanthomonas campestris* pv. *campestris*. *Mol. Gen. Genet.* 199:338–343.
47. Vidaver, A. K. 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. *Annu. Rev. Phytopathol.* 14:451–465.
48. Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259–268.
49. Vogel, H. J., and D. M. Bonner. 1956. Acetyl ornithinase of *Escherichia coli*: partial purification and some properties. *J. Biol. Chem.* 218:97–106.
50. Werneke, J. M., S. G. Sligar, and M. A. Schuler. 1985. Development of broad-host-range vectors for expression of cloned genes in *Pseudomonas*. *Gene* 38:73–84.
51. Wood, W. B. 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. *J. Mol. Biol.* 16:118–133.