MOLECULAR PLANT-MICROBE INTERACTIONS
VOL. 5, No. 4, pp. 322-329, 1992

Identification of a *lysA*-Like Gene Required for Tabtoxin Biosynthesis and Pathogenicity in *Pseudomonas syringae* pv. *tabaci* Strain PTBR2.024

Karen Engst and Paul D. Shaw

Department of Plant Pathology, University of Illinois at Urbana-Champaign, Urbana, IL 61801 U.S.A.
Received 28 February 1992. Accepted 6 April 1992.

***Pseudomonas syringae* pv. *tabaci* strain PTBR2.024 produces tabtoxin and causes wildfire disease on tobacco and green bean. PTBR7.000, a Tn*5* mutant of PTBR2.024, does not produce tabtoxin, is nonpathogenic on tobacco, and is prototrophic. A 3-kb fragment from a genomic library of the parent strain PTBR2.024 complemented both mutant phenotypes. This 3-kb fragment contains two open reading frames (ORFs), ORF1 and ORF2, and two truncated ORFs, ORF3 and ORF4. The Tn*5* insert in PTBR7.000 was mapped to ORF2, and complementation studies showed that an intact ORF2 was sufficient to restore tabtoxin production and pathogenicity. The deduced amino acid sequences of ORF2 and truncated ORF3 contain significant homology to bacterial lysine biosynthetic enzymes, diaminopimelate decarboxylase, and Δ^1-piperidine-2,6-dicarboxylate succinyl transferase, respectively. ORF2, however, is not required for lysine biosynthesis. We designated the sequence corresponding to ORF2 as gene *tabA* and propose that the product of *tabA* is an enzyme in the tabtoxin biosynthetic pathway that recognizes a substrate analogue of a compound in the lysine biosynthetic pathway.**

Additional keywords: *dapD*, GUG, *Pseudomonas syringae* pv. *tabaci* strain BR2.

Tabtoxin (Stewart 1971) is produced by several phytopathogenic pathovars of *Pseudomonas syringae* van Hall, including *P. s.* pv. *tabaci* (Wolf and Foster) Young *et al.* and *P. s.* pv. *coronafaciens* (Elliott) Young *et al.* This toxin is hydrolyzed to the biologically active form tabtoxinine-β-lactam (TβL) by peptidases present in host plants or other microorganisms (Levi and Durbin 1986). TβL inhibits glutamine synthetase (GS) (Thomas *et al.* 1983; Knight *et al.* 1986). In the host plant, this inhibition causes the accumulation of ammonia, which leads to the formation of chlorotic halos (Turner and Debbage 1982) that surround the necrotic lesions at sites of infection by *P. s.* pv. *tabaci* on tobacco (*Nicotiana tabacum* L.) and *P. s.* pv. *coronafaciens* on oats (*Avena sativa* L.) (Durbin 1982). Although TβL is associated with symptoms of wildfire disease on tobacco and halo blight disease on oats, it is thought not to be an essential component of the disease processes (reviewed in Durbin 1991). For example, *P. s.* pv. *angulata* Fromme and Murray is considered to be a spontaneous toxin-deficient (Tox^-) derivative of *P. s.* pv. *tabaci* (Braun 1937). It induces necrotic spots on tobacco leaves without producing chlorotic halos. Similarly, *P. s.* pv. *striafaciens* (Elliott) Young *et al.*, a spontaneous Tox^- derivative of *P. s.* pv. *coronafaciens* (Chatterjee and Vidaver 1986), is pathogenic on oats. These observations suggest that TβL production is not required for pathogenicity by the tobacco and oat isolates.

Nucleoide sequence data have been assigned the accession number M88485 by GenBank.

TβL, however, may play a pathogenic role in *P. s.* pv. *tabaci* strain BR2, a unique strain isolated from green bean (*Phaseolus vulgaris* L.) (Ribeiro *et al.* 1979). This strain contains a plasmid, pBPW1, that inhibits the ability of the bacterium to be pathogenic on tobacco. Strains of BR2 cured of plasmid pBPW1 are pathogenic on green bean and tobacco (Obukowicz and Shaw 1985). Salch and Shaw (1988) mutagenized one strain of BR2 free of pBPW1, PTBR2.024, with transposon Tn*5* and reported that two of the chromosomal mutants that are nonpathogenic (Pat^-) on tobacco are also defective in tabtoxin production. Similarly, Kinscherf *et al.* (1991) described 23 Tox^- mutants of strain BR2 that are also nonpathogenic on green bean. Three of the mutants resulted from Tn*5* insertions, and the remaining mutants were spontaneous. The Tox^- and Pat^- phenotypes of these spontaneous mutants were characterized and had chromosomal deletions in a region involved in tabtoxin production (Kinscherf *et al.* 1991). This region is also absent in the chromosomes of *P. s.* pv. *angulata* and *P. s.* pv. *striafaciens* (Kinscherf *et al.* 1991). These results demonstrated that although tabtoxin production is not a pathogenicity determinant for the tobacco and oat isolates, it is an essential factor of pathogenicity for *P. s.* pv. *tabaci* strain BR2. The biosynthetic pathway for tabtoxin is not well understood; however, isotope incorporation studies suggest that tabtoxin synthesis is associated with the bacterial lysine biosynthetic pathway (Unkefer *et al.* 1987; Roth *et al.* 1990).

In this study, we used mutant PTBR7.000, a Tn*5*-induced Tox^- Pat^- mutant of PTBR2.024 (Salch and Shaw 1988), to investigate the genotypic and phenotypic relationships between tabtoxin biosynthesis and pathogenicity. We report the identification of a gene (*tabA*) that is involved in tabtoxin biosynthesis and demonstrate that the expression of

this gene is also required for pathogenicity. We also provide the first genetic evidence for the association of tabtoxin and lysine biosynthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *Escherichia coli* (Migula) Castellani and Chalmers were grown in LB (Lennox 1955) or M9 minimal medium (Chang and Cohen 1978) at 37° C. *P. syringae* strains were grown in LB or VB (Vogel and Bonner 1956) medium at 30° C. *Salmonella typhimurium* (Loeffler) Castellani and Chalmers was grown in liquid VB medium at 37° C. Antibiotics were added to the media at the following concentrations for *E. coli* strains: tetracycline (Tet), 15 μg/ml; kanamycin (Kan), 50 μg/ml; ampicillin (Amp), 100–300 μg/ml. For *P. syringae* strains, the concentrations were Tet, 15 μg/ml; Kan, 100 μg/ml; Amp, 300 μg/ml.

Pathogenicity assays. Tobacco plants, cultivar Havana 48, were maintained in a greenhouse and used for pathogenicity tests. In some experiments, additional light was provided by quartz-halogen lamps. The inocula were grown in liquid VB medium overnight. Cell suspensions, approximately 50 μl, containing 10^6–10^8 colony-forming units (cfu)/ml, were inoculated into tobacco leaves by the infiltration method (Bertoni and Mills 1987). Cell suspensions containing 10^8 cfu/ml from strain BR2 or PTBR7.000 were used for induction of the hypersensitive response (HR) on plants (HR was observed within 12 hr after inoculation), whereas all experiments to complement the Pat$^-$ or Tox$^-$ phenotype were done with cell suspensions containing 10^6 cfu/ml. The presence or absence of symptoms on the plant leaves was recorded 2–7 days after inoculation.

Toxin production assays. Cells of *Pseudomonas* strains were grown in liquid VB medium overnight. Cell suspensions (5 μl) were applied to plates containing 5 ml of VB medium supplemented with histidine (0.1 mg/ml), 1% agar, and 0.15 ml of overnight cultures of the indicator strain, *S. typhimurium* strain TA1975. The plates were incubated overnight at 30° C. The presence or absence of zones of inhibition surrounding the *Pseudomonas* inoculum was recorded.

DNA isolation. Plasmid DNA was isolated by the methods of Maniatis *et al.* (1982) and Morelle (1989). Chromosomal DNA was isolated from a 5-ml overnight culture. The cell pellet was resuspended in 750 μl of buffer (50 mM glucose; 25 mM Tris-Cl, pH 8.0, and 10 mM EDTA), lysed by 120 μl of 0.25 M EDTA and 75 μl of 20% sodium

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference[b]
Pseudomonas syringae		
P. s. pv. *tabaci* PTBR2.004	Tox$^+$ Tox^r Nal^r HR$^+$ on tobacco, Pat$^+$ on green bean, derived from strain BR2	This laboratory
P. s. pv. *tabaci* PTBR2.024	Tox$^+$ Tox^r Pat$^+$ on tobacco and green bean, Nal^r Rif^r, plasmid-free strain of BR2	Obukowicz and Shaw 1985
P. s. pv. *tabaci* PTBR7.000	Tox$^-$ Tox^r Pat$^-$ and HR$^+$ on tobacco, Kan^r Nal^r Tn*5* insertion mutant of PTBR2.024	Salch and Shaw 1988
Escherichia coli		
MC1061	*hsdR mcrB araD139* Δ(*araABC-leu*)7679 Δ*lacX74 galU galK rpsL thi*	S. K. Farrand
403CBS140	*lysA*::Tn*5 thyA* Kan^r	J. E. Cronan
155X478	*proC32 purE42 metE70 lysA23 thi1 leu6 trpE68 lacZ36 mtl1 xyl5 ara14 azi6 tonA23 tsx67 str109*	J. E. Cronan
AT986	*dapD8 relA1 spoT1 thi-1* λ$^-$ *Hfr*	*E. coli* genetic stock center, Yale University
Salmonella typhimurium		
TA1975	*hsd* Rif^r	J. B. Johnston
Plasmids		
pWS3	Amp^r Kan^r Str^r	Werneke *et al.* 1985
pGEM11-Zf	Amp^r	Promega, Madison, WI
pRK415	Tet^r	D. K. Willis
pWS4kpn	Amp^r Str^r	Werneke *et al.* 1985
pWE702	22-kb *Eco*RI fragment from PTBR2.024 in pWS4kpn	This study
pWH704	*Hin*dIII fragment from pWE702 in pWS3	This study
pWX707	1.1- and 1.9-kb *Xho*I fragments from pWE702 in pWS3	This study
pWX708	3-kb fragment (0.8- and 2.2-kb *Xho*I fragments) from pWE702 in pWS3	This study
pGX708-1, pGX708-2	3-kb insert from pWX708 in pGEM11-Zf in different orientations	This study
pRBE708	3-kb insert from pGX708-2 in pRK415	This study
pWX709	1.9-, 0.8-, and 2.2-kb *Xho*I fragments from pWE702 in pWS3	This study
pWX710, pWX713	2.2-kb *Xho*I fragment from pWX708 in pWS3 in different orientations	This study
pWX711	0.8-kb *Xho*I fragment from pWX708 in pWS3	This study
pWH712	*Hin*dIII-*Xho*I fragment from pWX710 in pWS3	This study
pGNC714	*Nco*I-generated deletion in the 3-kb insert of pGX708-2	This study
pRBE714	Insert from pGNC714 in pRK415	This study
pGH715	*Hin*dIII fragment of pGX708-2 in the opposite orientation	This study
pGH716	pGX708-2 with the *Hin*dIII fragment deleted	This study

[a] Nal, nalidixic acid; Rif, rifampicin; Kan, kanamycin; Cam, chloramphenicol; Tet, tetracycline; Amp, ampicillin; Str, streptomycin; r, resistant; HR, hypersensitive response; Pat$^+$, pathogenic; Pat$^-$, nonpathogenic; Tox$^+$, produces tabtoxin; Tox$^-$, does not produce tabtoxin; Tox^r, tabtoxin resistant.

[b] S. K. Farrand, University of Illinois, Urbana; J. E. Cronan, University of Illinois, Urbana; J. B. Johnston, University of Illinois, Urbana; D. K. Willis, University of Wisconsin, Madison.

dodecyl sulfate (SDS), and diluted to the total volume of 3 ml. Cesium chloride (3 g) was directly added to this mixture, then Triton X-100 (36 μl of a 1% solution) and ethidium bromide (80 μl of a 10 mg/ml solution) were added. The mixture was centrifuged at 100,000 rpm for 4 hr in a TLN-100.3 rotor (Beckman Instruments, Inc., Fullerton, CA), and genomic DNA was isolated (Maniatis *et al.* 1982).

DNA hybridization. The DNA fragments used as hybridization probes were isolated by electroelution from agarose gels. DNA probes were labeled with [α-^{32}P]dCTP (ICN Biomedicals, Inc., Costa Mesa, CA) by nick translation or by random primer labeling with kits from Bethesda Research Laboratories (BRL, Gaithersburg, MD). DNA blots were generated with GeneScreen (New England Nuclear, Beverly, MA) under the transfer conditions recommended by the supplier. Hybridizations were done in 50% formamide at 42° C.

Transformation and transduction. Transformations of *E. coli* or *Pseudomonas* were done as described previously (Salch and Shaw 1988). Cosmid packaging and transduction to recipient cells were accomplished with the Packagene kit (Promega Biotec., Madison, WI) under the conditions recommended by the supplier. Tet^r (tetracycline resistant) and Kan^r transductants were selected on media containing the appropriate antibiotics.

Tn*5*seq1 mutagenesis. Tn*5*seq1 was constructed by Nag *et al.* 1988; it contains promoters from phages T7 and SP6 that allow bidirectional sequencing of DNA containing the transposon. *E. coli* MC1061(pWX708) was grown overnight in 2 ml of T broth containing tryptone, 10 g/L; NaCl, 5 g/L; maltose, 2 g/L; and ampicillin, 300 μg/ml. We harvested and resuspended the cells in 1 ml of 0.01 M $MgSO_4$ by shaking at 37° C for 30 min. Seventy microliters of a phage λ::Tn*5*seq1 suspension (2.4×10^7 plaque-forming units per microliter) was added to the cell suspension. After incubation at 30° C for 15 min, 9 ml of T broth containing ampicillin was added. All subsequent operations followed the procedures described by Nag *et al.* (1988).

DNA sequencing. Deaza sequencing mixes (Pharmacia, Piscataway, NJ) were used for double-stranded sequencing of both DNA strands as described by the supplier and by Hsiao (1991). Primers used for the bidirectional sequencing of the Tn*5*seq1 insertion mutants were supplied by U.S. Biochemical, Cleveland, OH, or by the University of Illinois Biotechnology Center, Genetic Engineering Facility.

Computer programs. DNA sequence analysis was aided by the DNA computer program DNA Strider (Marck 1988).

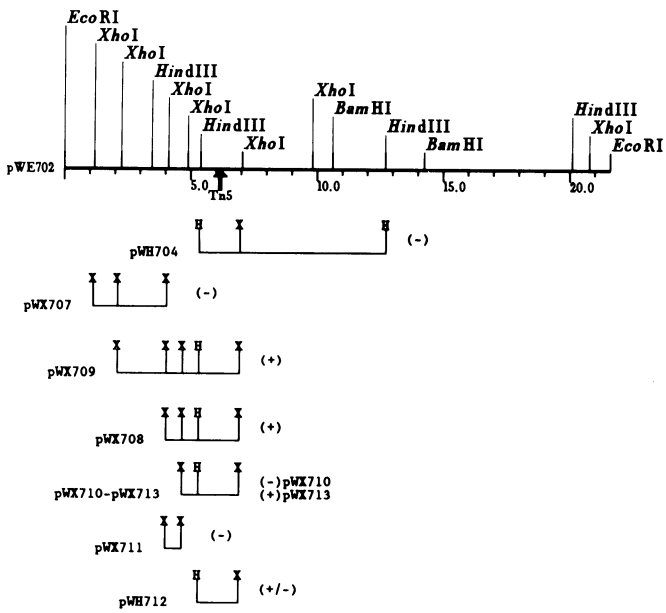

Fig. 1. Cloned fragments from the PTBR2.024 genomic library. Partial restriction map of pWE702. H = *Hin*dIII; X = *Xho*I. The small fragments indicate the subclones of the 22-kb insert in pWS3 that were introduced into mutant PTBR7.000 for complementation tests. A + indicates complementation of both the Pat$^-$ and Tox$^-$ phenotypes of PTBR7.000; a − indicates no complementation of either phenotype; a +/− indicates variable results (see text). pWX710 and pWX713 contain the 2.2-kb *Xho*I fragment in opposite orientations.

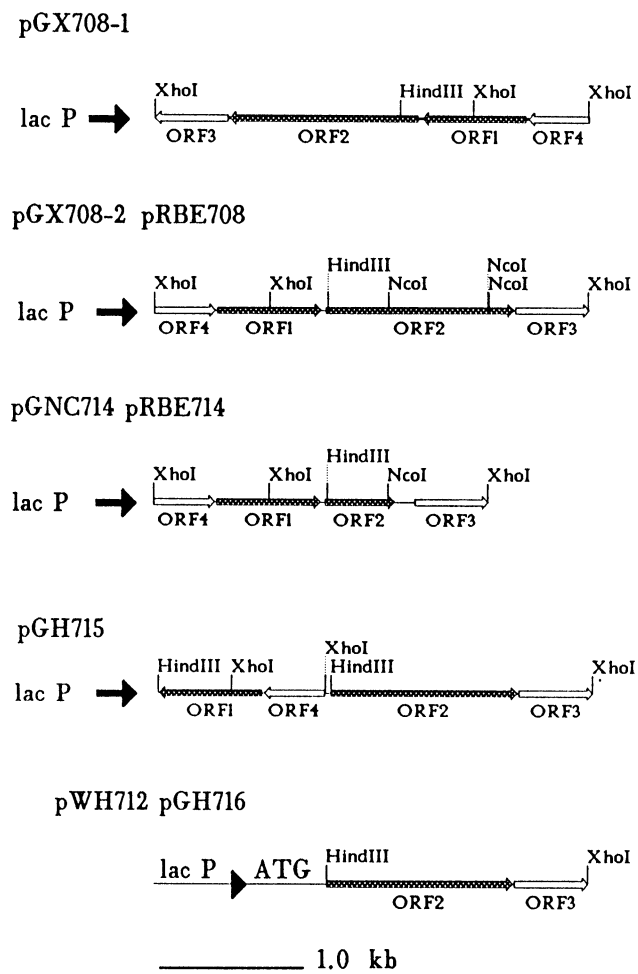

Fig. 2. Restriction maps of the constructs used for complementation tests and *in vitro* transcription and translation experiments. The shaded arrows indicate the open reading frames, and open arrows indicate truncated open reading frames. The solid arrows indicate the site of the Tn*5* insertion or the direction of transcription from the *lac* promoter on the vector. The solid arrowhead in pGH716 and pWH712 indicates the orientation of the vector promoter, and ATG indicates the translation initiation codon on the vector. pWS3 is the vector for pWH712; pGEM11-Zf for pGX708-1, pGX708-2, pGNC714, and pGH716; and pRK415 for pRBE708 and pRBE714.

PATMAT (Henikoff *et al.* 1990) was used to search for homologies between the deduced amino acid sequences of the ORFs and sequences in the Swiss-Prot (Release 17) and the EMBL (Release 26) data bases. The FASTA Align program and RDF2 (Pearson and Lipman 1988; Pearson 1990) were used to align known protein sequences from Swiss-Prot data base with the polypeptide sequences encoded by the ORFs and for the statistical analyses, respectively.

***In vitro* transcription and translation.** An *E. coli* S30 extract (Promega) was used for the coupled *in vitro* transcription and translation of DNA fragments (Figs. 1,2). SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyze the translation products (Sambrook *et al.* 1989).

RESULTS

Cloning of the Tn*5*-containing *Eco*RI fragment from the PTBR7.000 chromosome and identification of a genomic sequence homologous to this fragment. To identify the region in the PTBR7.000 chromosome responsible for the Pat$^-$ Tox$^-$ phenotype, we isolated the region containing the Tn*5* insert as a 28-kb *Eco*RI fragment. Restriction endonuclease mapping and Southern hybridization showed that Tn*5* had inserted into a 2.2-kb *Xho*I fragment contained within a 22-kb *Eco*RI fragment. The Tn*5* containing the *Eco*RI fragment was used as a probe to isolate the complementary 22-kb *Eco*RI fragment in pWE702 from the genomic library of PTBR2.024 (Salch and Shaw 1988). Figure 1 shows the restriction map of the 22-kb *Eco*RI fragment present in pWE702 as well as the site of the Tn*5* insertion.

Complementation tests. Various subclones were generated from the 22-kb *Eco*RI fragment (Fig. 1) and introduced into mutant PTBR7.000 for complementation tests. Results of tabtoxin and pathogenicity assays are shown in Figures 1 and 3. The fragments that restored tabtoxin production and pathogenicity to mutant PTBR7.000 were present in plasmids pWX708 and pWX709. The smallest fragment that complemented PTBR7.000 was the 1.7-kb *Hin*dIII-*Xho*I fragment in pWH712, but the results with this construct were not consistent from one experiment to another. In some experiments, toxin production was restored but not pathogenicity, and in other experiments the opposite results were observed. The second smallest fragment that complemented mutant PTBR7.000 was the 2.2-kb *Xho*I fragment in pWX713, but the symptoms on tobacco plants appeared 2–3 days later than on plants inoculated with the parent strain or with PTBR7.000 containing pWX708 or pWX709. In contrast, plasmids that did not contain the 2.2-kb *Xho*I or 1.7-kb *Hin*dIII-*Xho*I fragments (pWX707 and pWX711), as well as the plasmids that contained these two fragments but in a reversed orientation (pWX710 and pWH704), did not complement mutant PTBR7.000. Thus, in all cases except pWH712, either both or neither of the Pat$^-$ and Tox$^-$ phenotypes was complemented, and no quantitative differences in the results were observed. These results demonstrated that the DNA region responsible for restoring tabtoxin biosynthesis and pathogenicity was located within the 2.2-kb *Xho*I fragment and that the expression of this region required a vector promoter.

Sequence of the 3-kb *Xho*I fragments. The 3-kb insert in pWX708 was mutagenized by Tn*5*seq1, and 15 of the resulting mutants were sequenced from the insertion sites. This 2.927-kb fragment contains two open reading frames (ORFs), ORF1 and ORF2, and two truncated ORFs, ORF3 and ORF4 (Fig. 4). Computer-aided analysis showed that the predicted polypeptide encoded by ORF1 is 28 kDa; ORF2, 37 kDa; ORF3, 18 kDa; and ORF4, 15 kDa.

The role of ORF2 in tabtoxin biosynthesis and pathogenicity. The site of the original Tn*5* insertion responsible for the Pat$^-$ Tox$^-$ phenotype of strain PTBR7.000 was mapped to ORF2 by restriction endonuclease digestion. To confirm that ORF2 was essential for the complementation of PTBR7.000, we introduced an *Nco*I-generated deletion internal to ORF2 in the 3-kb fragment by digestion with that endonuclease and religation; the resulting fragment was cloned into pRK415 (Keen *et al.* 1988) to give pRBE714 (Fig. 2). As a control, the 3-kb *Xho*I fragment

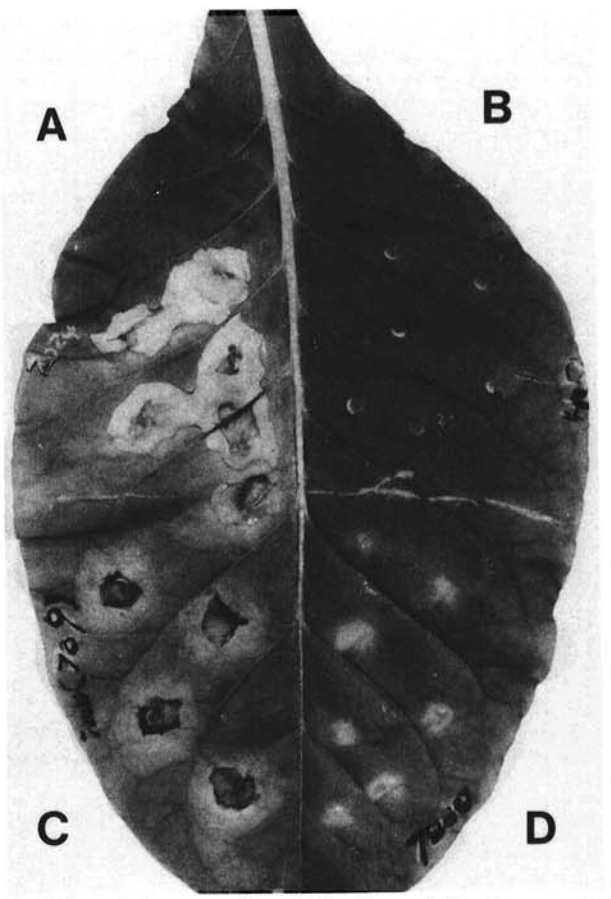

Fig. 3. Pathogenicity assays. Overnight cultures of *Pseudomonas* strains were diluted to 10^6 cfu/ml in distilled water. Small portions of these cell suspensions (approximately 50 μl) were inoculated into intact tobacco leaves by an infiltration method (Bertoni and Mills 1987). The leaf was inoculated with **A,** the parental strain, PTBR2.024; **B,** distilled water; **C,** PTBR7.000 containing pWX709; and **D,** the Tox$^-$ Pat$^-$ mutant, PTBR7.000. The small chlorotic spots surrounding the sites of PTBR7.000 inoculation are also shown by nonpathogens of tobacco, such as *Pseudomonas syringae* pv. *avenae* (a maize pathogen isolated by R. Chang) when inoculated at the same cell density.

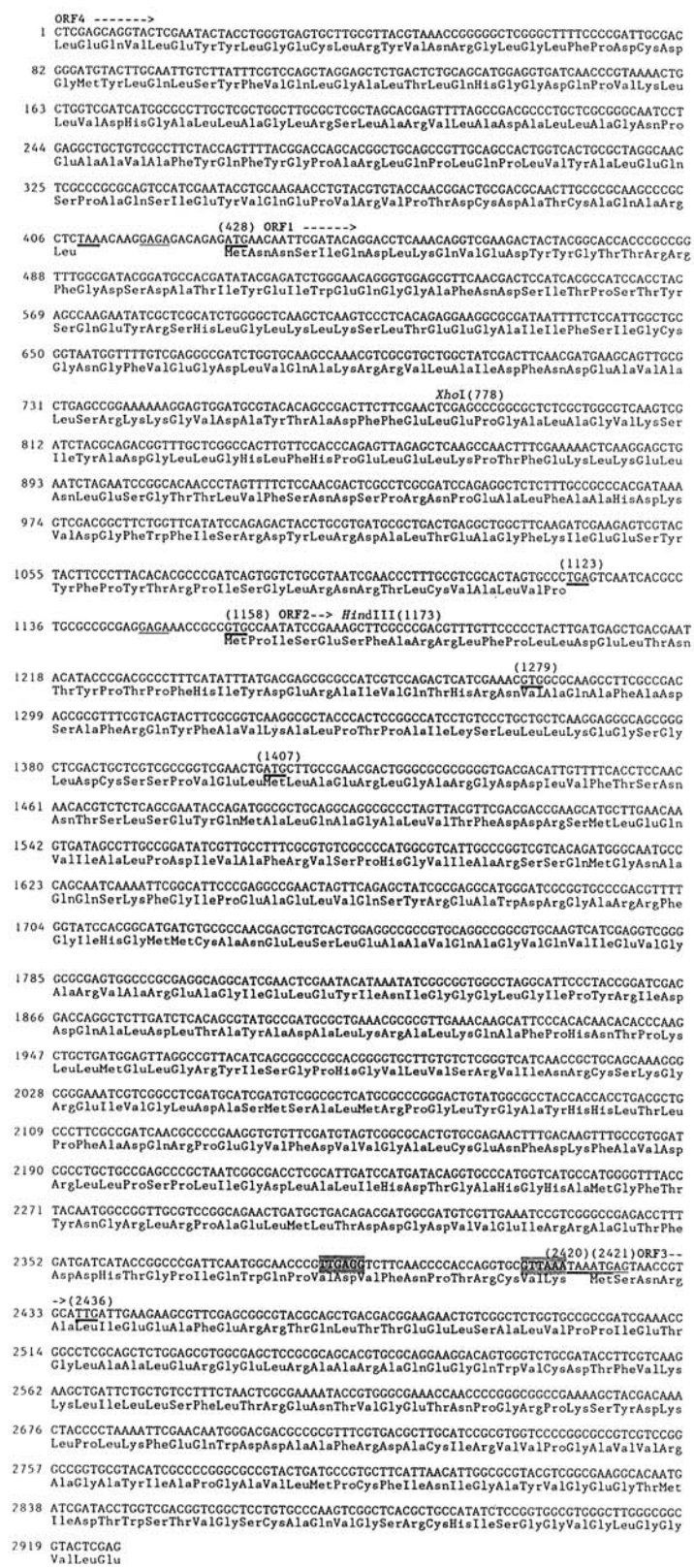

Fig. 4. Sequence of the 3-kb *Xho*I fragment. Potential ribosome binding sites are underlined, and possible translation start and stop codons are double underlined. The *Escherichia coli dapD* −10 and −35 sequences are GATAAA and TTGACA, respectively (Richaud *et al.* 1984). The sequences similar to these *E. coli dapD* regions are shadowed. The arrows indicate the direction of transcription.

was also cloned into the same vector to give pRBE708. pRBE708 restored tabtoxin production in PTBR7.000, but the ability to restore pathogenicity was inconsistent. pRBE708 was lost from PTBR7.000 with high frequency when grown in culture without tetracycline, so inconsistencies in the pathogenicity assays were attributed to plasmid loss *in planta*. pRBE714, however, consistently did not complement either phenotype. These results showed that the ORF2 product is essential for complementing the mutation in PTBR7.000.

***In vitro* transcription and translation.** To establish the gene organization of the ORFs on the 3-kb *Xho*I fragment, we generated various constructs (Fig. 2) and used them in coupled *in vitro* transcription and translation experiments. As shown in Figure 5, the 3-kb *Xho*I fragment in pWX708 (lane 3) yielded one polypeptide, subsequently identified as the ORF3 product, with an apparent molecular weight of 14 kDa. Polypeptides with the same mobility were also synthesized by pWX710 (lane 2) and pWH712 (lane 4). pGX708-2 contained the same 3-kb insert as pWX708 but in a different vector background that resulted in a translational fusion between the amino terminus of *lacZ* in the vector and ORF4. The 3-kb fragment in this construct yielded three distinct polypeptides with approximate molecular weights of 16, 24, and 51 kDa (lane 7). The 16-kDa and 24-kDa polypeptides were only synthesized by one other plasmid, pGNC714 (lane 8), which contained the *Nco*I deletions in ORF2. These two polypeptides were not synthesized by plasmid pWH712 (lane 4), which did not contain ORF1 and ORF4, nor by pGH715 (lane 9) and pGX708-1 (lane 6), which contained ORF1 and ORF4 in an orientation opposite to that in pGX708-2. These results suggested that the 16-kDa polypeptide was the ORF4 fusion product, and the 24-kDa polypeptide was the product of ORF1.

The 51-kDa polypeptide was also synthesized by pWH712 (lane 4), but not by pGX708-1 (lane 6) or by pGNC714 (lane 8), suggesting that this band was the product of ORF2. This polypeptide, however, was considerably larger than the predicted size of the ORF2 product, if the ATG at position 1407 (Fig. 4) served as the translation initiation codon. Similarly, ORF2 containing *Nco*I deletions in pGNC714 was expected to yield a 9-kDa polypeptide; instead, we observed a unique 18-kDa polypeptide (lane 8). Using these *in vitro* translation data and the location of a consensus ribosome-binding site (RBS) (Martin *et al.* 1988), we predicted that the translation initiation of ORF2 begins with GUG at position 1158. This led to a second prediction that the 51-kDa polypeptide synthesized by pWH712 was a translational fusion of ORF2 and *lacZ* on the vector, because this plasmid lacks the GUG translation initiation codon of ORF2 (Fig. 2). To test this hypothesis, we generated a similar translational fusion between *lacZ* and ORF2 by deleting the region containing the GTG codon in pGX708-2 to give pGH716 (Fig. 2). We also generated a plasmid to prevent this translational fusion by reversing the orientation of the *Hin*dIII fragment containing the GTG codon to give pGH715 (Fig. 2). As shown in Figure 5, a 51-kDa polypeptide was synthesized by pGH716 (lane 10) but not by pGH715 (lane 9). These results demonstrated that the 51-kDa polypeptide synthe-

sized from pWH712 was a translational fusion of *lacZ* and ORF2. There was an additional 16-kDa polypeptide band from pGH716 for which we have no explanation.

Homology between the amino acid sequences encoded by the ORFs and known polypeptides. Computer-aided amino acid analyses revealed that the deduced amino acid sequence of the ORF2 translation product is 26% homologous (Z score of 40) (Pearson 1990) to the amino acid sequences of the diaminopimelate decarboxylases (DAPDC) of *P. aeruginosa* (Schroeter) Migula, *E. coli* (Fig. 6), and *Corynebacterium glutamicum* (Kinoshita *et al.*) Abe *et al.* The nucleotide sequence of ORF2 is about 50% homologous to the DAPDC genes (*lysA*) of *E. coli* and *P. aeruginosa*.

The deduced 169 amino acids of the truncated polypeptide encoded by ORF3 showed 50% homology to the first 169 amino acids of Δ^1-piperidine-2,6-dicarboxylate succinyl transferase (PDST) of *E. coli* (Fig. 7). The nucleotide sequence was about 60% homologous to the first 507 nucleotides of the PDST gene (*dapD*) of *E. coli*. Regions similar to promoter sequences and to the RBS of *dapD* (Richaud *et al.* 1984) were also found upstream from potential ORF3 translation start codons (ATG at position 2421 and TTG at position 2436) (Fig. 4). The ATG translation initiation codon, however, overlaps the RBS. No homologies were detected between the putative ORF1 and ORF4 products and amino acid sequences of known polypeptides.

Complementation studies with *lysA* and *dapD* mutants. To examine if ORF2 and ORF3 encoded DAPDC and PDST enzymatic activities, we introduced plasmids pWE702, pWX708, pWX713, pWH712, or pGX708-2 (Figs. 1,2) into *E. coli lysA* mutants 403 and 155, and pWE702 and pGX708 into the *E. coli dapD* mutant, AT986. None of the plasmids complemented the *lysA* and *dapD* mutants.

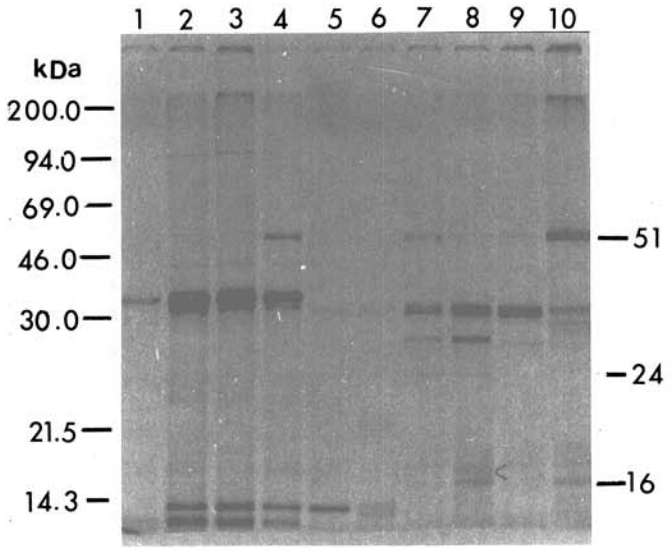

Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of coupled *in vitro* transcription and translation experiments. The constructs used for this experiment are shown in Figures 1 and 2. Lane 1, pWS3; 2, pWX710; 3, pWX708; 4, pWH712; 5, pGEM11-Zf; 6, pGX708-1; 7, pGX708-2; 8, pGNC714; 9, pGH715; and 10, pGH716. The numbers on the sides indicate the sizes of the polypeptides in kilodaltons. The 18-kDa polypeptide band is indicated by an arrowhead.

Conservation of the 3-kb fragment among *Pseudomonas* strains. To examine if the 3-kb fragment was conserved among pseudomonads, we used pWX708 to probe the *Xho*I digests of chromosomal DNA from various *Pseudomonas* strains. Hybridization to 2.2- and 0.8-kb *Xho*I fragments was detected in digests of the tabtoxin-producing strains, PTBR2.004, PTBR2.024, *P. s.* pv. *tabaci* 11528, and *P. s.* pv. *coronafaciens* (PC27). In contrast, no hybridization was observed in digests of *P. s.* pv. *striafaciens* (PS1); PT28.032 (a spontaneous Tox^- strain of *P. s.* pv. *tabaci* 11528); *P. s.* pv. *angulata* (PA45); *P. s.* pv. *syringae* van Hall (BSA002); *P. s.* pv. *mellea* (Johnson) Young *et al.* (PM1000); *P. s.* pv. *glycinea* (Coerper) Young *et al.* (PG59); *P. cepacia* (ex Burkholder) Palleroni and Holmes (PCE716); or *P. putida* (Trevisan) Migula (PPG277). None of these produces tabtoxin.

DISCUSSION

A 3-kb DNA fragment that restored pathogenicity and tabtoxin production to a Tn*5* insertion mutant (PTBR7.000) was isolated from *P. s.* pv. *tabaci* strain BR2.024. The fragment contained two complete ORFs, ORF1 and ORF2, and two truncated ORFs, ORF3 and ORF4. Restriction endonuclease mapping and sequencing identified ORF2 as the site of the transposon insertion. The truncated ORF3 in the 3-kb fragment lacks 303 nucleotides at the 3′ terminus (L. Liu, unpublished), which accounts for more than one-third of the carboxyl terminus of the potential translation product. It is unlikely that this truncated polypeptide would be functional. Furthermore, the inability of an *Nco*I-generated deletion in ORF2 to complement either the Pat^- or Tox^- phenotype is consistent with the hypothesis that the mutation in ORF2 was responsible for the loss of both phenotypes in mutant PTBR7.000. These results suggest that the expression of ORF2 is required for pathogenicity and tabtoxin production and indicate that, in *P. s.* pv. *tabaci* strain BR2, the two phenotypes depend on a common gene. We have designated the gene encoded by ORF2 *tabA*.

ORF1, ORF4, and *tabA* translation products were detected in *in vitro* transcription and translation experiments with pGX708-2 as the template but not with pWX708 or pGX708-1. These results show that there are no regions in pWX708 recognized by the *E. coli* system as promoters for *tabA*, ORF1, and ORF4. In pGX708-2, these three ORFs were transcribed as an operon under control of a promoter on the vector. In contrast, ORF3 was expressed independently in pWX708, indicating that a promoter for ORF3 was recognized by the *E. coli* system. We were not able to detect a band attributable to ORF3 in the translation products of the pGEM11-Zf constructs.

DNA homologous to *tabA* was detected only in the chromosome of tabtoxin-producing strains; this demonstrates the specificity of conservation of this region. Kinscherf *et al.* (1991) stated that tabtoxin is required for pathogenicity in strain BR2 on green bean. Our results, showing that TβL biosynthesis is essential for pathogenicity on tobacco, are consistent with their proposal but do not prove that TβL itself, the final product of the biosynthesis, was essential. The ability to produce tabtoxin is not the only patho-

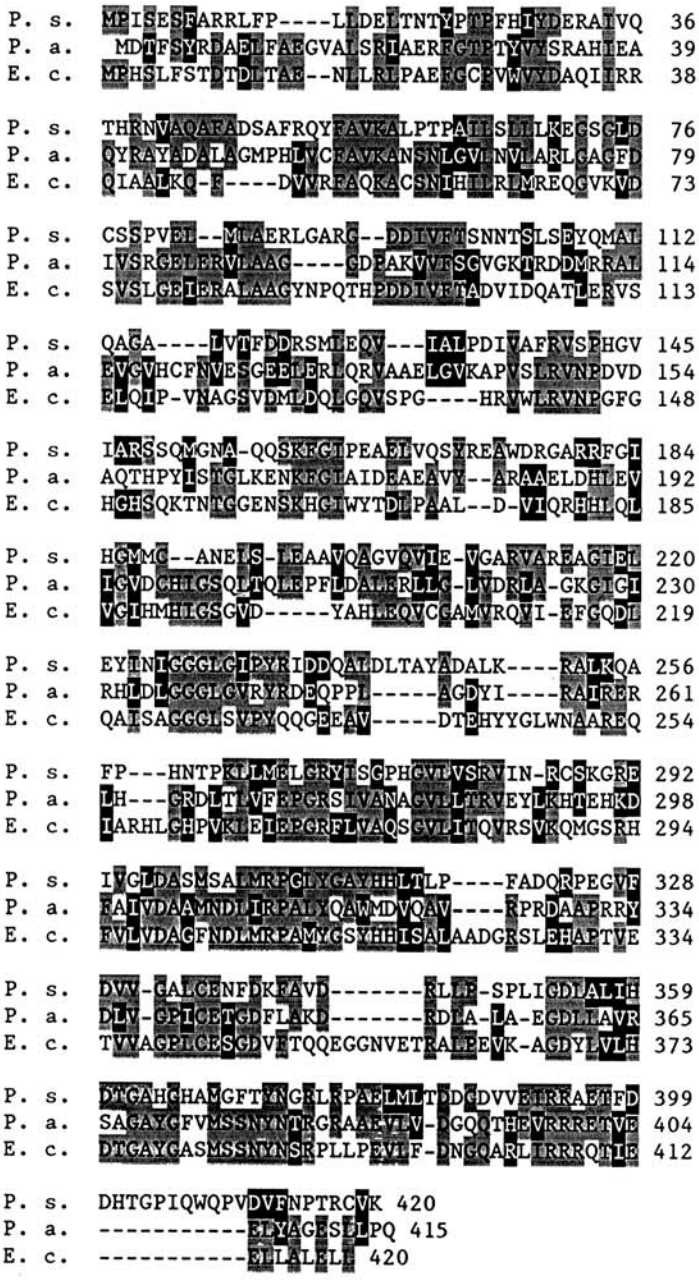

Fig. 6. Comparison of the deduced amino acid sequence of the ORF2 translation product (P. s.) with the *lysA* translation products of *Pseudomonas aeruginosa* (P. a.) and *Escherichia coli* (E. c.). Shaded regions represent identical amino acids; regions in reverse type represent conservative replacements. The amino acids considered equivalent were L, I, V, and M; F, Y, and W; S and T; K and R; D and E; A and G; N and Q.

Fig. 7. Comparison of the deduced amino acid sequence of the truncated ORF3 (P. s.) translation product with the *Escherichia coli dapD* translation product (E. c.). Details are the same as in Figure 6.

genic factor in strain PTBR2.024. This was demonstrated by the isolation of six independent Tox^+ Pat^- mutants of strain PTBR2.024 (Salch and Shaw 1988).

Although the deduced amino acid sequences of *tabA* and ORF3 have considerable homology to DAPDC and PDST, the *tabA* mutant, PTBR7.000, and the Tox^- mutants of *P. syringae* that lack the chromosomal sequences homologous to the 3-kb *Xho*I fragments were not auxotrophic for lysine; therefore, the *tabA* and ORF3 products are not required for lysine biosynthesis. Our inability to detect *lysA* by hybridization experiments with chromosomal DNA of *Pseudomonas* species might have been due to the high stringency conditions that were used. Martin *et al.* (1986) demonstrated that the *P. aeruginosa lysA* gene, whose product has 31% homology to the product of the *E. coli lysA* gene, complements *E. coli lysA* mutants. In contrast, the constructs encoding a functional *tabA* did not complement two independent *E. coli lysA* mutants, even though those constructs yielded the *tabA* translation product *in vitro*. Taken together, the evidence is consistent with a proposal that *tabA* does not encode a product involved in lysine biosynthesis. ORF3 resembles the *E. coli dapD* coding and upstream promoter sequences; however, a clone containing an intact ORF3 did not complement an *E. coli dapD* mutant, suggesting that the ORF3 product is not involved in lysine biosynthesis.

Radiolabeling experiments show that the tabtoxin biosynthetic pathway is associated with lysine biosynthesis, but labeled lysine is not incorporated into tabtoxin. Roth *et al.* (1990) proposed that tabtoxin biosynthesis branches off from the lysine synthetic pathway before the formation of L,L-diaminopimelate, and Unkefer *et al.* (1987) suggested that the initial steps in the tabtoxin biosynthetic pathway may use reactions analogous to the initial steps in the lysine pathway. Our results, which demonstrate the role of *tabA* in tabtoxin production and its sequence homology to *lysA*, provide the first genetic evidence to support these hypotheses. We propose that the products of *tabA* and possibly ORF3 have functions similar to DAPDC and PDST and that these products recognize substrates in the tabtoxin biosynthetic pathway that are analogous to *meso*-diaminopimelate and L-Δ^1-piperidine-2,6-dicarboxylate.

Because the increase in tabtoxin concentration in culture is not concurrent with cell growth, tabtoxin biosynthesis likely is regulated. Because *tabA* has high homology to *lysA*, which is expressed constitutively in *P. aeruginosa* (Martin *et al.* 1986), the rare translation start codon, GUG, for *tabA* might serve as a site for translational regulation of *tabA*. Another rare translation initiation codon, UUG, might have a similar function in ORF3 expression. Rare

translation initiation codons are known to play a role in translational gene regulation (Gualerzi and Pon 1990). The role of GUG and UUG as translation initiation codons and the possible involvement of ORF1, ORF3, and ORF4 in tabtoxin biosynthesis are currently under investigation.

ACKNOWLEDGMENTS

We thank S. Maloy for assistance during this research. Cultures were kindly provided by J. E. Cronan, S. K. Farrand, D. K. Willis, J.-C. Patte, and the *E. coli* genetic stock Center at Yale University. We also thank S. Von Bodman, S. K. Farrand, A. Kriz, and R. Sotak for helpful discussions and for reviewing the manuscript. K. S. Kim provided the titered phage; J. Landes maintained the tobacco plants.

LITERATURE CITED

Bertoni, G., and Mills, D. 1987. A simple method to monitor growth of bacterial populations in leaf tissue. Phytopathology 77:832-835.

Braun, A. C. 1937. A comparative study of *Bacterium tabacum* Wolf and Foster and *Bacterium angulatum* Fromme and Murray. Phytopathology 27:283-304.

Chang, A. C. Y., and Cohen, S. N. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. 134:1141-1156.

Chatterjee, A. K., and Vidaver, A. K. 1986. Genetics of pathogenicity factors: Application to phytopathogenic bacteria. Adv. Plant Pathol. 4:1-224.

Durbin, R. D. 1982. Toxins and Pathogenesis. Pages 423-441 in: Phytopathogenic Prokaryotes. Vol. 1. M. S. Mount and G. H. Lacy, eds. Academic Press, New York.

Durbin, R. D. 1991. Bacterial phytotoxins: Mechanism of action. Experientia 47:776-783.

Gualerzi, C. O., and Pon, C. L. 1990. Initiation of mRNA translation in prokaryotes. Biochemistry 29:5881-5889.

Henikoff, S., Wallace, J. C., and Brown, J. P. 1990. Finding protein similarities with nucleotide sequence databases. Pages 111-132 in: Methods in Enzymology. Molecular Evolution: Computer Analysis of Protein and Nucleic Acid Sequences. Vol. 183. R. F. Doolittle, ed. Academic Press, New York.

Hsiao, K.-C. 1991. A fast and simple procedure for sequencing double stranded DNA with sequenase. Nucleic Acids Res. 19:2787.

Keen, N. T., Tamaki, S., Kobayashi, D., and Trollinger, D. 1988. Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. Gene 70:191-197.

Kinscherf, T. G., Coleman, R. H., Barta, T. M., and Willis, D. K. 1991. Cloning and expression of the tabtoxin biosynthetic region from *Pseudomonas syringae*. J. Bacteriol. 173:4124-4132.

Knight, T. J., Durbin, R. D., and Langston-Unkefer, P. J. 1986. Effects of tabtoxinine-β-lactam on nitrogen metabolism in *Avena sativa* L. roots. Plant Physiol. 82:1045-1050.

Lennox, E. S. 1955. Transduction of linked genetic characters of the host bacteriophage P1. Virology 1:190-206.

Levi, C., and Durbin, R. D. 1986. The isolation and properties of a tabtoxin-hydrolysing aminopeptidase from the periplasm of *Pseudomonas syringae* pv. *tabaci*. Physiol. Mol. Plant Pathol. 28:345-352.

Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Marck, C. 1988. "DNA Strider": A "C" program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. 16:1829-1836.

Martin, C., Cami, B., Borne, F., Jeenes, D. J., Haas, D., and Patte, J.-C. 1986. Heterologous expression and regulation of the *lysA* genes of *Pseudomonas aeruginosa* and *Escherichia coli*. Mol. Gen. Genet. 203:430-434.

Martin, C., Cami, B., Yeh, P., Stragier, P., Parsot, C., and Patte, J.-C. 1988. *Pseudomonas aeruginosa* diaminopimelate decarboxylase: Evolutionary relationship with other amino acid decarboxylases. Mol. Biol. Evol. 5:549-559.

Morelle, G. 1989. A plasmid extraction procedure on a miniprep scale. Focus 11:7-8.

Nag, D. K., Huang, H. V., and Berg, D. E. 1988. Bidirectional chain-termination nucleotide sequencing: Transposon Tn*5*seq1 as a mobile source of primer sites. Gene 64:135-145.

Obukowicz, M., and Shaw, P. D. 1985. Construction of Tn*3*-containing plasmids from plant-pathogenic pseudomonads and an examination of their properties. Appl. Environ. Microbiol. 49:468-473.

Pearson, W. R. 1990. Rapid and sensitive sequence comparison with FASTP and FASTA. Pages 63-98 in: Methods in Enzymology. Molecular Evolution: Computer Analysis of Protein and Nucleic Acid Sequences. Vol. 183. R. F. Doolittle, ed. Academic Press, New York.

Pearson, W. R., and Lipman, D. J. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85:2444-2448.

Ribeiro, R. de L. D., Hagedorn, D. J., Durbin, R. D., and Uchytil, T. F. 1979. Characterization of the bacterium inciting bean wildfire in Brazil. Phytopathology 69:208-212.

Richaud, C., Richaud, F., Martin, C., Haziza, C., and Patte, J.-C. 1984. Regulation of expression and nucleotide sequence of the *Escherichia coli dapD* gene. J. Biol. Chem. 259:14824-14828.

Roth, P., Hädener, A., and Tamm, C. 1990. Further studies on the biosynthesis of tabtoxin (wildfire toxin): Incorporation of [2,3-$^{13}C_2$]pyruvate. Helv. Chim. Acta 73:476-482.

Salch, Y. P., and Shaw, P. D. 1988. Isolation and characterization of pathogenicity genes of *Pseudomonas syringae* pv. *tabaci*. J. Bacteriol. 170:2584-2591.

Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. Molecular Cloning: A Laboratory Manual. Vol. 3. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Stewart, W. W. 1971. Isolation and proof of structure of wildfire toxin. Nature (London) 229:174-178.

Thomas, M. D., Langston-Unkefer, P. J., Uchytil, T. F., and Durbin, R. D. 1983. Inhibition of glutamine synthetase from pea by tabtoxinine-β-lactam. Plant Physiol. 71:912-915.

Turner, J. G., and Debbage, J. M. 1982. Tabtoxin-induced symptoms are associated with the accumulation of ammonia formed during photorespiration. Physiol. Plant Pathol. 20:223-233.

Unkefer, C. J., London, R. E., Durbin, R. D., Uchytil, T. F., and Langston-Unkefer, P. J. 1987. The biosynthesis of tabtoxinine-β-lactam. J. Biol. Chem. 262:4994-4999.

Vogel, H. J., and Bonner, D. M. 1956. Acetyl ornithinase of *Escherichia coli*: Partial purification and some properties. J. Biol. Chem. 218:97-106.

Werneke, J. M., Sligar, G. S., and Schuler, M. 1985. Development of broad-host-range vectors for expression of cloned genes in *Pseudomonas*. Gene 38:73-84.