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Evaluation of the Role of Syringomycin in Plant Pathogenesis by Using Tn*5* Mutants of *Pseudomonas syringae* pv. *syringae* Defective in Syringomycin Production†

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Syringomycin is a necrosis-inducing phytotoxin produced by *Pseudomonas syringae* pv. *syringae*. To determine whether syringomycin production is a determinant in virulence or pathogenicity, we isolated nontoxigenic (Tox$^-$) Tn*5*-containing mutants and then quantitatively evaluated them for the ability to multiply and cause disease in immature sweet-cherry fruits. Transposon Tn*5* was delivered to Tox$^+$ strain B301D-R by using the suicide vector, pGS9, and the resultant kanamycin-resistant (Km^r) colonies were screened for changes in syringomycin production by testing for antibiosis against *Geotrichum candidum*. Southern blot analysis of *Kpn*I- and *Eco*RI-digested DNA showed that 15 (0.3%) Tox$^-$ mutants were isolated which had Tn*5* inserted into 1 of 14 distinct loci. Phenotypic characterization of the Tox$^-$ mutants identified three major groups, which were differentiated by pathogenicity and ability to cause a tobacco hypersensitive reaction (HR). The eight strains in group A were pathogenic (Path$^+$) in cherry fruit assays, but the disease index was 17 to 66% lower (significant at $P = 0.01$) than for the parental Tox$^+$ strain, B301D-R. The population dynamics of group A strains W4S770 and W4S116 in cherry fruits were, however, indistinguishable from that of strain B301D-R. The remaining seven Tox$^-$ strains were nonpathogenic; group B strain W4S2545 (Path$^-$ HR$^+$) and group C strain W4S468 (Path$^-$ HR$^-$) developed significantly lower populations (10^5 to 10^7 CFU per cherry fruit) 3 days after inoculation than strain B301D-R did (nearly 10^9 CFU per fruit). The data indicate that syringomycin is not essential for pathogenicity, but contributes significantly to virulence.

Phytotoxin production appears to be an important component of plant pathogenesis for most pathovars of *Pseudomonas syringae* (20). The phytotoxins produced by *P. syringae* are structurally diverse and, accordingly, cause specific physiological and biochemical effects in plant cells, which are manifested as either chlorosis or necrosis (20, 34). *P. syringae* pv. *syringae* is the only pathovar known to produce necrosis-inducing toxins; other pathovars produce toxins that cause chlorosis. *P. syringae* pv. *syringae* also differs from other pathovars of *P. syringae* in that it causes diseases in numerous plant species, including both monocots and dicots (7, 23).

Most strains of *P. syringae* pv. *syringae*, irrespective of host range, produce the phytotoxin syringomycin. The structurally related phytotoxin, syringotoxin, has thus far been shown to be produced only by citrus strains (17, 26). Syringomycin concentrations as low as 1 μg/ml applied to parenchymatous tissues elicit necroses resembling symptoms caused by the bacterium (23); nevertheless, there has been no conclusive evidence that syringomycin production contributes to the pathogenicity or virulence of *P. syringae*. A fluorescent antibody specific to syringomycin successfully detected the phytotoxin in diseased and toxin-treated peach leaves and stems (37); however, attempts to isolate the phytotoxin from diseased tissues at physiologically active concentrations have been unsuccessful (25). Moreover, in previous studies (2, 16, 23) in which nontoxigenic spontaneous and chemically induced mutants of *P. syringae* pv. *syringae* were reduced in virulence and frequently nonpathogenic, the possibility that an auxiliary mutation(s) was directly responsible for the attenuated virulence was not discounted.

Although syringomycin contains a positively charged peptide moiety consisting of arginine, phenylalanine, serine, and diaminobutyric acid in a 1:1:2:2 molar ratio (26, 49), its structure is unknown. The biosynthetic pathway to syringomycin is also unknown, but it probably resembles that of other peptide-containing antibiotics which are made by peptide synthetases and does not involve mRNA as a primary template (9, 15, 29). Syringomycin synthesis is regulated by nutritional and environmental factors in a manner similar to that of other secondary metabolites produced by *P. aeruginosa* and related bacteria (19). When produced during plant pathogenesis, syringomycin apparently binds to plant membranes, which are the primary target of activity (2, 49). Bidwai et al. (4) demonstrated that syringomycin at low concentrations stimulates ATPase activity in plasma membranes from red beet cells, possibly by stimulating the activity of a protein kinase which, in turn, mediates the phosphorylation of ATPase. Thus, syringomycin has unique structural, biosynthetic, and phytotoxic properties (which distinguish it from the chlorosis-inducing pseudomonad phytotoxins [20, 34]).

There have been few studies of the molecular genetic basis of phytotoxin production in *P. syringae*, despite evidence for governing roles in plant pathogenesis. Early work (9, 12, 18, 36, 39) tested the involvement of plasmids in phytotoxin production because of the tendency of some strains of *P. syringae* to become nontoxigenic in culture. Structural genes for coronatine production in strains of *P. syringae* pv. *atropurpurea* (40) and, apparently, *P. syringae* pv. *glycinea* (J. W. Willis and J. V. Leary, Phytopathology **74**:838, 1984) are plasmid encoded. However, genes from *P. syringae* pv.

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phaseolicola and *tabaci*, which are required for the biosynthesis of phaseolotoxin and tabtoxin, respectively, are chromosomal (36, 38, 39). Currier and Morgan (12) also concluded that plasmids were not involved in syringomycin production, because less than 50% of the syringomycin-producing strains of *P. syringae* pv. *syringae* contained plasmids and the elimination of plasmids from two producer strains had no effect on phytotoxin production. In addition, Gonzalez et al. (18) showed that conjugal transfer of a plasmid (pCG131) indigenous to strain HS191, but tagged with Tn*1*, did not functionally restore toxigenicity in syringomycin-negative mutants of HS191.

Because syringomycin is similar to syringotoxin in structure and biological activity (17, 26), parallel similarities in biosynthesis and genetic organization might be expected. Morgan and Chatterjee (35; M. K. Morgan and A. K. Chatterjee, Proc. 3rd Int. Working Group on *Pseudomonas syringae* Pathovars, in press) reported the isolation of 15 syringotoxin-negative Tn*5* mutants of *P. syringae* pv. *syringae* B457 which were genotypically divided into three groups based on insertion of Tn*5* in a 10.5-, 19.3-, or 21.8-kilobase (kb) *Eco*RI fragment. The 10.5- and 21.8-kb *Eco*RI fragments were adjacent to one another on the chromosome and were associated with the expression of proteins of molecular weight 470,000 (ST1) and 435,000 (ST2) (Morgan and Chatterjee, in press). These large proteins were suspected of being peptide synthetases on the basis of factors that coregulate the expression of the proteins and syringotoxin production. Furthermore, virulence of a syringotoxin-negative Tn*5* mutant of the citrus pathogen was severely attenuated (Morgan and Chatterjee, in press).

This study initiates molecular genetic analyses of chromosomal determinants of syringomycin production in *P. syringae* pv. *syringae* relative to the plant-bacterium interaction. Because *P. syringae* pv. *syringae* is a serious pathogen of deciduous fruit trees, causing stem cankers and necrotic lesions on fruits and leaves (7, 22), the highly virulent strain B301D (22) was used to investigate the relationship between toxigenicity and pathogenesis. The suicide plasmid vector pGS9 (41) was used to generate random Tn*5* insertion mutants of strain B301D-R, which were then screened for changes in toxigenicity based on biocidal inhibition of the fungus *Geotrichum candidum* (23). Inoculations of immature sweet-cherry fruits with Tox$^-$ Tn*5* mutants were used to quantitatively evaluate the contribution of syringomycin to pathogenicity and virulence. The usage of pathogenicity and virulence in this report is in accordance with the description of Chatterjee and Vidaver (9).

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and plasmids used in this study and their relevant characteristics are described in Table 1. *P. syringae* pv. *syringae* B301D, originally isolated from a diseased pear (*Pyrus communis* L.) flower in England (22), is highly virulent and is a consistent producer of large quantities of syringomycin in vitro (19). Strain B301D-R was obtained by selection for spontaneous resistance of strain B301D to rifampin (100 μg/ml). Reversion of B301D-R to sensitivity to rifampin occurred at a frequency of $<10^{-8}$. B301D-R was indistinguishable from B301D in pathogenicity tests in pear (*P. communis* cv. Bartlett) seedlings (described below) and in ability to produce syringomycin on potato dextrose agar (PDA). Thus, unless stated otherwise, strain B301D-R was used as the parental strain. Plasmids pRZ102, pCU101, and pGS9 were maintained in *Escherichia coli* HB101, C-1a, and WA803, respectively. *G. candidum* F-260 was used in bioassays for syringomycin production (23).

Media, antibiotics, and culture conditions. Strains of *P. syringae* pv. *syringae* were routinely cultured on King medium B (KB) agar (28). Strains were preserved in glycerol for short-term storage (21) and lyophilized for long-term storage. *E. coli* strains were grown at 30 or 37°C on Luria-Bertani (LB) agar or in LB broth (32), both of which were modified to contain NaCl at 5 g/liter; all *E. coli* strains were preserved in the same way as *P. syringae*. For use in syringomycin bioassays, *G. candidum* was incubated overnight in nutrient broth-yeast extract (NBY) (47) broth with rotary shaking (250 rpm) at 25°C. The fungus was maintained on NBY agar slants at 4°C.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli		
HB101	Pro$^-$ Leu$^-$ Thy$^-$ Thi$^-$ Sm^r	6
C-1a	Strain C, wild type	32
WA803	Met$^-$ Thi$^-$	48
P. syringae pv. *syringae*		
B301D	Wild type from pear	22
B301D-R	Spontaneous Rif^r derivative of B301D	This study
W4S116, W4S178, W4S328, W4S459, W4S468, W4S770, W4S783, W4S787, W4S1016, W4S1522, W4S1649, W4S1991, W4S2395, and W4S2545	B301D-R::Tn*5* Rif^r Km^r	This study
W4S4469	B301D-R::Tn*5* Rif^r Km^r Cm^r Pro$^-$	This study
W4S4390	B301D-R::Tn*5* Rif^r Km^r Thy$^-$	This study
W4S4383 and W4S4428	B301D-R::Tn*5* Rif^r Km^r Pro$^-$	This study
Plasmids		
pGS9	Tra$^+$ (N type), p15A replicion, Cm^r Km^r (Tn*5*)	41
pCU101	Tra$^+$ (N type), p15A replicon, Cm^r	41
pRZ102	ColE1 replicon, Km^r (Tn*5*)	27

N minimal (NM) agar medium (47) was used to screen for auxotrophs among the Km^r *P. syringae* transconjugants. The nutritional requirements of the auxotrophs were determined by the method of Davis et al. (14). Syringomycin production was assayed on PDA containing 1.5% glucose and 0.5% vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.) (24). When appropriate, antibiotics were added at the following concentrations (in micrograms per milliliter) to agar media: kanamycin, 50; streptomycin, 100; rifampin, 100; chloramphenicol, 100; and tetracycline, 15. The antibiotic concentrations were decreased by half for liquid cultures.

Tn*5* mutagenesis of *P. syringae* pv. *syringae*. Transposon Tn*5* was delivered by the suicide plasmid, pGS9 (41), contained in WA803; pGS9 is composed of a p15A replicon and contains the N conjugative system. The donor strain, WA803, was grown to late-log phase in LB broth containing kanamycin, and the recipient strain, B301D-R, was grown to late-log phase in NBY broth. The donor and recipient cells were sedimented by low-speed centrifugation and resuspended in fresh LB broth. The donor and recipient cell counts were adjusted to 10^7 and 10^8 CFU/ml, respectively. For matings, equal volumes (0.1 ml each) of the donor and recipient cells were spotted onto nitrocellulose membranes (pore size, 0.45 μm) placed on LB agar and were incubated for 8 to 10 h at 30°C. After mating, the bacteria were washed into sterile water and spread on LB agar containing kanamycin and rifampin. Plates were incubated for 48 to 72 h at 25°C, and resultant Km^r colonies were picked and grown on KB agar containing kanamycin. Approximately 30 membranes were prepared for each mating; each membrane was washed and plated separately for isolation of Km^r transconjugants. Transconjugants were checked for streptomycin resistance (Sm^r) and chloramphenicol sensitivity (Cm^s). In addition, Km^r colonies were screened for production of the fluorescent siderophore $pyoverdin_{pss}$ (10) on KB agar and for auxotrophy on NM agar medium.

Reversion frequencies of Tn*5* insertions were determined for auxotrophic Tn*5* mutants. Thy^- (W4S4390) and Pro^- (W4S4383 and W4S4428) mutants from single colonies were grown overnight in 25 ml of NBY broth at 25°C, sedimented by low-speed centrifugation, suspended in NM broth, and resedimented. The cells were then suspended in NM broth (2.5 ml), from which serial dilutions were made and plated onto NM and NBY agar. Frequencies of reversion to prototrophy were determined from differences in viable cell counts (as CFU). After 3 to 4 days of incubation, prototrophs were isolated and then tested for growth on LB agar containing kanamycin.

Identification of syringomycin-negative Tn*5* mutants. Km^r mutants of *P. syringae* pv. *syringae* B301D-R were screened on PDA for changes in production of syringomycin by using the bioassay method of Gross and DeVay (23). Km^r mutants were grown overnight on KB agar containing kanamycin and then transferred with sterile toothpicks onto PDA; each plate carried up to 11 isolates together with the wild type, B301D. Duplicate plates were prepared and incubated for 4 days at 25°C. After incubation, the plates were lightly oversprayed with a spore suspension of *G. candidum* (23), and zones of fungal growth inhibition were measured and recorded after 16 h. Tn*5* mutants altered in toxin production relative to strain B301D were retested once to verify changes in syringomycin production.

DNA isolations. Plasmids pRZ102 and pCU101 were isolated from the appropriate *E. coli* strains by the method of Birnboim and Doly (5). Plasmid DNA was purified by ultracentrifugation (220,000 × *g* for 15 h) on ethidium bromide-cesium chloride gradients by using a vertical rotor. Plasmid DNA was collected, precipitated, and suspended in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) (32).

Total cellular DNA of *P. syringae* was isolated and purified by a modification (8) of the method of Currier and Nester (13). Purified DNA was stored in TE buffer at 4°C.

Restriction endonuclease digestion and agarose gel electrophoresis. The restriction endonucleases *Eco*RI, *Kpn*I, and *Bgl*II were from New England BioLabs, Inc., Beverly, Mass., and were used as recommended by the manufacturer. Genomic DNA of Tox^- Tn*5* mutants of *P. syringae* was digested to completion (3.5 U/μg), and the DNA fragments were separated by electrophoresis (24 h at 8 mA) in a 0.8% agarose gel with a Tris-borate buffer (32). Molecular size standards (in kilobases) consisted of bacteriophage lambda DNA (New England BioLabs, Inc.) digested with *Eco*RI, *Kpn*I, or *Sal*I; a mixture of the digests generates 12 fragments ranging from 0.5 to 32.7 kb. Gels were photographed under UV light after being stained with ethidium bromide (0.5 μg/ml).

Southern transfer of DNA fragments and DNA-DNA hybridization. DNA fragments were transferred from agarose gels to GeneScreen Plus (New England Nuclear Corp., Boston, Mass.) membranes as specified by the manufacturer. Purified plasmids (either pCU101 or pRZ102) were labeled with [^{32}P]dCTP (New England Nuclear Corp.) by a modification (46) of the nick translation reaction; the specific activity of labeled DNA was approximately 5×10^7 cpm/μg. DNA-DNA hybridization took place in a modified Denhardt solution (32) containing 6× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate), 5× Denhardt solution (32), 20 mM Tris (pH 7.6), 0.1% sodium dodecyl sulfate, 2 mM EDTA, 100 μg of denatured salmon sperm DNA (type III; Sigma Chemical Co., St. Louis, Mo.), and 10 ng of [^{32}P]DNA probe per ml. Membranes were incubated for 6 to 24 h at 65°C with gentle shaking (50 rpm in a Gyrotory water bath shaker; New Brunswick Scientific Co., Inc., Edison, N.J.). Following hybridization, the membranes were washed at 65°C with constant agitation in a buffer composed of 6× SSC, 0.2% sodium dodecyl sulfate, and 5 mM EDTA. The washing buffer was changed at 30-min intervals over a period of 2 h. Standard autoradiography methods (32) were used to identify DNA fragments homologous to the probe. The sizes of the DNA fragments hybridizing with the Tn*5* probe were determined by the method of Southern (44).

Plant pathogenicity and hypersensitivity tests and measurements of bacterial growth in planta. Tn*5* mutants affected in syringomycin production were initially screened for pathogenicity by using 2-week-old pear seedlings. Pear seeds, germinated as described by Currier and Morgan (12), were planted in potting soil and grown in the greenhouse at approximately 25°C. The light intensity was supplemented by exposure to cool-white fluorescent lamps 150 cm above the benches for 12 h. Stems of 2-week-old seedlings with two sets of fully expanded leaves were stab-inoculated with an inoculum-laden needle (approximately 10^6 cells) between the cotyledon and first leaves. The inoculum of the bacterial strains was grown overnight on NBY agar. Inoculated pear seedlings were incubated at a high humidity (>90%) at 25°C. Symptoms were recorded 2 to 3 days after inoculation.

Quantitative evaluations of Tox^- Tn*5* mutants for virulence were performed by using immature sweet-cherry (*Prunus avium* L. cv. Bing) fruits as previously described (22). Cells used in inoculation were grown to log phase in

NBY broth, sedimented by centrifugation (11,700 × *g* for 20 min), and suspended to approximately 10^6 CFU/ml in sterile deionized water. Immature cherry fruits (picked 6 to 8 weeks after pollination) were surface sterilized (22), rinsed in deionized water, and air dried at room temperature. Each fruit was stab-inoculated with a 25-gauge needle at three sites to a depth of 5 mm through a 10-μl droplet of the bacterial suspension. Twelve fruits were inoculated per strain. Fruits serving as controls were inoculated with sterile deionized water. After inoculation, the fruits were supported in a test-tube rack and incubated at a high humidity (>90%) for 3 days at 25°C. Disease was evaluated 3 days after inoculation by measuring the diameter of the lesion according to the following rating system: 0 = no lesion; I = lesion diameter up to 1 mm; II = lesion diameter of 1.1 to 2.0 mm; III = lesion diameter of 2.1 to 3.0 mm; IV = lesion diameter of 3.1 to 4.0 mm; and V = lesion diameter greater than 4.0 mm. The disease index was calculated by using the formula of McKinney (33).

The population dynamics of *P. syringae* pv. *syringae* B301D-R and nontoxigenic Tn*5* mutants W4S116, W4S770, W4S2545, and W4S468 in immature cherry fruits were determined over a period of 3 days. Fruits were inoculated as described above, and at each 24-h interval three fruits were triturated with a mortar and pestle in 3 ml of sterile deionized water. Samples (100 μl) from appropriate dilutions were plated in duplicate onto KB agar containing rifampin (50 μg/ml), the plates were incubated at 25°C, and colonies were counted after 48 h. Three replications (three fruits per replication) were prepared for each treatment at a given time.

The hypersensitivity reaction (HR) was determined as previously described (22) by using tobacco (*Nicotiana tabacum* L. cv. White Gold) leaves.

RESULTS

Tn*5* mutagenesis of *P. syringae* pv. *syringae*. Km^r colonies of *P. syringae* pv. *syringae* B301D-R were obtained at an average frequency of 1.2×10^{-5} per initial donor cell of *E. coli* WA803(pGS9). All Km^r mutants were also Sm^r, which has been shown to be carried on Tn*5* but expressed only in certain nonenteric bacteria such as *Rhizobium* spp. (11, 42). In addition, approximately 6.6% of the Km^r Sm^r colonies were resistant to chloramphenicol (Cm^r). The Cm^r mutants apparently arose by Tn*5*-promoted vector integration into the recipient genome (11, 41). The presence of vector sequences in the Km^r Cm^r mutants was confirmed by Southern blot analysis with the progenitor of pGS9 (i.e., pCU101) as probe.

Of over 2,400 Tn*5* mutants tested, approximately 0.4% were auxotrophs. Analysis of the nutritional requirements indicated that Tn*5* transposition occurred randomly in strain B301D-R, since some of the auxotrophic mutants were identified as Cys^-, Glt^-, Pro^-, Thy^-, or Ura^-. Frequencies of reversion for Thy^- and Pro^- mutants to prototrophy were between 10^{-8} and 10^{-9}, indicative of stable insertion of Tn*5*.

Isolation of Tn*5* mutants affected in syringomycin production. Over 4,500 Km^r mutants of *P. syringae* pv. *syringae* B301D-R were screened on PDA plates for quantitative differences in syringomycin production. Strain B301D-R produced a fungicidal zone of inhibition which typically measured 7 mm from the edge of the bacterial colony to the edge of fungal growth (Fig. 1). The Tn*5* mutants were categorized into four classes representing four distinct zone patterns of inhibition. Class I Tox^- Tn*5* mutants produced no detectable syringomycin and occurred at a frequency of 0.3%; 15 Tox^- strains were identified, and all but 1 were found to be prototrophic (Table 1). *G. candidum* grew over the colonies of several strains of the class I Tn*5* mutants (Fig. 1). Class II Tn*5* mutants, occurring at a frequency of 2.8%, were hypoproducers, because they produced a syringomycin zone smaller than that of B301D-R. Class III Tn*5* mutants consisted of 54 hyperproducers of syringomycin, occurring at a frequency of 1.2%, which produced zones of about 20 mm as measured from the margins of the colonies. Colonies of the hyperproducers on PDA were characteristically less mucoid than and over twice the diameter of B301D-R colonies (Fig. 1). Class IV mutants, composing over 95% of the Tn*5* mutants, were indistinguishable from B301D-R in their ability to produce syringomycin.

Phenotypic characteristics of Tox^- mutants. Strain B301D-R was highly virulent in pear seedlings and caused a black lesion at the site of inoculation 24 h after inoculation. After 2 to 3 days, a black necrosis spread several millimeters up and down the stem and frequently into the midribs of leaves. The top portions of the seedlings usually were flaccid and completely discolored 4 days after inoculation. The class II strains (hypoproducers) and class III strains (hyperproducers) generally were indistinguishable from B301D-R in virulence. In contrast, all of the class I (nontoxigenic) strains were either nonpathogenic or substantially less virulent, causing necrosis only within the inoculation site and with no systemic symptoms.

The 15 Tox^- Tn*5* mutants were subdivided into four phenotypic groups (Table 2). Group A contained eight strains which were pathogenic to pear seedlings, but were less virulent than B301D-R. Group B contained only one strain, W4S2545, which was clearly nonpathogenic ($Path^-$), but caused an HR in tobacco leaves. The five strains in group C were $Path^-$ HR^-, and the single strain (W4S4469) in group D resembled the group C strains, except that it did not

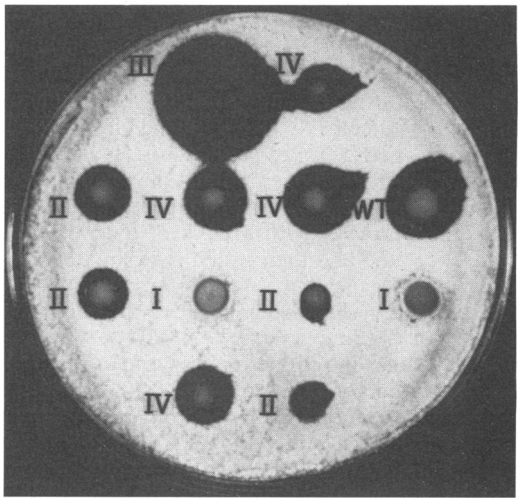

FIG. 1. *G. candidum* bioassay for screening Tn*5* mutants of *P. syringae* pv. *syringae* B301D-R for syringomycin production. The Tn*5* mutants were grown on PDA for 4 days at 25°C and then oversprayed with the fungus to observe zones of inhibition to growth. Tn*5* mutants were categorized into four groups according to the size of the zone of inhibition, as compared with that produced by the Rif^r wild-type strain B301D-R (WT): class I, syringomycin nonproducers (I); class II, syringomycin hypoproducers (II); class III, syringomycin hyperproducers (III); and class IV, normal producers of syringomycin (IV).

TABLE 2. Phenotypic characteristics of nontoxigenic mutant groups of *P. syringae* pv. *syringae* obtained by Tn*5* mutagenesis

Group[a]	Representative strain	Syringomycin production	Pathogenicity to pear seedlings	Tobacco hypersensitivity	Pyoverdin$_{pss}$ production
A	W4S770	−	+[b]	+	+
B	W4S2545	−	−	+	+
C	W4S468	−	−	−	+
D	W4S4469	−	−	−	−
	B301D-R	+	+	+	+

[a] Strains included in each group are as follows: group A contains W4S116, W4S178, W4S328, W4S459, W4S770, W4S783, W4S1522, and W4S2395; group B contains W4S2545; group C contains W4S468, W4S787, W4S1016, W4S1649, and W4S1991; and group D contains W4S4469.
[b] Virulence of all eight group A strains was noticeably less than that of B301D-R.

produce the fluorescent siderophore pyoverdin$_{pss}$ and was auxotrophic for proline.

Physical analysis of Tn*5* transpositions yielding Tox$^-$ phenotypes. Southern blot analysis verified the occurrence of single Tn*5* insertions in the genomes of the 15 nontoxigenic mutants and the absence of sequences in strain B301D homologous to Tn*5* (Fig. 2). Because Tn*5* does not contain a restriction site for either *Kpn*I or *Eco*RI, the copy number of Tn*5* per genome and the size of the fragment in which it was inserted were readily determined. The following *Kpn*I/*Eco*RI fragments (in kilobases minus the 5.7 kb of Tn*5*), respectively, hybridized to Tn*5* within the pRZ102 probe: W4S468, 8.2/5.3; W4S787, 5.4/8.4; W4S1016, 15.8/9.2; W4S2545, 5.6/13.0; W4S4469, 2.3/13.0; W4S1649, 14.1/15.8; W4S1991, 14.1/5.9; W4S116, 14.1/9.2; W4S178, 14.1/15.8; W4S328, 20.4/7.0; W4S459, 8.5/25.0; W4S770, 9.9/7.4; W4S783, 12.2/5.9; W4S1522, 13.2/22.5; and W4S2395, 9.9/11.4. Four strains (W4S1649, W4S1991, and W4S116, and W4S178) showed Tn*5* insertions into 14.1-kb *Kpn*I fragments, and two (W4S770 and W4S2395) had insertions into 9.9-kb *Kpn*I fragments (Fig. 2A). However, blots of *Eco*RI-digested genomic DNA (Fig. 2B) showed that the Tn*5* insertions were in unique fragments; the only exceptions were strains W4S1649 and W4S178, which were not differentiated genotypically but were distinct phenotypically (Table 2). These results showed that at least 14 of the nontoxigenic Tn*5* mutants are genotypically distinct from one another and that there are several genes involved in syringomycin production.

To determine whether IS*50* from Tn*5* had independently inserted into the genome of a given nontoxigenic mutant, we cleaved genomic DNA with *Bgl*II. Because Tn*5* contains two *Bgl*II restriction sites, complete digests yielded three *Bgl*II bands that hybridized to the Tn*5* probe (data not shown). There was no indication that IS*50* had independently inserted into the genome of any of the Tox$^-$ mutants, which can complicate genetic studies of some strains of *P. syringae* (1). Vector insertion was nevertheless demonstrated for strain W4S4469, which was Km^r Cm^r and hybridized to ^{32}P-labeled pCU101 as probe (data not shown). The pCU101 probe did not hybridize with any of the other Tox$^-$ mutants, which were all Km^r Cm^s.

Pathogenicity of Tox$^-$ Tn*5* mutants. The disease indices for all of the Tox$^-$ Tn*5* mutants in inoculated cherry fruits were significantly ($P = 0.01$) lower than that of B301D-R (Table 3). Furthermore, 7 of the 15 Tox$^-$ strains (group B, C, and D mutants) were nonpathogenic, causing the same response as inoculations with sterile water, as exemplified by strains W4S2545 (group B) and W4S468 (group C) (Fig. 3). In contrast, all of the group A strains were virulent, but had significantly lower disease indices, ranging from 17% (W4S116) to 66% (W4S770) lower than that for B301D-R (Table 3). For example, strain W4S770 caused a discoloration of inoculated areas which first became visible 2 days after inoculation. The lesion size subsequently expanded very slowly, and by day 3 the average diameter was 1.4 mm, with no diffuse necrosis surrounding the lesion (Fig. 3). In comparison, the lesions caused by B301D-R enlarged to an average diameter of 4.3 mm by day 3.

Population dynamics of Tox$^-$ Tn*5* mutants in cherry fruits. The population dynamics of the Tox$^-$ Tn*5* strains in imma-

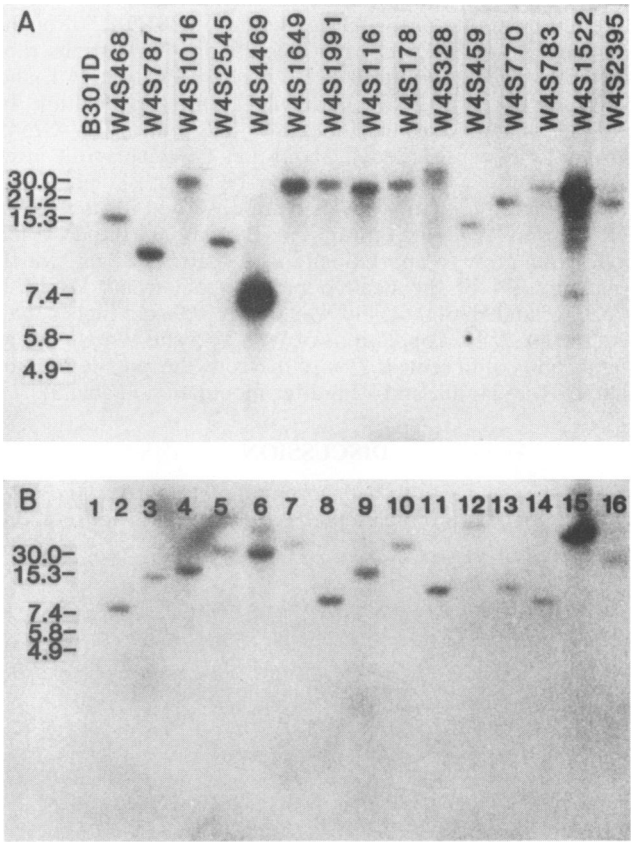

FIG. 2. Southern blot analysis of Tox$^-$ (class I) Tn*5* mutants of *P. syringae* pv. *syringae* B301D-R. Total genomic DNA was cleaved with *Kpn*I (A) and *Eco*RI (B). The DNA fragments were then separated by agarose gel electrophoresis, transferred to GeneScreen Plus membranes, and assayed for hybridization to Tn*5* with ^{32}P-labeled pRZ102 as the probe. Lane 1 contains B301D; lanes 2 to 16 contain the Tox$^-$ Tn*5* strains as identified. Apparently, the anomalous results observed for strain W4S1522 were caused by an overload of DNA. Size standards in kilobases are indicated on the left.

TABLE 3. Virulence and growth of *P. syringae* pv. *syringae* B301D-R and its derivative Tox$^-$ Tn*5* mutants in immature cherry fruits

Strain	Phenotypic characteristic	Disease index[a]	Bacterial population (log CFU/fruit) after:			
			0 h	24 h	48 h	72 h
B301D-R	Path$^+$ HR$^+$	86.9	3.0	7.2	7.6	8.9
W4S770	Path$^+$ HR$^+$	29.4**	3.5	7.5	7.5	8.1
W4S116	Path$^+$ HR$^+$	72.2**	3.1	7.3	7.7	8.9
W4S2545	Path$^-$ HR$^+$	0**	3.4	4.9**	5.4**	5.4**
W4S468	Path$^-$ HR$^-$	0**	4.0	4.6**	6.1**	6.9**
$LSD_{0.05}$[b]		7.5		1.2	0.9	1.0
$LSD_{0.01}$[b]		10.7		2.0	1.6	1.7

[a] Disease index was calculated after 72 h of incubation by the formula of McKinney (33): sum of disease ratings × 100/(total number of ratings × maximum disease grade) where disease grades are defined in the text.

[b] The protected least significant differences (LSD) between the B301D-R and mutant strains are shown at $P = 0.05$ (*) and $P = 0.01$ (**).

ture cherry fruits showed three distinct patterns of growth (Table 3; Fig. 4). The populations of the group A strains (Path$^+$ HR$^+$), exemplified by W4S770 (least virulent) and W4S116 (most virulent), were not significantly ($P = 0.01$) different from that of the parental strain, B301D-R, throughout the 72-h period following inoculation. Populations rapidly increased to about 10^7 CFU per fruit within 24 h and peaked at nearly 10^9 CFU per fruit 72 h after inoculation. In contrast, populations of the Path$^-$ HR$^+$ strain W4S2545 (group B) never exceeded about 10^5 CFU per fruit after growth from an initial population of 10^3 CFU per fruit. Intermediate population levels were observed for the Path$^-$ HR$^-$ strain W4S468 (group C). By day 3, the W4S468 population grew to approximately 10^7 CFU per fruit, which was only 1% of the peak population of strains W4S770, W4S116, and B301D-R, but was nearly 100-fold higher than that of W4S2545. Populations of W4S2545 and W4S468 were significantly different at $P = 0.01$ from the populations of B301D-R at 24, 48, and 72 h after inoculation (Table 3).

DISCUSSION

Analyses of Tox$^-$ transposon mutants of *P. syringae* pv. *syringae* B301D-R indicate that syringomycin is not required

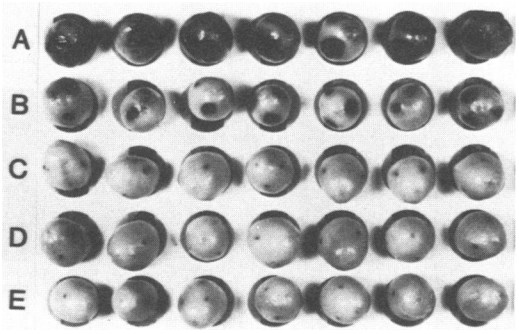

FIG. 3. Symptoms caused by representative Tox$^-$ (class I) Tn*5* mutants of *P. syringae* pv. *syringae* B301D-R in immature sweet-cherry fruits. Necrosis reactions were observed 3 days after injection with 10^4 CFU per wound, three wounds per fruit. Treatments: Tox$^+$ parental strain B301D-R (line A); group A strain W4S770 (line B); group B strain W4S2545 (line C); group C strain W4S468 (line D); and a sterile-water control (line E).

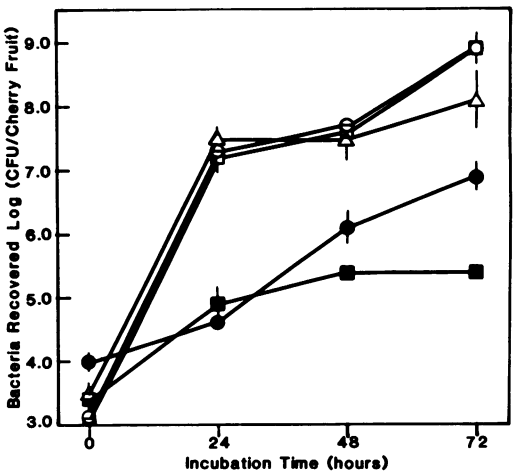

FIG. 4. Population dynamics of Tox$^-$ (class I) Tn*5* mutants of *P. syringae* pv. *syringae* B301D-R in immature sweet-cherry fruits. Average populations per fruit over a 72-h period are shown for group A strains W4S116 (○) and W4S770 (△), group B strain W4S2545 (■), group C strain W4S468 (●), and Tox$^+$ parental strain B301D-R (□). Each value was an average of three replications, with three cherry fruits per replication. Vertical bars indicate standard error of the mean of log populations.

for their growth in planta and pathogenicity, but does significantly contribute to virulence. This is based on the observations that 8 of the 15 Tox$^-$ mutants (i.e., group A) caused necrosis in both pear seedlings and immature sweet-cherry fruits and that the lesions were localized and were only about one-third the size of lesions caused by the Tox$^+$ parental strain. Apparently, syringomycin accentuates disease during pathogenesis by killing a large number of host cells, which is expressed as markedly more necrosis and larger lesion size. Nevertheless, seven of the Tox$^-$ mutants (groups B, C, and D) were nonpathogenic, indicating that syringomycin production is linked to pathogenicity in some instances. This substantiates earlier observations (16, 23) that syringomycin-negative mutants are often Path$^-$.

Phytotoxins produced by various other pathovars of *P. syringae* likewise function as virulence determinants and are not essential for pathogenicity. For example, Bender et al. (3) observed that Tn*5* mutants of *P. syringae* pv. *tomato* PT23.2, defective in coronatine production, caused necrotic lesions of 0.2 mm in diameter, which were only about one-third the size caused by the wild type in tomato leaves. However, in contrast to our syringomycin-negative (group A) mutants W4S770 and W4S116, populations of their wild-type strain, PT23.2, were ultimately and significantly higher ($P = 0.01$) than the populations of two coronatine-negative mutants. There is also evidence that phaseolotoxin and tabtoxin are virulence rather than pathogenicity determinants (34), although transposon mutants defective in phytotoxin production have not been used to quantitatively evaluate the contribution of toxin production to virulence.

Southern blot analyses of Tox$^-$ Tn*5* mutants showed that several genes are necessary for syringomycin production. Of the 14 Tox$^-$ mutants in groups A, B, and C, 13 resulted from insertion of Tn*5* in unique loci as judged by homology of ^{32}P-labeled Tn*5* to single, discrete *Kpn*I and *Eco*RI fragments. Even though Tn*5* mutagenesis was apparently random in strain B301D-R, the chromosome was not saturated with Tn*5*, since there were only two Tox$^-$ mutants, W4S178 and W4S1649, which appeared to contain Tn*5* in the same

*Kpn*I and *Eco*RI fragments. In comparison, Morgan and Chatterjee (35; in press) isolated 15 syringotoxin-negative mutants, at a frequency of about 0.28%, which were divided genotypically into only three groups based on Tn*5* insertions into *Eco*RI fragments of approximately 10.5, 19.3, and 21.8 kb. Moreover, none of the syringotoxin-negative mutants appeared to resemble our group B and C Tox$^-$ mutants, since they all were Path$^+$, although reduced in virulence (Morgan and Chatterjee, in press). In addition, the 10.5- and 21.8-kb *Eco*RI fragments were near one another on the chromosome and were required for expression of two large proteins, ST1 and ST2, which are possibly peptide synthetases. Apparently, the 10.5- and 21.8-kb fragments shared homology with genomic fragments from strains of *P. syringae* pv. *syringae* that produced syringomycin and not with DNA from other pathovars such as *P. syringae* pv. *phaseolicola* and *tabaci*, which, respectively, produce phaseolotoxin and tabtoxin (9; Morgan and Chatterjee, in press). It remains to be determined whether some of the genes required for syringomycin production, particularly those identified in the group A Tox$^-$ mutants, encode similarly large polypeptides and are in close proximity to one another on the chromosome. Precedence for a clustering of genes required for phytotoxin production also has been demonstrated for phaseolotoxin (38).

Loss of syringomycin production by *P. syringae* pv. *syringae* could have arisen from mutations in one of several types of genes, including genes involved in syringomycin biosynthesis, precursor formation, and excretion, as well as genes that regulate these processes (9). Thus, it is not unexpected that considerable genotypic and phenotypic diversity exists among the Tox$^-$ (class I) Tn*5* mutants. Because syringomycin, like syringotoxin, is a peptide-containing antibiotic and is probably synthesized via a peptide synthetase system analogous to that of *Bacillus* spp. (9, 15, 29), Tox$^-$ mutants arising from inactivation of structural genes for enzymes required for syringomycin synthesis would be preferred for critical tests of the involvement of syringomycin in pathogenesis. Considerable work, however, is needed to elucidate the specific functions of the genes inactivated by Tn*5* in the Tox$^-$ mutants. Auxotrophy was nevertheless not responsible for any of the Tox$^-$ phenotypes placed in mutant groups A, B, and C, although the sole group D mutant, W4S4469, was Pro$^-$, and this was presumably at least partly responsible for the observed pleiotrophic effects on phenotype (Table 2).

Many, if not most, of the hypoproducers (class II) and hyperproducers (class III) of syringomycin probably arose as a result of mutations in genes that regulate the biosynthesis of syringomycin or its precursors. Altered levels of peptide synthetases and the precursors necessary for formation of syringomycin could dramatically affect the yield. Indeed, auxotrophs accounted for about 3% of the hypoproducers of syringomycin; syringomycin yields from these mutants were apparently diminished to less than half that from the wild type. Moreover, a preponderance of the auxotrophs among the class II mutants failed to produce the fluorescent siderophore pyoverdin$_{pss}$, which also contains a small peptide (10). Membrane alterations could also contribute to diminished or enhanced secretion of syringomycin. The fact that all of the hyperproducer colonies showed an altered morphology typified by larger and perhaps less-mucoid colonies is indicative of a significant change in the cell envelope of strain B301D. Morgan and Chatterjee (35) did not obtain Tn*5* mutants which produced noticeably higher levels of syringotoxin, but they did observe that many of the hypoproducers displayed altered colony morphologies and were prone to lysis induced by treatment with lysozyme-EDTA and changes in osmoticum.

The five Tox$^-$ strains in group C are especially intriguing, because they appear to have arisen from Tn*5* insertion in *hrp* genes (i.e., hypersensitive reaction and pathogenicity) as defined by Lindgren et al. (30). The *hrp* genes are required for both inducing the HR in nonhost plants and expressing pathogenicity in susceptible plants. The group C mutants meet the criteria of being Hrp mutants, since they fail to elicit either resistance, expressed as hypersensitivity in tobacco leaves, or susceptible responses in plants. It was unexpected that syringomycin production was linked to expression of *hrp* genes. Such a linkage has not been previously reported for any other pseudomonad phytotoxin, including syringotoxin and phaseolotoxin. Lindgren et al. (30) proposed that Hrp mutants of *P. syringae* pv. *phaseolicola* failed to elicit the HR because they could not produce a compound necessary for its induction. If syringomycin was the end product associated with *hrp* gene activity in *P. syringae* pv. *syringae*, then logic would suggest that the phytotoxin would be responsible for induction of the HR. Alternatively, the *hrp* genes might be functionally responsible for production of a factor necessary for several processes involved in pathogenicity, including, perhaps, toxigenesis and formation of an elicitor of the HR. Another possibility is that the *hrp* genes are not structural genes required for syringomycin biosynthesis, but instead are regulatory genes which somehow control a number of genes required for pathogenicity, including the biosynthetic genes for syringomycin.

The growth kinetics of group C strain W4S468 in immature cherry fruits resembled that of known Hrp mutants of *P. syringae* pv. *phaseolicola* in bean leaves (30, 43). For example, the Hrp mutants of *P. syringae* pv. *phaseolicola* in phenotypic class I showed limited growth in the primary leaves of bean plants, with peak populations approximately 10^4-fold lower than those of the wild-type strain in the study of Lindgren et al. (30). However, peak populations only 100-fold lower than those of the wild type were observed by Somlyai et al. (43) for Hrp Tn*5* mutants of a different strain inoculated into trifoliate bean leaves. Our data likewise show that populations of the Hrp mutant, W4S468, in cherry fruits were about 100-fold lower than those of the parental strain, B301D-R. Because there was no plant response with strain W4S468, limited growth occurred as compared with almost no growth for the Path$^-$ HR$^+$ strain, W4S2545 (Fig. 4). Strain W4S2545 apparently induced the HR in cherry fruits, and this in turn restricted bacterial growth.

Genes that are required for pathogenicity but not for induction of the HR were also associated with syringomycin production in *P. syringae* pv. *syringae* B301D, as evidenced by the occurrence of the group B strain, W4S2545. Although the Path$^-$ HR$^+$ phenotype has not been obtained as frequently in *P. syringae* as the Hrp phenotype following Tn*5* mutagenesis, extensive searches for this phenotype among Tn*5* mutants of strains from several *P. syringae* pathovars have demonstrated its wide distribution (11, 30, 31, 43). The relationship of the Path$^-$ HR$^+$ phenotype to toxigenicity is puzzling, since analysis of group A mutants demonstrated that syringomycin is a virulence rather than a pathogenicity determinant. One possible explanation is that individual genes for pathogenicity and syringomycin production are linked within an operon. A cosmid clone from an *Eco*RI genomic library of B301D was recently identified which contained a 2.3- to 2.8-kb DNA sequence that coordinately

restored syringomycin production and pathogenicity in W4S2545 to wild-type levels after marker exchange (Xu and Gross, unpublished data). These results verify a linkage between toxigenicity and pathogenicity in strain B301D.

The attenuated virulence of all the syringomycin-negative Tn*5* mutants is strong evidence that the phytotoxin has a significant role in the pathogenicity of *P. syringae* pv. *syringae*. Simple screenings for Tox^- mutants proved to be highly efficient in selecting strains that were severely altered in plant reaction, which is indicative of interactions between genes imparting pathogenicity or genes that have regulatory features in common. It is nevertheless possible that the Tox^- mutants produce syringomycin in the plant environment. If such a situation exists for the group A mutants, one could argue that syringomycin contributes more to virulence than indicated by the measurements of disease indices in this study. Because syringomycin cannot be directly isolated from infected plant tissues (25), further genetic studies are needed to substantiate the extent of the involvement of syringomycin in plant pathogenesis. For example, reporter gene fusions (45) to genes encoding proteins mediating syringomycin synthesis could prove particularly useful in studying the expression of syringomycin genes in planta. Consequently, further studies of the Tox^- mutants are needed to physically characterize the genes affecting toxigenicity, to identify the specific functions or gene products of the mutagenized genes, and to determine how the syringomycin genes interact with other genes, such as the *hrp* genes, which are necessary for pathogenicity.

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LITERATURE CITED

1. **Anderson, D. M., and D. Mills.** 1985. The use of transposon mutagenesis in the isolation of nutritional and virulence mutants in two pathovars of *Pseudomonas syringae*. Phytopathology **75**:104–108.
2. **Backman, P. A., and J. E. DeVay.** 1971. Studies on the mode of action and biogenesis of the phytotoxin syringomycin. Physiol. Plant Pathol. **1**:215–233.
3. **Bender, C. L., H. E. Stone, J. J. Sims, and D. A. Cooksey.** 1987. Reduced pathogen fitness of *Pseudomonas syringae* pv. *tomato* Tn*5* mutants defective in coronatine production. Physiol. Mol. Plant Pathol. **30**:273–283.
4. **Bidwai, A. P., L. Zhang, R. C. Bachmann, and J. Y. Takemoto.** 1987. Mechanism of action of *Pseudomonas syringae* phytotoxin, syringomycin: stimulation of red beet plasma membrane ATPase activity. Plant Physiol. **83**:39–43.
5. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
6. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**:95–113.
7. **Bradbury, J. F.** 1986. Guide to plant pathogenic bacteria, p. 175–177. CAB International Mycological Institute, Kew, England.
8. **Buchholz, W. G., and M. F. Thomashow.** 1984. Host range encoded by the *Agrobacterium tumefaciens* tumor-inducing plasmid pTiAg63 can be expanded by modification of its T-DNA oncogene complement. J. Bacteriol. **160**:327–332.
9. **Chatterjee, A. K., and A. K. Vidaver.** 1986. Genetics of pathogenicity factors: application to phytopathogenic bacteria, p. 153–170. *In* D. S. Ingram and P. H. Williams (ed.), Advances in plant pathology, vol. 4. Academic Press, Inc., New York.
10. **Cody, Y. S., and D. C. Gross.** 1987. Characterization of pyoverdin$_{pss}$, the fluorescent siderophore produced by *Pseudomonas syringae* pv. *syringae*. Appl. Environ. Microbiol. **53**:928–934.
11. **Cuppels, D. A.** 1986. Generation and characterization of Tn*5* insertion mutations in *Pseudomonas syringae* pv. *tomato*. Appl. Environ. Microbiol. **51**:323–327.
12. **Currier, T. C., and M. K. Morgan.** 1983. Plasmids of *Pseudomonas syringae*: no evidence of a role in toxin production or pathogenicity. Can. J. Microbiol. **29**:84–89.
13. **Currier, T. C., and E. W. Nester.** 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. **76**:431–441.
14. **Davis, R. W., D. Botstein, and J. R. Roth (ed.).** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Demain, A. L., Y. Aharonowitz, and J.-F. Martin.** 1983. Metabolic control of secondary biosynthetic pathways, p. 49–72. *In* L. C. Vining (ed.), Biochemistry and genetic regulation of commercially important antibiotics. Addison-Wesley Publishing Co., Reading, Mass.
16. **DeVay, J. E., F. L. Lukezic, S. L. Sinden, H. English, and D. L. Coplin.** 1968. A biocide produced by pathogenic isolates of *Pseudomonas syringae* and its possible role in the bacterial canker disease of peach trees. Phytopathology **58**:95–101.
17. **Gonzalez, C. F., J. E. DeVay, and R. J. Wakeman.** 1981. Syringotoxin: a phytotoxin unique to citrus isolates of *Pseudomonas syringae*. Physiol. Plant Pathol. **18**:41–50.
18. **Gonzalez, C. F., S. K. Layher, A. K. Vidaver, and R. H. Olsen.** 1984. Transfer, mapping, and cloning of *Pseudomonas syringae* pv. *syringae* plasmid pCG131 and assessment of its role in virulence. Phytopathology **74**:1245–1250.
19. **Gross, D. C.** 1985. Regulation of syringomycin synthesis in *Pseudomonas syringae* pv. *syringae* and defined conditions for its production. J. Appl. Bacteriol. **58**:167–174.
20. **Gross, D. C., and Y. S. Cody.** 1985. Mechanisms of plant pathogenesis by *Pseudomonas* species. Can. J. Microbiol. **31**: 403–410.
21. **Gross, D. C., Y. S. Cody, E. L. Proebsting, Jr., G. K. Radamaker, and R. A. Spotts.** 1983. Distribution, population dynamics, and characteristics of ice nucleation-active bacteria in deciduous fruit tree orchards. Appl. Environ. Microbiol. **46**: 1370–1379.
22. **Gross, D. C., Y. S. Cody, E. L. Proebsting, Jr., G. K. Radamaker, and R. A. Spotts.** 1984. Ecotypes and pathogenicity of ice-nucleation-active *Pseudomonas syringae* isolated from deciduous fruit tree orchards. Phytopathology **74**:241–248.
23. **Gross, D. C., and J. E. DeVay.** 1977. Population dynamics and pathogenesis of *Pseudomonas syringae* in maize and cowpea in relation to the *in vitro* production of syringomycin. Phytopathology **67**:475–483.
24. **Gross, D. C., and J. E. DeVay.** 1977. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. Physiol. Plant Pathol. **11**:13–28.
25. **Gross, D. C., and J. E. DeVay.** 1977. Role of syringomycin in holcus spot of maize and systemic necrosis of cowpea caused by *Pseudomonas syringae*. Physiol. Plant Pathol. **11**:1–11.
26. **Gross, D. C., J. E. DeVay, and F. H. Stadtman.** 1977. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by *Pseudomonas syringae*. J. Appl. Bacteriol. **43**:453–463.
27. **Jorgensen, R. A., S. J. Rothstein, and W. S. Reznikoff.** 1979. A restriction enzyme cleavage map of Tn*5* and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177**:65–72.
28. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
29. **Kleinkauf, H., and H. von Dohren.** 1987. Biosynthesis of peptide antibiotics. Annu. Rev. Microbiol. **41**:259–289.
30. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity on nonhost

plants. J. Bacteriol. **168**:512–522.
31. **Malik, A. N., A. Vivian, and J. D. Taylor.** 1987. Isolation and partial characterization of three classes of mutant in *Pseudomonas syringae* pathovar *pisi* with altered behaviour towards their host, *Pisum sativum*. J. Gen. Microbiol. **133**:2393–2399.
32. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
33. **McKinney, H. H.** 1923. Influence of soil temperature and moisture on infection of wheat seedlings by *Helminthosporium sativum*. J. Agric. Res. **26**:195–217.
34. **Mitchell, R. E.** 1984. The relevance of non-host-specific toxins in the expression of virulence by pathogens. Annu. Rev. Phytopathol. **22**:215–245.
35. **Morgan, M. K., and A. K. Chatterjee.** 1985. Isolation and characterization of Tn*5* insertion mutants of *Pseudomonas syringae* pv. *syringae* altered in the production of the peptide phytotoxin syringotoxin. J. Bacteriol. **164**:14–18.
36. **Obukowicz, M., and P. D. Shaw.** 1985. Construction of Tn*3*-containing plasmids from plant-pathogenic pseudomonads and an examination of their biological properties. Appl. Environ. Microbiol. **49**:468–473.
37. **Paynter, V. A., and R. Alconero.** 1979. A specific fluorescent antibody for detection of syringomycin in infected peach tree tissues. Phytopathology **69**:493–496.
38. **Peet, R. C., P. B. Lindgren, D. K. Willis, and N. J. Panopoulos.** 1986. Identification and cloning of genes involved in phaseolotoxin production by *Pseudomonas syringae* pv. "*phaseolicola*." J. Bacteriol. **166**:1096–1105.
39. **Quigley, N. B., D. Lane, and P. L. Bergquist.** 1985. Genes for phaseolotoxin synthesis are located on the chromosome of *Pseudomonas syringae* pv. *phaseolicola*. Curr. Microbiol. **12**: 295–300.
40. **Sato, M., K. Nishiyama, and A. Shirata.** 1983. Involvement of plasmid DNA in the productivity of coronatine by *Pseudomonas syringae* pv. *atropurpurea*. Ann. Phytopathol. Soc. Jpn. **49**: 522–528.
41. **Selvaraj, G., and V. N. Iyer.** 1983. Suicide plasmid vehicles for insertion mutagenesis in *Rhizobium meliloti* and related bacteria. J. Bacteriol. **156**:1292–1300.
42. **Selvaraj, G., and V. N. Iyer.** 1984. Transposon Tn*5* specifies streptomycin resistance in *Rhizobium* spp. J. Bacteriol. **158**: 580–589.
43. **Somlyai, G., M. Hevesi, Z. Banfalvi, Z. Klement, and A. Kondorosi.** 1986. Isolation and characterization of non-pathogenic and reduced virulence mutants of *Pseudomonas syringae* pv. *phaseolicola* induced by Tn*5* transposon insertions. Physiol. Mol. Plant Pathol. **29**:369–380.
44. **Southern, E. M.** 1979. Measurement of DNA length by gel electrophoresis. Anal. Biochem. **100**:319–323.
45. **Stachel, S. E., G. An, C. Flores, and E. W. Nester.** 1985. A Tn*3* *lacZ* transposon for the random generation of β-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. EMBO J. **4**:891–898.
46. **Thomashow, M. F., R. Nutter, A. L. Montoya, M. P. Gordon, and E. W. Nester.** 1980. Integration and organization of Ti plasmid sequences in crown gall tumors. Cell **19**:729–739.
47. **Vidaver, A. K.** 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. Appl. Microbiol. **15**:1523–1524.
48. **Wood, W. B.** 1966. Host specificity of DNA produced by *Escherichia coli*: bacterial mutations affecting the restriction and modification of DNA. J. Mol. Biol. **16**:118–133.
49. **Zhang, L., and J. Y. Takemoto.** 1987. Effects of *Pseudomonas syringae* phytotoxin, syringomycin, on plasma membrane functions of *Rhodotorula pilimanae*. Phytopathology **77**:297–303.