JOURNAL OF BACTERIOLOGY, Feb. 1988, p. 617–622
0021-9193/88/020617-06$02.00/0

Vol. 170, No. 2

Molecular Cloning of Genes That Specify Virulence in *Pseudomonas solanacearum*

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Received 2 July 1987/Accepted 5 November 1987

The suicide plasmid pSUP2021 was used to introduce Tn*5* into the *Pseudomonas solanacearum* wild-type strain K60. We isolated eight avirulent mutants after screening 6,000 kanamycin-resistant transconjugants by inoculating eggplant (*Solanum melongena* L. cv. Black Beauty) and tobacco (*Nicotiana tabacum* L. cv. Bottom Special) seedlings. The Tn*5*-containing *Eco*RI fragments from the eight mutants were unique, suggesting that numerous genes specify virulence in this species. These *Eco*RI fragments were cloned into pBR322 or pUC12, and one of the clones, pKD810, was transformed into K60. All of the kanamycin-resistant, ampicillin-sensitive transformants were avirulent. Three randomly selected avirulent transformants were shown to carry the Tn*5*-containing fragment in place of the wild-type fragment and to exhibit the same hybridization pattern as the original KD810 mutant did. With pKD810 as a probe, we identified cosmids carrying the wild-type virulence genes by using a genomic library of K60 prepared in pLAFR3. Two of the homologous cosmids, pL810A and pL810C, when introduced into KD810 by transformation, restored virulence and normal growth of this mutant in tobacco. Altogether, these data indicate that the gene(s) interrupted by Tn*5* insertion in KD810 is essential for the virulence of *P. solanacearum*. Further characterization of this gene is now being completed by subcloning, transposon mutagenesis, and complementation analysis.

Pseudomonas solanacearum E. F. Sm. causes bacterial wilt, one of the most destructive plant diseases worldwide. Plants that are affected belong to more than 30 families, including many important crops (6). Wilt induction by virulent strains of *P. solanacearum* (e.g., K60) has been attributed in part to the production of slime, which is composed mostly of extracellular polysaccharide (EPS) (11). Avirulent, slimeless variants are produced rapidly in culture and can be detected easily by their colony appearance on a medium containing tetrazolium chloride (11). Some of these variants (e.g., B1) not only lack EPS, but also have a defective lipopolysaccharide (9, 21) and exhibit numerous physiological changes compared with the wild-type parents. In strain B1, one of the most interesting changes is the acquisition of the ability to cause a hypersensitive reaction (HR) in tobacco leaves (12, 16). The HR is a plant defense reaction and is characterized by very rapid (12-h) collapse of mesophyll cells that come in contact with a potential pathogen, effectively delimiting the infected area. Strains that are virulent on tobacco, such as K60, cause no overt symptoms within 48 h after inoculation, but are not contained within the initial site of inoculation and, in time, may cause wilting of the entire plant. The pleiotropic nature of the spontaneous-HR-inducing variants has made it difficult to use them for a detailed molecular analysis of changes associated with loss of virulence.

The development of techniques for mutagenesis with transposable elements has made it possible to generate more defined, avirulent mutants of *P. solanacearum* (2, 4, 14, 19). Suicide plasmids, such as pSUP2021, can be used to mobilize Tn*5* into wild-type strains of *P. solanacearum*, such as K60. The resulting disrupted gene can be cloned in *Escherichia coli* by conventional techniques, and the corresponding virulence gene can be identified in a gene library of the wild type. The information acquired from gene replacement and complementation can be used to verify the functions of the gene being cloned. We have used this approach to clone eight distinct genetic loci that appear to specify virulence in *P. solanacearum*. In this article we describe the molecular analysis of one of these clones.

MATERIALS AND METHODS

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

Media and antibiotics. *P. solanacearum* was routinely cultured in CPG medium (9) at 28°C, and mutants were grown in CPG medium containing 0.05% 2,3,5-triphenyltetrazolium chloride and 1.8% agar (TZC medium) (11). For transformation, bacteria were grown in minimal broth or minimal agar (MM) by the method of Boucher et al. (2). MAS minimal medium was used to screen auxotrophs, and MANY medium was used for matings, as described by Anderson and Mills (1). A modified selective medium, Sm-1 (8), was used to isolate Tn*5* mutants and was prepared by adding the following to TZC medium: crystal violet, 2.5 μg/ml; polymyxin B sulfate, 2 μg/ml; tyrothrycin, 20 μg/ml; chloramphenicol, 125 μg/ml; and cycloheximide, 50 μg/ml. In addition, the following antibiotics were added as required: kanamycin, 25 μg/ml; tetracycline, 15 μg/ml; and ampicillin, 50 μg/ml.

Chemicals and reagents. Restriction enzymes, enzymes and buffers for T4 DNA ligation, and a nick translation reagent kit were obtained from Bethesda Research Laboratories, Inc. Lysozyme, calf intestine alkaline phosphatase, and RNase were from Sigma Chemical Co. Gigapack Plus extract was from Stratagene. Zeta-probe membrane was from Bio-Rad Laboratories. Nitrocellulose was from Schleicher & Schuell, Inc.

Transposon mutagenesis. The recipient strain, *P. solanacearum* K60, and the donor strain, *E. coli* SM10(pSUP2021),

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TABLE 1. Bacteria and plasmids used in this work

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
HB101	*proA2 recA13 hsdS20 ara-14* r_K^- m_K^- F^- *galK2 rpsL20* (Str) *supE44 leu-6*	13
TB1	r_K^- m_K^+ *lacZ* (M15)	J. Jessee
SM10	*thi thr leu supF chr* RP4-2 (Tc::Mu)	18
P. solanacearum		
K60	Vir^+ EPS^+ Km^s wild type	A. Kelman
KD810	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD600	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD688	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD1358	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD1506	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD1602	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD300	K60::Tn*5* Vir^- EPS^+ Km^r	This paper
KD500	K60::Tn*5* Vir^- EPS^- Km^r	This paper
KD400	K60::Tn*5* Vir^+ EPS^- Km^r	This paper
KD700	K60::Tn*5* Vir^+ EPS^- Km^r	This paper
C810-A, C810-B, C810-C	KD810 carrying pL810, Km^r Tc^r	This paper
Plasmids		
pBR322	Ap^r Tc^r	13
pUC12	Ap^r	20
ColE1::Tn*5*	Km^r	13
pLAFR3	IncP1 Tc^r *rlx*$^+$	15
pSUP2021	(pBR325 *mob*)::Tn*5* Km^r Amp^r	18
pKD810	pUC12 carrying 13.6-kb *Eco*RI fragment from KD810 Am^r Km^r	This paper
pL810-A, pL810-B, pL810-C	pLAFR3 containing K60 genomic DNA, Tc^r	This paper

were grown to log phase (10^8) in MANY medium and LB broth, respectively, and mixed at a ratio of 5:1. The suspensions were centrifuged for 5 min at full speed in a Beckman Microfuge (model 11), and the pellets were incubated at 15°C for 6 h to allow mating between donor and recipient before the cells were plated on Sm-1 containing kanamycin (25 μg/ml). The plates were incubated at 28°C; Km^r colonies were visible after 48 h. Km^r colonies were then transferred to TZC and MAS plates for further characterization.

DNA isolation. To isolate total genomic DNA, strain K60 was grown in CPG medium to 10^8 cells per ml and harvested by centrifugation. The pellet was washed once with TE buffer (10 mM Tris, 10 mM EDTA [pH 8.0]), and the cells were suspended in the same buffer but containing 1 mg of lysozyme per ml. After incubation for 30 min at 37°C, the cells were lysed by addition of sodium dodecyl sulfate to 0.5% and NaCl to 2 M. The lysate was incubated for an additional 30 min at 55°C and then extracted twice with phenol-chloroform (1:1). The DNA was further purified by isopycnic centrifugation in CsCl-ethidium bromide.

To isolate recombinant plasmid DNA from strain K60, the procedures of Kado and Liu (10) and Morales and Sequeira (14) were followed. Mini- and large-scale isolations of plasmid DNA from *E. coli* were done essentially as described by Maniatis et al. (13).

Molecular cloning of Tn*5* flanking sequences. Total cellular DNA was digested with *Eco*RI and subjected to preparative electrophoresis in a 0.5% SeaPlaque, low-melting agarose (FMC Corp., Marine Colloids Div.) gel in Tris-acetate buffer. The DNA bands in the appropriate size range were excised and heated to 68°C in 0.3 M sodium acetate buffer (pH 5.5) at a volume sufficient to give a final agarose concentration of 0.2%. Agarose was removed by phenol extraction. DNA was precipitated with ethanol, dried, and redissolved in distilled water at a concentration of about 100 μg/ml. Then 5 μl of this DNA solution was mixed with 0.5 μg of *Eco*RI-digested and dephosphorylated pBR322 and pUC12 DNA in the presence of T4 DNA ligase overnight at 12°C (13). The ligated DNA was used to transform *E. coli* HB101 or TB1 cells by the procedure described below. Transformants were selected on LB medium containing kanamycin and ampicillin.

Construction of a genomic library of strain K60. Total genomic DNA was partially digested with *Eco*RI. Reaction products containing *Eco*RI fragments in the size range of 15 to 30 kilobases (kb) were collected from a linear sucrose gradient (5% to 20%) after being centrifuged at 36,000 rpm for 6 h in a Beckman SW41 rotor. Approximately 2.5 μg of DNA was then ligated with 2.5 μg of the cosmid vector pLAFR3 which had been digested with *Eco*RI and treated with alkaline phosphatase as described above. The recombinant plasmid DNA was packaged in lambda phage heads in vitro by using the Gigapack packaging extracts, and phage particles were used to transduce competent *E. coli* TB1 cells to tetracycline resistance on LB medium.

Transformation of *P. solanacearum*. The cosmid clones carrying the wild-type virulence sequences, as determined by Southern hybridization analysis, were used to transform the Tn*5*-containing avirulent mutants by the following procedure. Log-phase cells of the appropriate mutant of *P. solanacearum* were pelleted by centrifugation, washed once with a solution containing 100 mM $CaCl_2$, 50 mM $MgCl_2$, and 10 mM $MnCl_2$ at 4°C, and resuspended in the same solution. These cells, which can be stored at −70°C for several weeks without losing competency, were mixed with cosmid DNA (0.01 to 1.00 μg) and then spotted on MM plates containing 0.1 M $CaCl_2$. The plates were kept at 4°C for 1 h and then incubated at 28°C for 2 days. After incubation, the cells were suspended in distilled water and plated out on TZC medium containing kanamycin and tetracycline.

To transform the wild-type strain K60 with Tn*5*-containing plasmid DNA, we used the method described by Boucher et al. (3).

DNA hybridization techniques. Nick translation, Southern blotting, DNA hybridization, and colony hybridization were performed as recommended by Maniatis et al. (13). DNA was hybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 0.5% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml at 68°C for 16 h; washing solutions contained 2× SSC and 0.5% sodium dodecyl sulfate at 65°C.

Plant pathogenicity tests. The method to test for pathogenicity was similar to that used by Anderson and Mills (1). The initial screening was carried out with individual seedlings of eggplant (*Solanum melongena* L. cv. Black Beauty) grown aseptically in test tubes containing 10 ml of full-strength Hoagland mineral solution in agar. The plants were grown for 2 weeks at 28°C under fluorescent lighting in a growth chamber. For inoculation, the primary leaves were wounded by being cut with a scalpel and then rubbed with cotton swabs saturated with a heavy suspension of *P. solanacearum* (about 10^9 cells per ml). The inoculated plants were left in the growth chamber for an additional 4 to 5 days and then scored for disease development. Each strain that gave a negative response was tested again on four plant seedlings. We subjected mutants to further checks by inoculating individual tobacco (*Nicotiana tabacum* L. cv. Bottom Special) seedlings by the stem inoculation procedure described by Bowman and Sequeira (5).

The growth of *P. solanacearum* mutants in leaf tissues was determined after infiltrating tobacco leaves with bacterial suspensions, as described by Sequeira and Hill (17).

RESULTS

Isolation and characterization of Tn*5*-induced mutants. The transposable element Tn*5* was conjugally transferred to *P. solanacearum* K60 from *E. coli* SM10 by means of the suicide plasmid pSUP2021. This plasmid is a derivative of pBR325 that carries a kanamycin resistance (Km^r) determinant in Tn*5* and is unable to replicate in bacteria other than *E. coli* (18). SM10 has the RP4 *tra* genes integrated in its chromosome; consequently it can mobilize plasmids such as pSUP2021, which carry the IncP1 *mob* site. The Sm-1 medium containing kanamycin effectively selected *P. solanacearum* Tn*5* exconjugants and did not permit growth of the donor cells. Approximately 6,000 Km^r Amp^s exconjugants were obtained. Of these, about 1.5% failed to grow on MAS minimal medium and were not studied further. The remaining Km^r Amp^s exconjugants were screened for loss of virulence by the eggplant seedling assay. Eight mutants failed to cause disease in eggplant seedlings (Table 1). In CPG medium, the eight avirulent mutants had growth rates comparable to those of the parent strain, K60. However, when these mutants were infiltrated into tobacco leaves, the growth pattern was as expected for the avirulent phenotype (Fig. 1). Populations of the avirulent strains declined slowly after infiltration. In contrast, populations of the virulent parent strain increased rapidly after an initial lag period of 12 to 24 h. All the avirulent mutants caused a typical HR^- reaction on tobacco leaves.

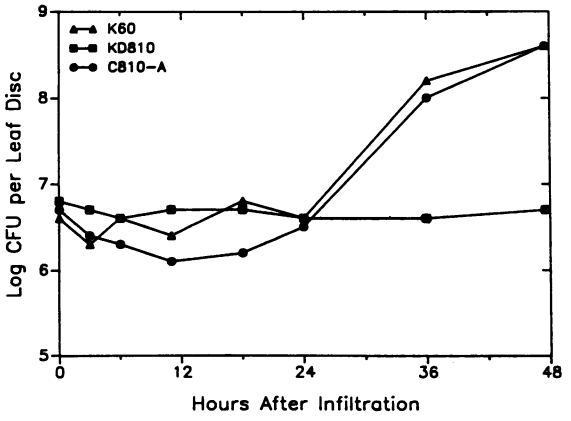

FIG. 1. Growth pattern of *P. solanacearum* wild-type strain K60, avirulent mutant KD810, and complemented mutant C810-A in tobacco leaves. Leaf disks were 0.78 cm^2. Results are based on the average of three determinations.

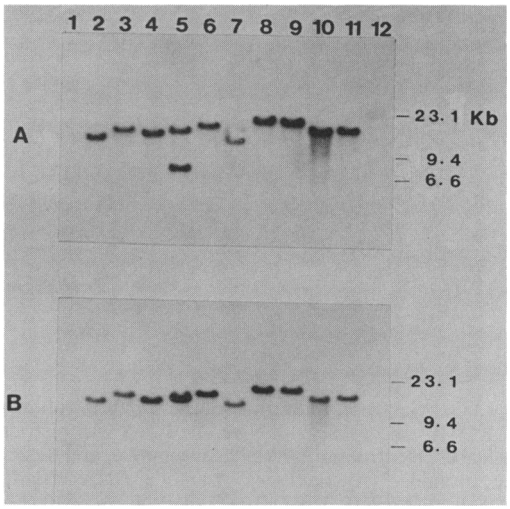

FIG. 2. Southern hybridization analysis of DNA of Km^r transconjugants digested with *Eco*RI. The lanes contain DNA of *P. solanacearum*: 1, K60; 2, KD1358; 3, KD1506; 4, KD688; 5, KD810; 6, KD300; 7, KD400; 8, KD500; 9, KD600; 10, KD700; 11, KD1602. DNA was electrophoresed in 0.5% agarose, transferred to Zeta-Probe membranes, and hybridized with ^{32}P-labeled ColE1::Tn*5* (A) or the 2.7-kb *Bgl*II fragment of Tn*5* (B). Lambda DNA digested with *Hin*dIII (lane 12) was used as a DNA size marker.

When grown on TZC medium, two of the avirulent mutants, KD300 and KD500, produced no extracellular polysaccharide (P. Xu et al., manuscript in preparation). The colony morphology of the remaining six mutants, KD688, KD810, KD600, KD1358, KD1506, and KD1602 resembled that of the parental strain, K60. In addition, two Km^r mutants, KD400 and KD700, had a rough colony morphology and did not produce EPS on TZC medium or in planta, but retained virulence on tobacco (Xu et al., in preparation).

Total genomic DNA was isolated from each of these 10 mutants and digested with *Eco*RI, which does not cleave within Tn*5*. The *Eco*RI-digested DNA was electrophoresed in an agarose gel, transferred to nitrocellulose, and probed with ^{32}P-labeled ColE1::Tn*5* DNA (Fig. 2A) or with the internal *Bgl*II fragment of Tn*5* (Fig. 2B). Each of the mutants contained a single Tn*5* insertion, and each of these insertions occurred in a different part of the genome, as evidenced by the unique sizes of Tn*5*-carrying *Eco*RI fragments which ranged from 12 to 18 kb. Different-sized fragments were also observed for each mutant when they were digested with *Eco*RI and *Bam*HI (data not shown). Two hybridizing *Eco*RI

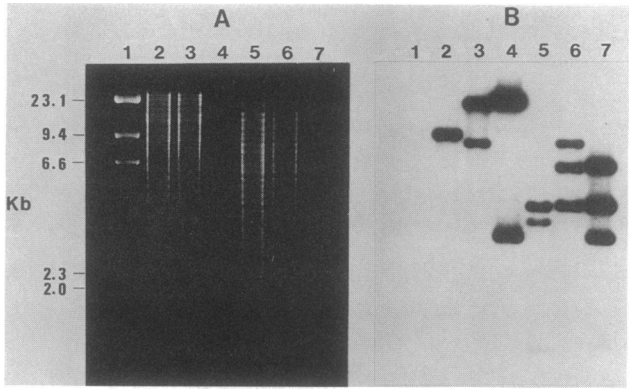

FIG. 3. Gel-electrophoretic and Southern hybridization analyses of K60, KD810, and pKD810 DNA. The DNA was restricted with endonucleases, electrophoresed in 0.5% agarose, and detected with ethidium bromide. (A) Lanes: 1, Lambda DNA (*Hin*dIII) size marker; 2, K60 (*Eco*RI); 3, KD810 (*Eco*RI); 4, pKD810 (*Eco*RI); 5, K60 (*Eco*RI-*Bam*HI); 6, KD810 (*Eco*RI-*Bam*HI); 7, pKD810 (*Eco* RI-*Bam*HI). (B) Autoradiogram of panel A probed with ^{32}P-labeled pKD810.

fragments were visible in KD810. When probed at high stringency with the internal *Bgl*II fragment of Tn*5*, which lacks IS*50* sequences, only one of the bands was hybridized (Fig. 2B). This suggests that the other band (7.4 kb) was the result of an IS*50* insertion or contained partially deleted plasmid sequences.

Molecular cloning of the Tn*5*-carrying *Eco*RI fragments. DNA isolated from the eight Tn*5*-induced, avirulent mutants was digested with *Eco*RI, and ligated to *Eco*RI-cleaved pBR322 or pUC12. The recombinant plasmids were transformed into *E. coli* HB101 or TB1, and transformants were identified by selection for Km^r and Amp^r. Each clone was found to have a unique restriction map; no two clones contained restriction fragments of the same size (data not shown). One of the avirulent mutants, KD810, was selected at random and studied in detail. Ligation of the 13.6-kb *Eco*RI fragment carrying Tn*5* in KD810 with pUC12 resulted in the plasmid pKD810, which was used as a probe for Southern hybridization analysis of DNA from K60 and KD810 (Fig. 3). As expected, *Eco*RI fragments of 7.8 and 13.6 kb were detected in DNAs of K60 and KD810, respectively. When digested with *Eco*RI and *Bam*HI, all the fragments in DNA from KD810 that hybridized with pKD810 were identical, except for the 7.4-kb fragment mentioned above and the 2.7-kb pUC12 vector (Fig. 3), indicating that no gross DNA rearrangements had occurred in the cloning process.

Marker exchange. To determine whether the loss of virulence was induced by Tn*5* insertion, marker exchange experiments were carried out by transforming pKD810, the plasmid carrying the 13.6-kb Tn*5*-containing *Eco*RI fragment from mutant KD810, into strain K60. Twenty independent Km^r transformants were scored for pathogenicity on tobacco leaves. The majority (70%) of these transformants failed to cause disease and were sensitive to ampicillin, but 30% retained the disease-causing ability of the parental strain and were resistant to ampicillin.

Total DNA was isolated from three randomly selected avirulent transformants, digested with *Eco*RI plus *Bam*HI, electrophoresed, blotted, and probed with pKD810. All of the avirulent transformants showed a hybridization pattern that was identical to that of the original KD810 mutant, except for the absence of the IS*50*-containing fragment (data not shown). These results indicated that marker exchange had occurred between the wild-type sequence in the transforming DNA and the K60 genome by a homologous double crossover event.

The positive correlation between the avirulent phenotype and insertion of Tn*5* in the same *Eco*RI fragment in these transformants strongly suggested that a virulence gene is located in the portion of the genome interrupted by Tn*5* insertion and not in the portion interrupted by IS*50* in KD810. Additional supporting evidence was derived from the observation that transformants remained virulent when pKD810 was integrated by a single crossover event (data not shown). In either case, the wild-type gene was preserved intact in these transformants, and they retained the virulent phenotype.

Identification of cosmid clones containing the corresponding virulence gene. Total DNA from strain K60 was partially digested with *Eco*RI and ligated into cosmid vector pLAFR3. Agarose gel electrophoresis of cosmid DNA from 12 randomly chosen clones showed that the average insert size was 25 kb. The K60 library contained 1,200 clones, which ensured a greater than 99.9% probability that any given gene was represented.

Cosmid clones homologous to eight of the Tn*5*-containing *Eco*RI fragments were identified in the K60 genomic DNA library by colony hybridization. Unique sets of cosmids were found to be homologous to each of the Tn*5* clones. When the 13.6-kb Tn*5*-containing *Eco*RI fragment from pKD810 was used as a probe, three cosmid clones were identified (see Fig. 5 for restriction maps). Southern hybridization of these cosmids with the ^{32}P-labeled 13.6-kb *Eco*RI fragment as a probe indicated a 7.8-kb *Eco*RI fragment, which was present in all three cosmids, as the wild-type hybridizing sequence (data not shown).

The three cosmids were individually transformed into KD810 by selection for Tc^r and Km^r cells, and transformants were tested for virulence on tobacco. All the transformants carrying pL810-A or pL810-C caused wilting of tobacco (Fig. 4), but none of the transformants carrying pL810-B or pLAFR3 were capable of complementing the avirulent phenotype of KD810. The simplest explanation for this observation is that the virulence gene, or operon, is located in the 2.1- and 9.8-kb *Eco*RI fragments that are common to pL810-A and pL810-C, but is absent from pL810-B (Fig. 5). Evi-

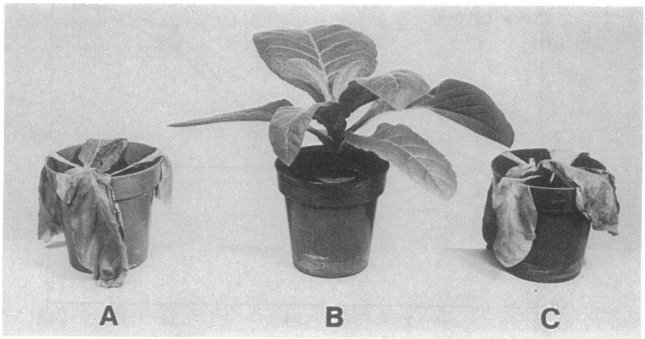

FIG. 4. Symptoms obtained in *N. tabacum* L. cv. Bottom Special seedlings 7 days after inoculation with *P. solanacearum* strains. (A) K60 (wild type); (B) KD810 (Tn*5*-induced mutant); (C) C810-A (KD810 complemented by transformation with the cosmid pL810-A).

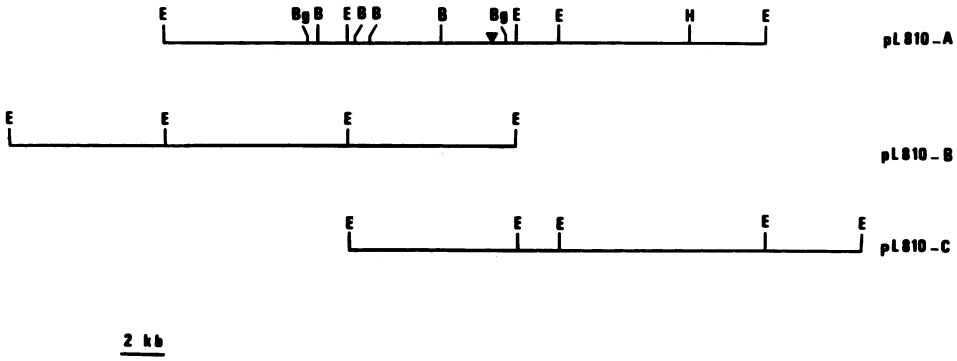

FIG. 5. Restriction endonuclease cleavage map of cosmids that are homologous with pKD810. Symbols: ▼, location of the Tn*5* insertion in pKD810. E, *Eco*RI; B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII.

dently, the presence of the common 7.8-kb *Eco*RI fragment is not sufficient for expression of the virulence gene.

The entire complementing plasmid could be easily recovered from Tc^r and Km^r transformants carrying any of the three pL810 clones or pLAFR3. The restriction patterns of the plasmid recovered from transformants did not change, indicating that no DNA rearrangements had occurred during growth in the host and that restoration of virulence was probably due to complementation and not to gene replacement. The methods used, however, would not have detected a low frequency of DNA exchange between homologous sequences in the chromosome and complementing plasmid.

When compared with the parental wild-type, virulent strain (K60), mutant KD810 showed reduced growth in host tissues but not in CPG medium. In contrast, the growth pattern of C810-A in tobacco leaves was identical to that of K60 (Fig. 1). These data provide further evidence that pL810-A is capable of fully complementing the virulence function that is missing in the mutant KD810.

DISCUSSION

The use of the suicide plasmid pSUP2021 allowed us to obtain a large number of mutants that resulted from apparently random insertion of Tn*5* in the genome of *P. solanacearum* K60. Of 6,000 Km^r transconjugants screened, 8 mutants were avirulent and prototrophic. Tn*5* insertions occurred at different loci and appear not to be closely linked, since common cosmid clones in the K60 gene library were not identified by hybridization with the different Tn*5*-containing *Eco*RI fragments as probes. The relatively high frequency of these types of mutants suggests, as previously reported by Boucher et al. (2), that numerous genes are essential for virulence in this pathogen. In this regard, our data differ from those of Boucher et al. (2), who reported that many genes controlling pathogenicity in *P. solanacearum* are clusted on a megaplasmid (3). We have used pKD810 to probe transfers of gel-electrophoresed genomic DNA prepared by the cell lysis method, which allows visualization of intact megaplasmid DNA. Hybridization to sheared chromosomal and plasmid DNA, as well as to intact plasmid DNA, was observed, suggesting that this gene may be plasmid borne (unpublished data). Further experiments are needed to clarify this point.

The value of transposon mutagenesis for the molecular analysis of virulence in *P. solanacearum* has been amply demonstrated by Boucher et al. (3) and Staskawicz et al. (19). Our work now extends the use of Tn*5* mutagenesis to the wild-type strain, K60, which has been used for more than three decades for studies of physiological and biochemical factors that are perceived to be important in pathogenesis. These include the production of pectinases, cellulases, auxins, and EPS (6). The availability of Tn*5*-generated avirulent mutants of K60 should allow an examination of the possible role of these factors in virulence of this strain.

The colony morphology of six of the avirulent mutants was indistinguishable from that of the wild-type strain, K60, which produces slimy, slightly pigmented colonies on TZC plates. The other two avirulent mutants produced afluidal, butyrous colonies with deep red pigmentation, similar to those produced by spontaneous variants such as B1. Since the production of EPS has been correlated with virulence in this plant-pathogenic bacterium, as well as in others (7, 11, 19), it is likely that insertion of Tn*5* in these two mutants (KD300 and KD500) occurred at a virulence locus involved in EPS production. This is in agreement with the results of Staskawicz et al. (19), who reported that all afluidal, Tn*5*-generated mutants of *P. solanacearum* S-82 were avirulent. It should be noted, however, that we obtained two K60 mutants (KD400 and KD700) that retained virulence to eggplant and tobacco at a range of inoculum levels, even though they appeared slimeless in plate culture. These mutants did not produce EPS in culture or in planta (Xu et al., in preparation). Boucher et al. (2) also have reported on Tn*5*-induced mutants of *P. solanacearum* that remained virulent to tomato plants but had the typical afluidal phenotype in culture. The role of EPS in pathogenicity of *P. solanacearum*, therefore, is questionable.

The eggplant leaf inoculation assay that was devised to screen for avirulent mutants is simple and reliable. Eggplant seeds germinate rapidly, and seedlings grow well on a simple agar medium containing mineral salts. The leaf inoculation procedure is rapid and efficient; the results are reproducible, and the plants, growing in test tubes, require very little space in the growth room. With this assay, more than 500 Tn*5*-containing transconjugants could be tested by one person in a single working day. It is important to note that with all the K60 mutants, loss of virulence for eggplant was always accompanied by loss of virulence for tobacco (data not shown). Similarly, restoration of virulence for eggplant by complementation was always accompanied by similar restoration of virulence for tobacco. Although in this report we have presented the data on marker exchange and complementation for only one mutant, similar work has been successfully completed with two additional mutants, KD400 and KD700 (data not shown). Thus, it is likely that all of the

Tn*5* insertions obtained in this study are causal to the avirulent phenotype observed. Moreover, the genes affected by Tn*5* insertion have a general function in virulence of *P. solanacearum* and are not likely to be involved in host specificity, because virulence to two different hosts was affected. It should be noted that strains of K60 exist in our culture collection that have spontaneously lost virulence on tomato but retained it on tobacco. This would argue for the existence of separate host-specific genes for virulence in *P. solanacearum*.

Marker exchange and complementation were used to characterize the virulence gene that was interrupted by Tn*5* insertion in one of the mutant strains, KD810. The introduction of pKD810, a plasmid containing the *Eco*RI fragment with the virulence gene interrupted by Tn*5* insertion, into the wild-type parent, K60, resulted in loss of virulence of many of the transformants. The phenotype of these Km^r transformants mutants was identical to that of KD810. In three randomly selected transformants, the mutation at the virulence locus replaced the wild-type sequence by homologous recombination. Only a small proportion of the Km^r transformants retained virulence for the host plant, and in all those that were examined, the target wild-type fragment was intact.

Our data clearly demonstrate that pLAFR3 and the corresponding derivatives containing DNA insertions from *P. solanacearum* were able to stably replicate and express in *P. solanacearum* cells. As a result, the cosmid clone containing the wild-type gene was able to fully restore virulence in strain KD810. At present, we are carrying out saturation transposon mutagenesis of the C810-A cosmid to identify the minimal DNA sequence that is required for expression of the virulence gene(s) in this clone. Parallel studies with the other mutants are also being completed.

We have described the identification and molecular cloning of genes that specify pathogenicity in *P. solanacearum*. It should now be possible to determine what functions are encoded by these genes by fine-structure molecular analysis and by examining the effects of these mutations on the physiology of this host-parasite interaction.

ACKNOWLEDGMENTS

We thank E. Barlow for assistance in the determination of growth rates of *P. solanacearum* in tobacco leaves. We also thank S. Vicen for valuable assistance in the preparation of the illustrations and K. D. Willis for helpful critical review of the manuscript.

This work was supported by project 1474 from the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by grant PCM-8403343 from the National Science Foundation.

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