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Cloning and Expression of the Tabtoxin Biosynthetic Region from *Pseudomonas syringae*

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Pseudomonas syringae BR2, a causal agent of bean wildfire, was subjected to Tn5 mutagenesis in an effort to isolate mutants unable to produce the β-lactam antibiotic tabtoxin. Three of the tabtoxin-minus (Tox$^-$) mutants generated appeared to have physically linked Tn5 insertions and retained their resistance to the active toxin form, tabtoxinine-β-lactam (TβL). The wild-type DNA corresponding to the mutated region was cloned and found to restore the Tn5 mutants to toxin production. The use of cloned DNA from the region as hybridization probes revealed that the region is highly conserved among tabtoxin-producing pathovars of _P. syringae_ and that the region deletes at a relatively high frequency (10^{-3}/CFU) in BR2. The Tox$^-$ deletion mutants also lost resistance to tabtoxinine-β-lactam. A cosmid designated pRTBL823 restored toxin production and resistance to BR2 deletion mutants. This cosmid also converted the tabtoxin-naive _P. syringae_ epiphyte Cit7 to toxin production and resistance, indicating that pRTBL823 contains a complete set of biosynthetic and resistance genes. Tox$^-$ derivatives of BR2 did not produce disease symptoms on bean. Clones that restored toxin production to both insertion and deletion mutants also restored the ability to cause disease. However, tabtoxin-producing Cit7 derivatives remained nonpathogenic on bean and tobacco, suggesting that tabtoxin production alone is not sufficient to cause disease.

Tabtoxin is the precursor of a monocyclic β-lactam antibiotic that is produced by several pathovars and isolates of *Pseudomonas syringae* (for reviews, see references 12, 13, and 51). Historically thought to be the active toxin itself, tabtoxin has been shown to be a dipeptide precursor that must undergo hydrolysis by a peptidase to yield the biologically active form, tabtoxinine-β-lactam (TβL) (15, 31, 50). Unlike the majority of known β-lactam antibiotics such as penicillin, which affect bacterial cell wall synthesis, TβL appears to specifically inhibit glutamine synthetase (GS) (41, 46, 47). TβL is a general GS inhibitor, having been shown to affect the enzyme in bacteria (46), plants (47), and fungi (13a). There has been recent interest in the clinical uses of other monocyclic β-lactam antibiotics known as monobactams (6, 44). While it seems unlikely that a toxic compound such as TβL would be of any direct medical utility, understanding the tabtoxin biosynthetic pathway might well lead to the isolation of useful intermediates, as well as uncover possible overlaps in β-lactam antibiotic evolution.

While tabtoxin-producing bacteria have been studied for decades, a number of controversies have accumulated over the years. For example, several mechanisms have been suggested as the basis for the resistance of tabtoxin-producing bacteria to TβL, including GS adenylation (28), a specific β-lactamase activity (29), and a tabtoxin-specific transacetylase activity (1; see Discussion). There have also been questions concerning the precise relationship between the TβL-producing pathogens and some closely related toxin-deficient isolates. The best studied of tabtoxin-producing phytopathogens is *P. syringae* pv. tabaci, the causal agent of wildfire of tobacco (*Nicotiana tabacum*) (for a review, see reference 34). Symptoms of this disease include a characteristic chlorotic halo that surrounds necrotic infection centers on leaves. This chlorosis can be mimicked by inoculation of purified TβL alone (9, 40) and is associated with an accumulation of ammonia due to the inhibition of the plant GS (48). Another tobacco disease, angular leaf spot, is caused by an organism previously known as "*Pseudomonas angulata*." This nomenspecies and *P. syringae* pv. tabaci are distinguishable from each other only in that the former does not produce tabtoxin. "*P. angulata*" still forms necrotic lesions on tobacco but without the chlorosis typical of wildfire disease (8). This implies that tabtoxin, while affecting disease symptoms and possibly relative virulence, is not absolutely necessary for the pathogenic interaction between *P. syringae* pv. tabaci and tobacco.

Another tabtoxin-producing bacterium, *P. syringae* BR2, causes a disease of bean (*Phaseolus vulgaris*) similar to tobacco wildfire. This organism is closely related to *P. syringae* pv. tabaci but cannot be classified in the pathovar tabaci because it is not pathogenic on tobacco (37). Interestingly, no disease parallel to angular leaf spot has been recorded for bean wildfire, indicating the possibility that either Tox$^-$ derivatives of this organism do not naturally occur or that if they do occur, they are not pathogenic. The latter possibility, if true, would suggest that tabtoxin production might be a strain-specific disease factor, affecting virulence in some bacteria but absolutely required for disease in others.

In this paper, we describe the isolation of Tox$^-$ derivatives of BR2 and their restoration to tabtoxin production, as well as symptom development on bean, by a chromosomal region containing genes for tabtoxin biosynthesis and resistance. We show that this region is missing from related Tox$^-$ pathogens. We also examine the disease potential of an epiphytic *P. syringae* isolate converted to a Tox$^+$ phenotype by the presence of the cloned region.

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Bacterial strains		
E. coli		
HB101	F$^-$ *recA13 rspL hsdS20* (*hsdR hsdM*) *thi-1 leuB6 proA2 ara-14 lacY1 galK2 xyl-5 mtl-1 supE44* λ^-	7
DH5α	F$^-$ *recA1 hsdR17 endA1 thi-1 gyrA96 relA1 supE44* ϕ80Δ*lacZDM15* λ^-	Bethesda Research Laboratories
MIKE	Wild-type isolate from sewage	R. D. Durbin
P. syringae		
BR2(R)	Tox$^+$ Tox^r, causal agent of bean wildfire, originally designated BW2	37
0152	Tox$^+$ Tox^r, causal agent of soybean wildfire	37
Cit7	Rif^r, nonpathogenic	32, 36
P. syringae pv. tabaci		
11528(R)	Tox$^+$ Tox^r, a causal agent of wildfire of tobacco (ATCC type strain)	R. D. Durbin
BEL76 and VIR78	Tox$^+$ Tox^r, causal agents of wildfire of tobacco	R. D. Durbin
Pa9(R), Pa8, and Pa14	Tox$^-$ Tox^s, "*P. angulata*" isolates, causal agents of angular leafspot of tobacco	W. C. Nesmith
P. syringae pv. coronafaciens Pc27(R)	Tox$^+$ Tox^r, a causal agent of halo blight of oats	M. P. Starr
T47	Tox$^+$ Tox^r, a causal agent of halo blight of oats	S. S. Hirano
P. syringae pv. striafaciens 2480	Tox$^-$ Tox^s, a causal agent of bacterial stripe of oats (NCPPB strain)	S. S. Hirano
P. syringae pv. garcae 2710	Tox$^+$ Tox^r, a causal agent of bacterial scorch of coffee (NCPPB strain)	S. S. Hirano
P. syringae pv. syringae B728a	Rif^r, bean pathogenic isolate	S. S. Hirano; 53
Plasmids		
pBluescriptKS+	Amp^r	Stratagene, La Jolla, Calif.
pHC79	Amp^r Tet^r *cos*$^+$	20
pGS9	Tn*5* Cam^r	39
pLAFR3	Tet^r *cos*$^+$ *rlx*$^+$	42
pRK415	Tet^r, pRK404 derivative containing the pUC19 polylinker	22
pRK2013	Kan^r, mobilizing plasmid	10, 18
pRK7813	Tet^r, *cos*$^+$ *rlx*$^+$, pRK404 cosmid derivative	21
pTOX9	*tbl-9*::Tn*5* Tet^r, pLAFR3 clone	This study
pWTE22	Tet^r, 22-kb BR2(R) chromosomal insert in pRK415	This study
pTBL2	Amp^r, ~35-kb BR2(R) chromosomal insert in pHC79	This study
pRTBL823	Tet^r, ~31-kb BR2(R) chromosomal insert in pRK7813	This study
pWT2.1	Amp^r, 2.1-kb *Pvu*II fragment from pWTE22 cloned into pBluescriptKS+	This study
pWT2.8	Amp^r, 2.8-kb *Pvu*II fragment from pWTE22 cloned into pBluescriptKS+	This study
pWT5.3	Amp^r, 5.3-kb *Pvu*II fragment from pWTE22 cloned into pBluescriptKS+	This study

[a] Abbreviations: Rif, rifampin; Tet, tetracycline; Kan, kanamycin; Cam, chloramphenicol; Amp, ampicillin; r, resistant; s, sensitive; Tox$^+$, produces tabtoxin; Tox$^-$, does not produce tabtoxin. The designation (R) following strain names is used to denote spontaneous rifampin-resistant (Rif^r) mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this work are described in Table 1. *P. syringae* strains were maintained in KB medium (23) at 28°C, and *Escherichia coli* strains were grown in Luria-Bertani medium (35) at 37°C unless otherwise indicated. Minimal media M9 (35) and TM (48) were prepared as previously described except that 0.4% glycerol was substituted for glucose. Antibiotics were added to growth media at the following concentrations for *E. coli*: ampicillin at 50 μg/ml; tetracycline at 15 μg/ml; and kanamycin at 50 μg/ml. For selection of resistant *P. syringae*, the antibiotic concentrations were as follows: tetracycline at 10 μg/ml; kanamycin at 10 or 15 μg/ml; and rifampin at 100 μg/ml.

Plant assays. Pathogenicity on beans was tested by localized infiltration of 10-fold serial dilutions of a bacterial culture into leaves of cultivar Bush Blue Lake 274 (Northrup King Co., Minneapolis, Minn.) as previously described (53). Growth conditions for bean plants and an alternative inoculation method using sterile cheesecloth are described in the legend to Fig. 1. The ability of bacterial strains to induce the hypersensitive reaction (HR) on the nonhost tobacco cultivar Havana 142 (seed provided by K. Knoche and R. D. Durbin, University of Wisconsin-Madison) was determined by the rapid inoculation technique of Staskawicz et al. (43). Tobacco plants were grown at 28°C with a 12-h day-night cycle. In planta growth was measured by the method of Bertoni and Mills (4) as previously modified (53).

Purification and bioassay of TβL. TβL was isolated and partially purified essentially as previously described (14, 47). The purification procedure was stopped after the first cation exchange concentration step, and the partially purified TβL was stored in 100% methanol at −20°C. Yield from a 1-liter starting culture was approximately 29 mg of TβL, as ascertained with an amino acid analyzer (Beckman Instruments, Anaheim, Calif.). Bioassays for detection of tabtoxin or TβL production have been previously described (19); *E. coli* MIKE (a wild-type isolate from sewage) was used as the standard indicator strain. The sensitivity of other bacteria to TβL was determined by incorporating the strain to be tested into the overlay and inoculating with a known tabtoxin-producing strain. Alternatively, 20 μl of either a cell culture or purified TβL could be absorbed into a 6-mm sterile blank antibiotic disk (Schleicher & Schuell), which was then inverted onto the top agar containing the strain to be tested. Plates were incubated at 28°C and examined after 24 and 48 h.

To assure that the inhibitory substance produced by restored strains was indeed TβL, bacteria were grown in Woolley's medium (55) for 3 days, and the culture was adjusted to 4 μM Zn^{2+} (to promote the cleavage of tabtoxin to TβL), shaken for 90 min., and passed through a 0.2-μm-pore-size syringe filter (Nalge Co., Rochester, N.Y.). The filtrate was then assayed with the amino acid analyzer.

Tn*5* mutagenesis, conjugations, genomic DNA isolation, and general techniques. *P. syringae* BR2(R) was mutagenized with Tn*5*, using pGS9 (39), by following the procedure of Willis et al. (53). Tox^- mutants were defined as Kan^r bacteria that no longer produced zones in bioassay but still grew on TM minimal medium. The rate of appearance of auxotrophs (no growth on TM) was monitored as a parameter of mutational efficiency and was fairly constant at 0.5 to 1.0%. Triparental matings were performed using pRK2013 as a mobilizing plasmid (52).

For the isolation of genomic DNA, a 1-ml aliquot of a stationary culture of bacteria was spun down in a microcentrifuge, washed once with 1 ml of 0.5 M NaCl, and resuspended in 0.5 ml of 10 mM Tris-HCl (pH 8.0)–1 mM EDTA (TE); 50 μg of proteinase K and Sarkosyl to 1% were added, and the cell suspension was incubated at 37°C for 3 h to overnight. The resulting lysate was extracted with an equal volume of phenol–chloroform (1:1), washed two times with 1.5 volumes of chloroform alone, and ethanol precipitated. These precipitates were dissolved in 150 μl of TE. This DNA preparation was routinely scaled up proportionately for 10 ml of cells, and the DNA was purified through CsCl gradients according to standard techniques.

General molecular techniques for cloning, DNA hybridization, etc., were standard methods (35).

RESULTS

Isolation of mutants defective in tabtoxin production. A total of 7,000 Kan^r transconjugants were analyzed for their ability to produce tabtoxin after matings with the Tn*5* delivery vehicle pGS9. As shown in Table 2, 23 Kan^r strains were determined to be deficient in tabtoxin by bioassay. The toxin deficiencies were apparently not the result of poor growth on the minimal bioassay plates, as presumptive auxotrophs (i.e., clones unable to grow on TM plates) generated during mutagenesis did produce small zones of inhibition. This result is believed to reflect residual toxin production by the nongrowing cells deposited by the replicator.

As summarized in Table 2, the 23 mutants were examined for resistance to TβL. Of these, 20 were found to be TβL sensitive (Tox^s) while 3 (KW109, KW116, and KW123) retained resistance to TβL (Tox^r).

Pathogenicity of Tn*5* mutants and their ability to elicit the HR. The ability of the mutants to cause symptoms on bean was tested by inoculating bacterial suspensions onto leaves of cultivar Bush Blue Lake 274. By using a localized infiltration inoculation method (53), parental strain BR2(R) produced necrosis surrounded by a chlorotic halo at inoculum densities ranging from 10^4 through 10^8 CFU/ml. By contrast, inoculation of the Tox^- mutants resulted in necrosis at only the highest inoculum density (10^8 CFU/ml), but unlike the wild-type reaction no chlorotic halo surrounded the necrotic tissue. This response was not viewed as a true disease symptom since even nonpathogens can elicit a plant reaction at this cell density (26, 53). When bacteria were swabbed onto leaves, neither class of Tox^- mutant (Tox^r or Tox^s) produced lesions (Fig. 1 and Table 2).

TABLE 2. Characteristics of tabtoxin-deficient mutants of BR2(R)[a]

Strain(s)	Toxin production	Toxin resistance	Pathogenicity	HR	No. found
BR2(R)	+	R	+	+	
KW109, KW116, and KW123	−	R	−	+	3
KW105 and KW110[b]	−	S	−	+	20

[a] +, wild-type zone of toxin inhibition, a pathogenic response, or a positive HR; −, no detectable toxin production or nonpathogenic; R, resistant to TβL; S, sensitive to TβL.

[b] Strains KW105 and KW110 are representative strains of this class.

By infiltration of bacterial suspensions we tested the ability of two Tox^- mutants, KW109 (Tox^r) and KW110 (Tox^s), to grow in bean leaves. Both KW109 and KW110 grew at the same rate and reached the same final cell density as the wild-type parent, BR2(R) (data not shown). This result suggests that the lack of symptoms caused by these mutants was not due to an alteration of their ability to grow in the plant environment.

In addition to lesion formation on host plants, phytopathogenic pseudomonads are usually characterized by the ability to cause a macroscopic necrotic response on nonhost plants when the bacteria are inoculated at concentrations greater than 10^7 CFU/ml. This response has been designated the HR (27) and has been correlated with metabolic activity of the bacteria within the plant (for reviews, see reference 26 and 54). The ability of the Tox^- mutants of BR2(R) to cause the HR in tobacco was tested by infiltrating dilutions of bacterial cultures in water into leaves of tobacco cultivar Havana 142. None of the mutants were impaired for HR induction; all 23 produced the same degree of tissue collapse and local necrosis as did the wild-type strain (Table 2).

Molecular characterization of presumptive mutants. Total genomic DNA was isolated from the Tox^- mutants and digested with restriction enzymes (e.g., *Eco*RI and *Kpn*I) that have no recognition sites within Tn*5*. Southern hybridizations were performed using the internal *Hin*dIII fragment of Tn*5* as a probe to determine whether any of the apparent Tn*5* insertions were linked on common restriction fragments. Somewhat surprisingly, only the three Tox^r mutants (KW109, KW116, and KW123) appeared to share any linkage; these three Tn*5* insertions were clustered within a 22-kb *Eco*RI fragment (Fig. 2B). All of the other 20 mutants appeared to represent independent Tn*5* insertions (data not shown).

Restoration of tabtoxin production to the KW109, KW116, and KW123 mutants. Cloning of the Tn*5* and flanking DNA from mutant KW109 was accomplished by directly selecting

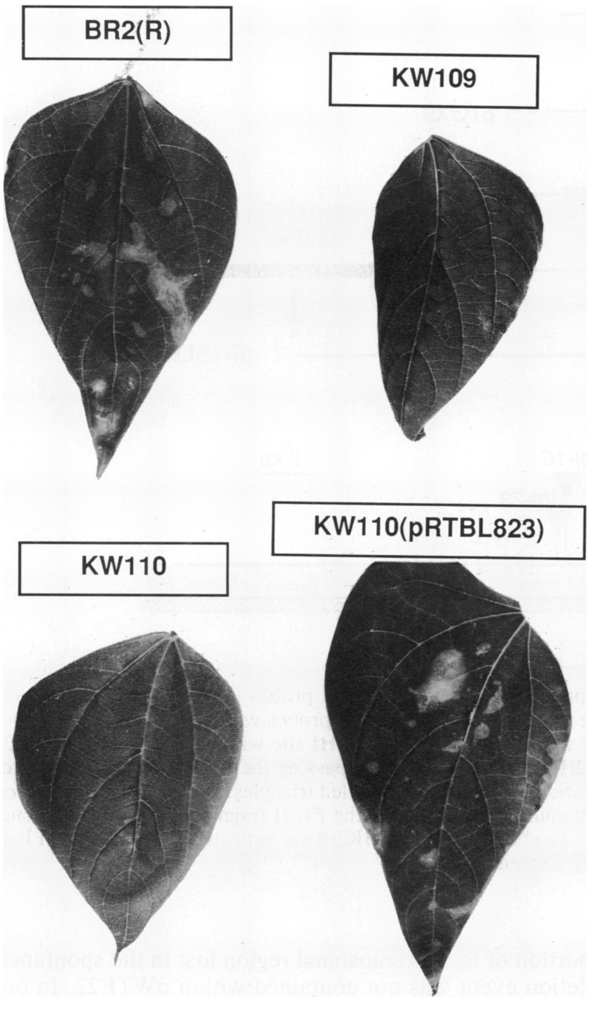

FIG. 1. Reaction on a trifoliate leaf of *P. vulgaris* cultivar Bush Blue Lake 274 after bacterial inoculation. Shown are the phenotypes of the Tox$^+$ strain BR2(R), the Tox$^-$ Tn*5* insertion mutant KW109, the deletion mutant KW110, and KW110 containing pRTBL823. Inoculation was performed using a sterile folded square of cheesecloth dipped in a bacterial culture adjusted to 10^7 CFU/ml. The applicator was then rubbed gently on leaves (midrib length, 8 to 10 cm) of intact bean plants (approximately 6 days postemergence). Bean plants were maintained at 28°C with a 12-h light cycle. Symptoms were recorded after 5 to 7 days.

cosmid transductants for Kan^r after ligating phosphatased, *Sau*3A partially digested KW109 DNA into pLAFR3 and in vitro λ packaging. A cosmid was obtained, designated pTOX9, that contained Tn*5* and approximately 16 kb of the flanking chromosomal DNA (Fig. 2). We used pTOX9 as a hybridization probe of *Eco*RI-digested DNA from the three Tox$^-$ Tox^r mutants (data not shown). The hybridization pattern of BR2(R) revealed a single strong homology signal about 22 kb in length as well as a weaker signal of 17 kb. Within KW109, KW116, and KW123, the 22-kb band was replaced by a larger band of approximately 28 kb, apparently reflecting the addition of Tn*5* (5.8 kb).

The previous experiments suggested that the Tn*5* insertions within KW109, KW116, and KW123 were linked and had occurred within a single 22-kb *Eco*RI fragment of the wild-type BR2(R) chromosome. Thus, a genomic library was made in pLAFR3 from BR2(R) DNA that had been completely digested with *Eco*RI. A subclone of pTOX9 was used as a probe to detect appropriate clones in colony hybridizations. The 22-kb fragment was isolated from one of the homologous cosmids and subcloned into the smaller (10.5 kb) vehicle pRK415. The resulting plasmid, called pWTE22, was readily mobilized into derivatives of BR2(R) by triparental mating. The presence of pWTE22 in transconjugants of the three Tox$^-$ Tox^r Tn*5* insertion mutants (KW109, KW116, and KW123) restored tabtoxin production as determined by bioassay. The presence of TβL in the culture filtrate of KW109(pWTE22) was confirmed by using the amino acid analyzer (data not shown). The restoration of tabtoxin production appears to be the result of *trans* complementation: first, all tested Tet^r transconjugants of the Tox$^-$ Tox^r Tn*5* mutants were restored to toxin production; second, hybridization of pWTE22 to total DNA prepared from the transconjugants produced bands consistent with the presence of an unaltered multicopy plasmid (data not shown).

Using pWTE22 as a probe in Southern hybridization experiments confirmed the linkage of the Tn*5* insertions in KW109, KW116, and KW123 within a 22-kb *Eco*RI fragment (Fig. 3A). Using the same probe with *Pvu*II-digested DNA showed that the Tn*5* insertions within KW109 and KW116 occurred in the conserved (see below) 5.3-kb *Pvu*II fragment (Fig. 2 and 3B) while an adjoining 4.2-kb *Pvu*II fragment was altered by Tn*5* in KW123 (Fig. 3B). Since we established that the mutations within KW109, KW116, and KW123 were independent, although physically linked, we have designated these mutations *tbl-9*::Tn*5*, *tbl-16*::Tn*5*, and *tbl-23*::Tn*5*, respectively.

The appearance of the 17-kb *Eco*RI fragment in Fig. 3A is due to a secondary chromosomal homology within the probe DNA, and this fragment is not associated with toxin production. The source of the secondary homology within pWTE22 is discussed in more detail below.

Molecular characterization of Tox$^-$ Tox^s mutants. *Eco*RI-digested total genomic DNA from the 20 Tox$^-$ Tox^s Kan^r mutants was probed with pWTE22, and the hybridization pattern was compared with that of wild-type BR2(R). Surprisingly, all of the mutants of this type lacked the 22-kb *Eco*RI fragment. There was, however, a novel 11-kb *Eco*RI fragment not found in the genomic DNA of parental strain BR2(R) (Fig. 3A). *Pvu*II fragments mapping to the right half of the 22-kb *Eco*RI fragment (indicated by the open box in Fig. 2B) were completely absent in genomic DNA from all of the Tox$^-$ Tox^s mutants (Fig. 3B).

Taken together, these data strongly suggest that all of the Tox$^-$ Tox^s mutants arose as a result of spontaneous deletions of the region required for tabtoxin production and resistance, with random genomic Tn*5* insertions to account for their Kan^r phenotype. The 11-kb fragment might represent a fusion fragment composed of the leftmost part of the pWTE22 *Eco*RI fragment (Fig. 2B) and part of a second fragment at the opposite terminus of the deletion.

In order to establish that the loss of the common *Pvu*II fragments was not caused by the Tn*5* mutagenesis procedure, we plated 2,900 single colonies from a stationary culture of unmated BR2(R) grown in KB plus rifampin and screened them for loss of tabtoxin production. Six Tox$^-$ Tox^s mutants were identified, yielding a frequency for the spontaneous loss of tabtoxin production and resistance of 2.1×10^{-3}/CFU plated. Southern blot analysis of these six mutants revealed that the *Pvu*II fragments associated with the tabtoxin biosynthetic region (Fig. 2B) were absent (data

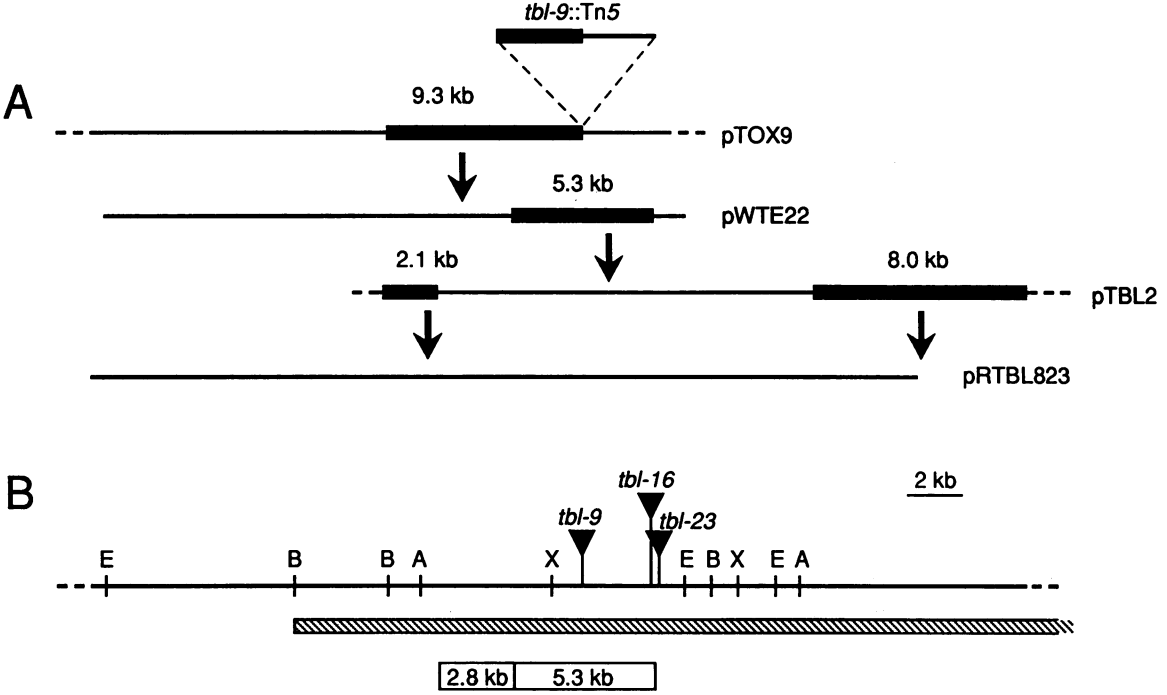

FIG. 2. (A) Genealogy of plasmids described in the text. Thick lines represent restriction fragment probes used for the identification of each succeeding clone. The sizes of the various probes are given above the lines. Fragments used as probes were as follows: 9.3-kb probe, a *Bam*HI fragment defined by the *Bam*HI site to the left of the *tbl-9*::Tn*5* insertion in B and the *Bam*HI site within Tn*5*; the 5.3-, 2.8-, 2.1-, and 8.0-kb probes were all *Pvu*II fragments. (B) Restriction map of the BR2(R) chromosome encompassing the region required for tabtoxin production. The locations of the *tbl-9*::Tn*5*, *tbl-16*::Tn*5*, and *tbl-23*::Tn*5* mutations are indicated by filled triangles. Restriction sites for *Eco*RI (E), *Bam*HI (B), *Asp*718 (A), and *Xba*I (X) are shown. The open boxes containing sizes represent the *Pvu*II fragments conserved among all tabtoxin-producing pseudomonads. The extent of the deletion within Tox$^-$ Tox^s derivatives of BR2(R) is indicated by the hatched bar. It should be noted that the genealogy (A) and restriction map (B) are spatially aligned.

not shown). The rate of this deletion event was in close agreement with the deletion frequency found within Kan^r transconjugants (20 of 7,000 or 2.9 × 10^{-3}/CFU).

Fragment conservation among tabtoxin-producing strains. In order to investigate the potential conservation of genetic loci required for tabtoxin production, pTOX9 was used as a hybridization probe for *Pvu*II-digested DNA from a variety of tabtoxin-producing *P. syringae* strains. *Pvu*II fragments with lengths of 5.3 and 2.8 kb (Fig. 2B) were present in all of the Tox$^+$ strains examined (Fig. 4), including *P. syringae* BR2 (R), *P. syringae* pv. tabaci, *P. syringae* pv. coronafaciens, *P. syringae* pv. garcae, and *P. syringae* 0152. These hybridization signals were apparently the result of a true conservation of homologous restriction fragments rather than the fortuitous comigration of different restriction fragments. Subclones containing the 5.3- or 2.8-kb *Pvu*II fragment from pWTE22 were constructed in the vector pBluescriptKS+ and designated pWTP5.3 or pWTP2.8, respectively. A single *Pvu*II fragment of either 5.3 or 2.8 kb in all of the Tox$^+$ strains examined shared homology with pWTP5.3 or pWTP2.8, respectively (Fig. 5). These *Pvu*II fragments were not present in *P. syringae* strains that did not produce tabtoxin, including "*P. angulata*" (Fig. 4 and 5) and *P. syringae* pv. striafaciens (Fig. 4), two bacterial strains thought to be closely related to *P. syringae* pv. tabaci and *P. syringae* pv. coronafaciens, respectively (8, 16, 38, 45, 49).

Cloning of the functional tabtoxin biosynthetic region. Plasmid pWTE22 did not restore the Tox$^-$ Tox^s deletion mutants, KW104 and KW110, to either tabtoxin production or symptom development on bean. These results indicated that a portion of the chromosomal region lost in the spontaneous deletion event was not contained within pWTE22. In order to isolate the tabtoxin biosynthetic region in its entirety, we constructed a BR2(R) genomic library in the small (6.5-kb) *E. coli* vector pHC79. Using the 5.3-kb *Pvu*II fragment as a probe in colony hybridization resulted in the identification of a cosmid, designated pTBL2 (Fig. 2), that conferred complete resistance to TβL upon *E. coli* DH5α. This cosmid could not be mobilized into *Pseudomonas* spp. but was used instead as a source of DNA probes to identify a cosmid that could be transferred. We used the two *Pvu*II fragments (2.1 and 8.0 kb) near the opposite ends of the chromosomal insert within pTBL2 (Fig. 2A) as probes of a BR2(R) genomic library constructed in the 11.5-kb wide-host-range cosmid pRK7813. Cosmid pRTBL823, containing homology to both probes, was isolated. Introduction of pRTBL823 led to restoration of tabtoxin production and TβL resistance to all three of the Tn*5*-generated mutants and three of the deletion mutants as measured by bioassay. The restoration of these properties to mutants carrying unlinked Tn*5* insertions by a cosmid that does not share homology with the sites of the various insertions confirms that the Tox$^-$ Tox^s phenotype of the mutants was not due to Tn*5* but was the result of the deletion of the biosynthetic region. Unlike pTBL2, the presence of pRTBL823 in *E. coli* DH5α provided only an intermediate level of resistance to TβL as measured by bioassay.

The use of pRTBL823 as a genomic probe allowed us to detect additional DNA lost in the spontaneous deletion of the tabtoxin biosynthetic region. This event appears to

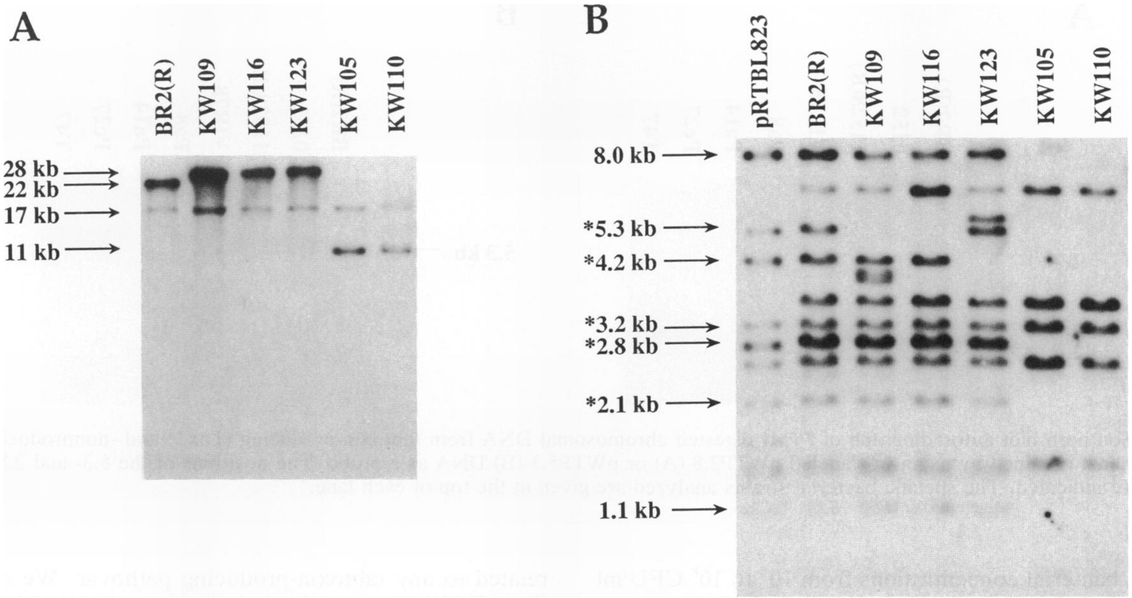

FIG. 3. (A) Autoradiograph of a Southern analysis of *Eco*RI-digested DNA from the bacterial strains designated, obtained by using ^{32}P-labeled pWTE22 DNA as a probe. A similar result was obtained when pTOX9 DNA was used as a probe. (B) Autoradiograph of chromosomal DNA digested with *Pvu*II and probed with ^{32}P-labeled pRTBL823 DNA. Sizes in kilobases of restriction fragments discussed in the text are indicated on the left. Fragments that share homology with pWTE22 are indicated by an asterisk. The pattern seen by using pTOX9 as a probe of *Pvu*II-digested chromosomal DNA was identical to that shown by pWTE22 except for the absence of a signal from the 4.2-kb *Pvu*II fragment.

involve in excess of 27 kb of the chromosome of the Tox$^-$ Tox^s mutants of BR2(R) (Figs. 2B and 3B).

Location of secondary chromosomal homology. As mentioned above, a second homologous band of approximately 17 kb appeared in hybridizations between plasmids containing the toxin region and *Eco*RI-digested chromosomal DNA from both wild-type and mutant strains of BR2(R). This was true even for pWTE22, which contains a discrete cloned *Eco*RI fragment. Experiments indicated that this signal was not the result of homology with either Tn*5* or pLAFR3. Repeated DNA sequences have been found near deletion termini in other prokaryotic backgrounds, and such repeats would be expected to cause a multiple signal if any individual repeat was used as a probe. Such appears to be the case in BR2(R).

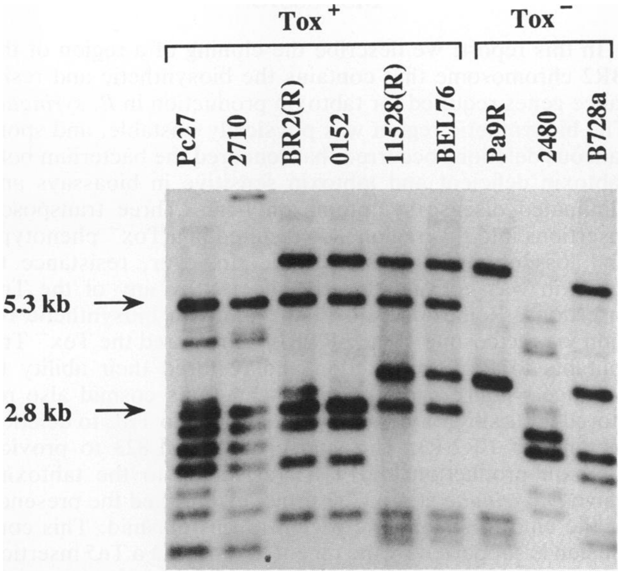

FIG. 4. Southern blot autoradiograph of *Pvu*II-digested chromosomal DNA from tabtoxin-producing (Tox$^+$) and -nonproducing (Tox$^-$) bacterial strains, obtained by using ^{32}P-labeled pTOX9 DNA as a probe. The positions of the 5.3- and 2.8-kb *Pvu*II fragments present in all Tox$^+$ isolates examined to date are indicated on the left. The specific bacterial strains analyzed are shown at the top of each lane.

Plasmid pWTP2.8 containing the conserved 2.8-kb *Pvu*II fragment is described above; pWT2.1 consists of the adjoining 2.1-kb *Pvu*II fragment (mentioned above as one of the probe fragments of pTBL2; Fig. 2A) cloned into pBluescriptKS+. These two plasmids were used as probes of *Eco*RI-digested chromosomal DNA (Fig. 6). Plasmid pWTP2.1 hybridized to two fragments in wild-type and Tn*5* mutant DNA but only to the smaller of the two fragments in DNA from deletion mutants. In contrast, pWTP2.8 produced only a single signal in hybridizations with wild-type and Tn*5* mutant DNA and no signal with DNA from deletion mutants. These data are consistent with the presence of DNA repeats, one lying within the deleted region and the other elsewhere on the chromosome. The deleted repeat lies to the left of the conserved 2.8-kb *Pvu*II fragment on our map and overlaps the adjoining 2.1-kb *Pvu*II fragment. The size of the repeat is not known, but results indicate that the secondary homology in *Pvu*II digests lies entirely within a 3.2-kb fragment (Fig. 3B), setting this as an upper size limit.

Restoration of disease symptoms on bean. KW109 (pWTE22) was tested for restoration of symptoms on bean leaves by localized infiltration inoculation. A necrotic area surrounded by a chlorotic halo was seen at inoculum concentrations ranging from 10^6 to 10^8 CFU/ml. Under the same experimental conditions, BR2(R) exhibited a pathogenic

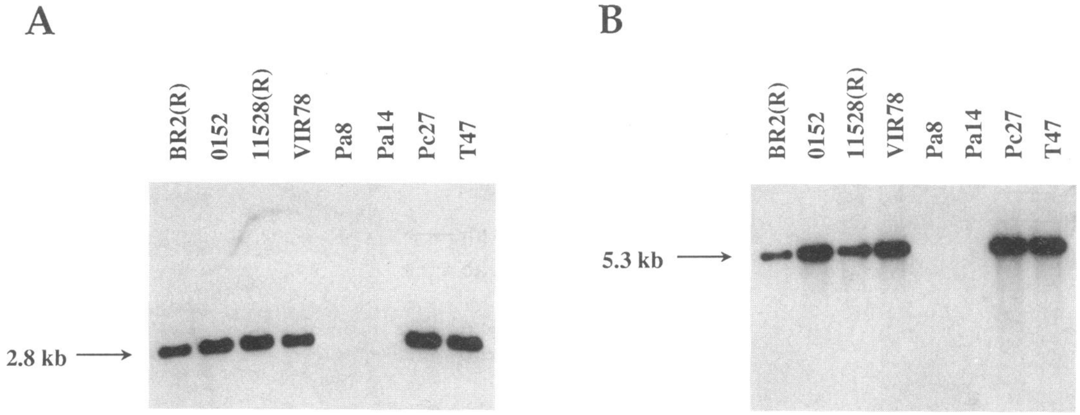

FIG. 5. Southern blot autoradiograph of *Pvu*II-digested chromosomal DNA from tabtoxin-producing (Tox$^+$) and -nonproducing (Tox$^-$) bacterial strains, obtained by using ^{32}P-labeled pWTP2.8 (A) or pWTP5.3 (B) DNA as a probe. The positions of the 5.3- and 2.8-kb *Pvu*II fragments are indicated. The specific bacterial strains analyzed are given at the top of each lane.

reaction at bacterial concentrations from 10^5 to 10^8 CFU/ml (data not shown). KW109 under these conditions gave a necrotic reaction without a chlorotic halo only at the highest inoculum concentration (10^8 CFU/ml). From this result, it is apparent that pWTE22 restored symptom production to KW109 but not to a level that was fully equivalent to that of the parental strain BR2(R). The observed difference between BR2(R) and KW109(pWTE22) is most likely due to the known instability of plasmid vectors derived from pRK404, such as pRK415, in the absence of antibiotic selection (11). However, there was no difference observed between BR2(R) and KW109(pWTE22) with regard to the time course of the appearance of chlorosis at those inoculum concentrations that gave a pathogenic response. In a similar manner, pWTE22 restored both KW116 and KW123 to pathogenicity on bean.

As expected, symptom development on bean was restored not only to the Tn*5* mutant (KW109) but also to a deletion mutant (KW110) by the presence of pRTBL823. The latter result is shown in Fig. 1.

Expression of tabtoxin in a nonpathogenic epiphyte. Strain Cit7 is one of the best characterized *P. syringae* epiphytes and has been shown to be nonpathogenic on all plant species tested (32, 33, 36). It does not produce tabtoxin and is not related to any tabtoxin-producing pathovar. We confirmed that pRTBL823 contained the entire tabtoxin biosynthetic region by the introduction of the cosmid into this tabtoxin-naive genetic background. The resulting transconjugants of Cit7 produced tabtoxin as determined by both bioassay and amino acid analyzer assay of culture filtrates (data not shown) and were also resistant to TβL. Unaltered pRTBL823 DNA was recovered from the Tox$^+$ Cit7 transconjugants, confirming that gene expression was in *trans*. Significantly, the toxin-producing epiphyte did not cause symptoms on tobacco or bean, although some chlorosis did appear on tobacco at the highest inoculum level. Apparently other factors are required to make Cit7 a pathogen on these hosts.

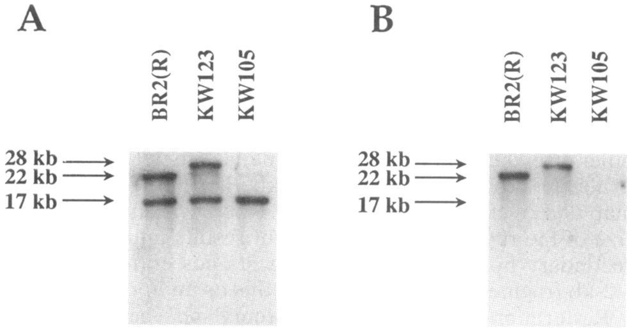

FIG. 6. Analysis of the source of the secondary chromosomal homology within pWTE22, using ^{32}P-labeled pWT2.1 (A) or pWT2.8 (B) as a probe. Total DNA from the strains indicated at the top of the lanes was digested with *Eco*RI. The sizes in kilobases of relevant fragments are indicated.

DISCUSSION

In this report, we describe the cloning of a region of the BR2 chromosome that contains the biosynthetic and resistance genes required for tabtoxin production in *P. syringae*. The biosynthetic region was physically unstable, and spontaneous deletions occurred that rendered the bacterium both tabtoxin deficient and tabtoxin sensitive in bioassays and eliminated disease symptoms on bean. Three transposon insertions into this region also resulted in a Tox$^-$ phenotype and loss of symptoms on bean. However, resistance to tabtoxin was not significantly affected by any of the Tn*5* insertions. Reintroduction of the tabtoxin biosynthetic region via the cosmid clone pRTBL823 restored the Tox$^-$ Tn*5* mutants to toxin production and restored their ability to produce wildfire symptoms on bean. This cosmid also restored tabtoxin production and resistance to TβL to deletion mutants of BR2(R). The ability of pRTBL823 to provide tabtoxin production and TβL resistance to the tabtoxin-naive *P. syringae* strain Cit7 firmly established the presence of the entire biosynthetic region in this plasmid. This conclusion is supported by the recent finding that a Tn*5* insertion within the homologous tabtoxin biosynthetic region of *P. syringae* pv. tabaci results in the accumulation of a novel amino acid that is a possible intermediate in the biosynthesis of tabtoxin (17, 30).

The conservation of restriction fragments among various tabtoxin-producing isolates strongly suggests that a common biosynthetic region exists in all such strains. In support of this conclusion, we have recently found that the introduction via recombination of the *tbl-9*::Tn*5* mutation into two distinct Tox^+ pathovars (*P. syringae* pv. coronafaciens and *P. syringae* pv. tabaci) results in a Tox^- Tox^r phenotype that is identical to that shown by the original mutant, KW109 (24). In addition, we have recently cloned the tabtoxin biosynthetic region from *P. syringae* pv. coronafaciens Pc27 and have shown that this region is able to restore the BR2 deletion mutant KW110 to tabtoxin production and resistance to TβL (25). The mechanism by which this physically and functionally conserved biosynthetic region arose in such diverse genetic backgrounds is not understood at present.

The molecular mechanism leading to the spontaneous deletion of the tabtoxin region is, as yet, unknown. The excision event was apparently precise since an 11-kb *Eco*RI junction fragment remained in all of the BR2 deletion mutants that we have examined. The loss of this region may be analogous to the self-deletion of antibiotic genes in *Streptomyces* spp. (5). The presence of sequences elsewhere in the BR2(R) genome that share homology with sequences near one end of the deletion hint that recombinational excision may have been involved in the deletion event. Such a mechanism might involve the *P. syringae* BR2(R) analog of the *E. coli recA* gene product (56). We have recently cloned the BR2(R) *recA* gene, and we are in the process of constructing recombination-deficient derivatives of BR2(R) in order to ascertain the effect of this genetic background on the deletion of the tabtoxin region.

Anzai et al. (1, 2) have recently reported the cloning and expression of a transacetylase gene (*ttr*) from *P. syringae* pv. tabaci that provides *E. coli* with resistance to TβL. The expression of this gene in plants was reported to render the transgenic tobacco resistant to tobacco wildfire. We have compared plasmid pARK10, containing the *ttr* gene (1), with pRTBL823 by Southern analysis and found no cross-hybridization between the two plasmid inserts (25). Homology to pARK10 was unaltered in comparisons of restriction digests of chromosomal DNA from BR2(R) and its Tox^s deletion derivative KW110. In fact, all of the tabtoxin-sensitive *P. syringae* isolates that we tested, including "*P. angulata*," *P. syringae* pv. phaseolicola, and *P. syringae* pv. tomato, contained sequences that hybridized to the pARK10 insert. At present, our data does not support the conclusion that the *ttr* gene is the source of resistance to TβL exhibited by tabtoxin-producing strains. We are at a loss to explain the apparent ability of pARK10 to provide both *E. coli* and tobacco with resistance to TβL. However, it is interesting that the published sequence of the putative resistance gene *ttr* lacks both an identifiable promoter and ribosome binding site (2). It is possible that the TβL resistance phenotype associated with *ttr* may be due to a fortuitous gene fusion product.

Our results indicate that tabtoxin is required by BR2(R) for both chlorosis and lesion formation on bean. Toxin production was not required for growth in planta, and as yet, we have been unable to genetically separate toxin production from the disease symptoms caused by this strain. All mutations that affected tabtoxin production, whether spontaneous deletion or transposon induced, also affected lesion formation, and in all cases, restoration of tabtoxin production also restored pathogenic symptoms. Other factors may be required for BR2 to be pathogenic on bean, but apparently these are in addition to tabtoxin production.

The requirement of tabtoxin production for lesion formation by BR2 contrasts with our recent analysis of tabtoxin-deficient mutants of *P. syringae* pv. tabaci ATCC 11528 and *P. syringae* pv. coronafaciens Pc27. Tox^- derivatives of these two genetic backgrounds, whether transposon or deletion mutants, retained the ability to form lesions, and the symptoms produced in planta were indistinguishable from those caused by the naturally occurring Tox^- strains "*P. angulata*" and *P. syringae* pv. striafaciens, respectively (3). In contrast, the ability to produce tabtoxin did not cause the epiphyte Cit7 to exhibit pathogenic symptoms on either bean or tobacco, apparently demonstrating the need for a genetic predisposition to cause disease on these hosts.

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