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Expression *in Vitro* and During Plant Pathogenesis of the *syrB* Gene Required for Syringomycin Production by *Pseudomonas syringae* pv. *syringae*

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The *syrB* gene required for syringomycin production by *Pseudomonas syringae* pv. *syringae* B301D was subjected to Tn*3*HoHo1 mutagenesis to generate random transcriptional fusions to a promoterless *lac* operon. Approximately 80 Tn*3*HoHo1 insertions were mapped to the 3.1-kilobase *syrB* locus, and the direction of transcription for *syrB* was determined from those inserts expressing β-galactosidase activity. Besides nontoxigenicity, Tn*3*HoHo1 mutations caused deficiencies in proteins SR4 and SR5, postulated to constitute part of the syringomycin synthetase complex in strain B301D. Three inserts (i.e., 132, 257, and 329) that were marker exchanged into the genome of strain B301D-R each expressed ~1,300 β-galactosidase units after incubation on an agar medium conducive to syringomycin production. Tn*3*HoHo1 insert 132 was marker exchanged into strains B301D-R and B3A-R to yield BR132 and B3AR132, respectively. The *syrB*::*lacZ* fusion in strain BR132 expressed β-galactosidase activity in the nutritionally defined SRM liquid medium (D. C. Gross, Journal of Applied Bacteriology 58:167-174, 1985) as compared to nonexpression of the same fusion in strain B3AR132. In contrast, both *syrB*::*lacZ* recombinant strains expressed activity in a medium containing a potato extract; activity was expressed about 24 hr before the initial detection of toxin production in parental strains B301D-R and B3A-R. Like syringomycin production itself, the *syrB*::*lacZ* fusion was regulated in a positive manner by iron, since quantities of Fe^{3+} >2 μM were required for maximum expression. In immature cherry fruits, the *syrB*::*lacZ* fusions in strains BR132 and B3AR132 were expressed soon after inoculation, although peak β-galactosidase activity of ~400 units occurred at day 1 for strain B3AR132 versus day 3 for BR132. Addition of a water-soluble extract from cherry leaves to SRM liquid medium induced strain B3AR132 to express β-galactosidase activity. Signal molecules in cherry tissues were inferred to induce the *syrB*::*lacZ* fusion in strain B3AR132.

Additional keywords: antibiotic production, gene fusions, phytotoxin, plant signal molecule, *Prunus avium*, transposon mutagenesis.

Syringomycin production is an important element of virulence for most phytopathogenic strains of *Pseudomonas syringae* pv. *syringae* van Hall, a bacterium that causes necrotic lesions on leaves and stems of diverse dicot and monocot species. The necrotic symptoms are at least partly attributed to the activity of syringomycin, a phytotoxin that extensively disrupts ion transport across the plasmalemma of plant cells (Bidwai *et al.* 1987). Minute quantities of syringomycin applied to isolated epidermes and intact leaves caused measurable stomatal closure, presumably via perturbation of transport processes of guard cells (Mott and Takemoto 1989). The contribution of syringomycin production to bacterial virulence was established recently by pathogenicity tests of nontoxigenic (Tox^-) Tn*5* mutants of *P. s.* pv. *syringae* B301D-R in immature sweet cherry (*Prunus avium* L.) fruits (Xu and Gross 1988a, 1988b). For example, strain W4S770, which contains Tn*5* inserted into the *syrB* gene required for syringomycin production, caused only small necrotic lesions 3 days after inoculation. The disease index for W4S770 was only about half that of either parental strain B301D-R or a restored strain obtained by marker exchange of the cosmid-cloned *syrB* gene into W4S770.

Besides having an acute effect on virulence, the *syrB* gene is associated with the formation of two large proteins, SR4 and SR5 (~350 and ~130 kDa, respectively), that were hypothesized to function as syringomycin synthetases (Xu and Gross 1988b). Physical analysis of the *syrB* gene showed it to be between 2.4 to 3.3 kilobases (kb) and therefore too short to directly encode such large polypeptides, leaving open the possibility that *syrB* might encode a positive regulatory protein which controls expression of either SR4 or SR5 or both. Because syringomycin is a cyclic lipodepsinonapeptide antibiotic within the polypeptin class of antibiotics (Segre *et al.* 1989), its biosynthesis likely requires the formation of a large multienzyme complex analogous to the synthetase system described for production of the structurally related polymyxins by *Bacillus* (Kleinkauf and Von Döhren 1987). Furthermore, positive regulation of antibiotic production in most bacteria is tightly controlled by iron concentration (Weinberg 1977). Expression of the five large proteins associated with syringomycin production in *P. s.* pv. *syringae* B301D is indeed controlled positively by iron, closely corresponding with the positive regulatory effect of iron concentration on syringomycin production itself (Gross 1985; Xu and Gross 1988b).

Despite evidence from genetic studies for the requisite production of syringomycin for full virulence, there is no biochemical evidence for syringomycin production during plant pathogenesis. Attempts at recovery of syringomycin

directly from diseased plant tissues or tissues treated with purified syringomycin were either inconclusive or unsuccessful, presumably because the phytotoxin was either irreversibly bound to plant membranes or enzymatically degraded and metabolized by the plant (Gross and DeVay 1977b; Sinden *et al.* 1971). Perhaps the best evidence for syringomycin production *in planta* was obtained by Paynter and Alconero (1979), who produced a fluorescent antibody specific to syringomycin and observed fluorescence in both infected and toxin-treated tissues of peach (*P. persica* (L.) Batsch) stems and leaves. The fluorescent antibody-syringomycin complex was observed in the parenchyma, tracheid, and phloem cells of infected field samples. The time course for syringomycin production during plant pathogenesis also is unknown, although the toxin generally is presumed to be produced shortly after infection. *In vitro* comparisons of toxin production in potato-dextrose broth (PDB) by representative toxigenic strains, including B301D, nevertheless showed no appreciable syringomycin production until after 2 days of incubation when cells were in a stationary phase of growth (Gross and DeVay 1977a). This suggests that syringomycin would not be produced *in planta* by *P. s.* pv. *syringae* during the initial stages of infection, but rather after infection is extensive within tissues.

To study the expression of syringomycin production by *P. s.* pv. *syringae* during phytopathogenesis, the *syrB* gene was mutagenized with Tn*3*HoHo1 to construct fusions to a promoterless *lac* operon. A transcriptional fusion between the *syrB* and *lacZ* genes that yielded high β-galactosidase activity was used to measure expression of *syrB* both *in vitro* and *in situ*. These studies showed that environmental factors conducive to syringomycin production were likewise favorable to expression of the *syrB*::*lacZ* fusion. Pathogenicity tests in immature cherry fruits established that the *syrB* gene was expressed shortly after inoculation and thereafter remained active throughout disease development. The induction of the *syrB*::*lacZ* fusion in strain B3A-R in intact cherry fruit tissues and in SRM medium (Gross 1985) supplemented with a cherry leaf extract suggested that a plant factor or signal promotes expression of genes required for syringomycin production in at least some strains of *P. s.* pv. *syringae*.

MATERIALS AND METHODS

Strains, plasmids, and media. The bacterial strains and plasmids used in this study are described in Table 1. The procedures for routine culture and maintenance of *P. s.* pv. *syringae* strains were described elsewhere (Xu and Gross 1988a). *Escherichia coli* strain HB101, and its derivatives, and strain C2110 were routinely grown at 37° C in Luria-Bertani (LB) broth or on LB agar (Maniatis *et al.* 1982). N minimal (NM) agar medium (Vidaver 1967) was used for selection of *P. s.* pv. *syringae* transconjugants after matings with *E. coli*. PDB and potato-dextrose agar (PDA) were supplemented with 1% glucose and 0.4% vitamin-free casamino acids (Difco Laboratories, Detroit, MI) (Gross and DeVay 1977a) for syringomycin production by *P. s.* pv. *syringae*. PDB was deferrated with 8-hydroxyquinoline as described previously (Gross 1985), and iron-free glassware was prepared by the method of Waring and Werkman (1942). SRM liquid medium (Gross 1985) was used for tests of syringomycin production or induction of *syrB*::*lacZ* fusions under defined conditions. The iron concentration of SRM liquid medium was routinely adjusted to 10 μM using filter-sterilized $FeCl_3$, resulting in an excess of iron required for maximum syringomycin production (Gross 1985). SRM liquid medium also was used to culture *P. s.* pv. *syringae* strains for analysis of high molecular weight proteins associated with syringomycin synthesis. Antibiotics (Sigma Chemical Co., St. Louis, MO), when needed, were added to media in the following concentrations (μg/ml): chloramphenicol, 34; kanamycin, 25; nalidixic acid, 60; piperacillin, 25; rifampicin, 50; streptomycin, 100; and tetracycline, 12.

Table 1. Bacterial strains and plasmids used in this study

Designation	Relevant characteristics[a]	Source or reference
Escherichia coli		
HB101	Pro⁻ Leu⁻ Thy⁻ Thi⁻ Sm^r *recA13*⁻	Bolivar *et al.* 1977
C2110	*polA* Nal^r	Stachel *et al.* 1985
Pseudomonas syringae pv. *syringae*		
B301D	Wild type from pear	Cody *et al.* 1987
B301D-R	Spontaneous Rif^r derivative of B301D	Xu and Gross 1988a
B3A	Wild type from peach	Gross 1985
B3A-R	Spontaneous Rif^r derivative of B3A	This study
W4S770	*syrB*::Tn*5* derivative of B301D-R Km^r	Xu and Gross 1988a
BR132	*syrB*::Tn*3*HoHo1 (insert 132) derivative of B301D-R Pp^r	This study
BR253	*syrB*::Tn*3*HoHo1 (insert 253) derivative of B301D-R Pp^r	This study
BR146	*syrB*::Tn*3*HoHo1 (insert 146) derivative of B301D-R Pp^r	This study
BR257	*syrB*::Tn*3*HoHo1 (insert 257) derivative of B301D-R Pp^r	This study
BR329	*syrB*::Tn*3*HoHo1 (insert 329) derivative of B301D-R Pp^r	This study
B3AR132	*syrB*::Tn*3*HoHo1 (insert 132) derivative of B3A-R Pp^r	This study
Plasmids		
pHoHo1	Pp^r Tn*3-lacZYA* pMB8 replicon	Stachel *et al.* 1985
pSShe	TnpA$^+$ Cm^r pACYC184 replicon	Stachel *et al.* 1985
pRK2013	IncP Km^r TraRK2$^+$ Δ*repRK2 repE1*$^+$	Ditta *et al.* 1980
pGS72	IncP Tc^r Km^r TraRK2$^+$ *cos*$^+$	Selvaraj and Iyer 1985
pLAFR3	IncP Tc^r *cos*$^+$ *rlx*$^+$	Staskawicz *et al.* 1987
pSR1	pLAFR3 cosmid containing *syrB* in an ~31-kb *Hin*dIII DNA fragment from B301D-R	This study
pYM1	pGS72 containing the 16-kb *Hin*dIII fragment from pSR1	This study
pYM1.132	pYM1 containing Tn*3*HoHo1 insert 132 in *syrB*	This study

[a] Sm^r, Nal^r, Rif^r, Km^r, Pp^r, Cm^r, and Tc^r = resistance to streptomycin, nalidixic acid, rifampicin, kanamycin, piperacillin, chloramphenicol, and tetracycline, respectively; kb = kilobase.

DNA techniques. Plasmid DNA was isolated by the method of Birnboim and Doly (1979). DNA cloning methods and the procedure for physical mapping of clones were as described by Maniatis *et al.* (1982).

Tn*3*HoHo1 mutagenesis of *syrB* and marker exchange mutagenesis of *P. s.* pv. *syringae*. The cosmid clone, pSR1, containing an ~31-kb insert carrying the *syrB* gene was obtained from a *Hin*dIII library of B301D-R genomic DNA prepared in pLAFR3 by methods described earlier (Xu and Gross 1988b). A 16-kb *Hin*dIII fragment from pSR1 was subcloned into pGS72 to yield pYM1 that fully complemented the Tn*5*-containing *syrB* mutant, W4S770, for syringomycin production.

Tn*3*HoHo1 mutagenesis of pYM1 was according to the method of Stachel *et al.* (1985). Strain HB101 containing plasmids pSShe and pHoHo1 was transformed (Maniatis *et al.* 1982) with pYM1. *E. coli* transformants were selected on LB agar containing chloramphenicol, piperacillin, and tetracycline and then mated with strain C2110 by triparental conjugation with the helper strain HB101(pRK2013) (Ditta *et al.* 1980). C2110 transconjugants were selected on LB agar containing nalidixic acid, piperacillin, and tetracycline. Individual colonies containing pYM1 with a Tn*3*HoHo1 insertion were mated on LB agar with *P. s.* pv. *syringae* W4S770 in a triparental mating with HB101(pRK2013); transconjugants were selected on NM agar medium containing piperacillin, rifampicin, and tetracycline. Piperacillin-, tetracycline-, and rifampicin-resistant colonies were transferred to PDA containing piperacillin and tetracycline and screened for syringomycin production after incubation for 5 days at 25° C. Colonies that failed to complement strain W4S770 for syringomycin production were further analyzed for insertion of Tn*3*HoHo1 within *syrB*. The location and orientation of Tn*3*HoHo1 inserts were mapped using the restriction enzymes *Eco*RI and *Bam*HI.

Marker exchange mutagenesis was performed by transferring pYM1 derivatives containing a given *syrB*::Tn*3*HoHo1 insert to strain B301D-R or B3A-R by triparental mating as described above. Transconjugants were selected on NM agar containing rifampicin, piperacillin, and tetracycline. After incubation at 25° C for 4 days, colonies were picked onto PDA and then subcultured for two to three cycles of growth on PDA. Colonies that were tetracycline-sensitive and piperacillin-resistant were checked for syringomycin production on PDA. Relative β-galactosidase activities were determined for *syrB*::*lacZ* mutants by spotting them on PDA containing X-gal (60 μg/ml), followed by incubation for 2–3 days before observing colonies for the production of a blue color. Quantitative levels of β-galactosidase activities were determined by using *o*-nitrophenyl β-D-galactopyranoside (Sigma) as described below after growth in SRM liquid medium for 5 days at 25° C.

Disruption of the chromosomal *syrB* gene by a given Tn*3*HoHo1 insert was verified by Southern blot analysis according to standard procedures (Maniatis *et al.* 1982) using pHoHo1 as the ^{32}P-labeled probe.

Assays for syringomycin production and expression of *syrB*::*lacZ* fusions *in vitro*. Conditions for the culture of strains B301D-R and B3A-R in SRM liquid medium for tests of syringomycin production have been described by Gross (1985). Syringomycin production on agar media and its yield in liquid media were determined by standard methods (Gross and DeVay 1977a) using the fungus *Geotrichum candidum* Link strain F-260 for all bioassays.

Expression of β-galactosidase activity by the *syrB*::*lacZ* mutants BR132 and B3AR132 was measured at 24-hr intervals over a 5-day period in SRM liquid medium and in PDB. The inocula were grown at 25° C in nutrient broth-yeast extract (NBY) medium to late exponential phase and washed twice with equal volumes of sterile deionized water. Cells were adjusted to a final concentration of 10^8 colony-forming units (cfu) per milliliter in sterile deionized water. To each 4.95-ml portion of medium in 16-mm-diameter test tubes, 50 μl of cells was added and then incubated at 25° C without shaking. Three replicate cultures were prepared for both strains for each interval.

The effect of iron concentration on the induction of the *syrB*::*lacZ* fusion was tested in deferrated PDB (divided into 4.95-ml portions in 16-mm-diameter test tubes) supplemented with filter-sterilized $FeCl_3$ to final concentrations of 1, 2, 5, and 10 μM. Inoculum for strains BR132 and B3AR132 was prepared as described above, and 50 μl of cells (10^8 cfu/ml) was added to each 4.95-ml portion of media. Triplicate cultures were prepared for both *syrB*::*lacZ* mutants at each iron concentration. Cultures were incubated at 25° C without shaking for 5 days.

Bacterial cells grown *in vitro* were assayed for expression of β-galactosidase activity by the method described by Miller (1972) as modified by Stachel *et al.* (1985). After incubation of cultures for the prescribed time, cells from 0.5 ml of culture were pelleted in microcentrifuge tubes, suspended to the original volume in Z-buffer (Miller 1972), repelleted, and resuspended in Z-buffer (1.5 ml). The bacterial cell density of a 1-ml portion of the washed cells was measured at 600 nm. A 0.5-ml portion of the washed cells was lysed by vortexing for 10 sec in the presence of sodium dodecyl sulfate (SDS) (0.05%, 20 μl) and chloroform (20 μl) followed by a 10-min incubation at 28° C. The assay reaction was started by adding 100 μl of *o*-nitrophenyl β-D-galactopyranoside (4 mg/ml) and then incubated at 28° C from 5 to 10 min depending on the intensity of the reaction. The reaction was terminated (1 M Na_2CO_3, 250 μl), the cellular debris was removed by pelleting in a microcentrifuge, and the absorbance of the supernatant was determined at 420 nm. Specific units of β-galactosidase activity were calculated using the formula given by Stachel *et al.* (1985).

Isolation and electrophoretic resolution of high molecular weight proteins. Cellular proteins were isolated from *P. s.* pv. *syringae* and then separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 4.5 to 9% polyacrylamide gradient slab gels as described by Xu and Gross (1988b). Protein preparations were prepared from parental strain B301D-R and its *syrB* mutants, W4S770, BR132, and BR253, grown for 5 days at 25° C without shaking in SRM liquid medium. The high molecular weight standard proteins described previously (Xu and Gross 1988b) were used to estimate the size of proteins associated with syringomycin synthesis. After electrophoresis for 20 hr at 50 V constant voltage, the gels

were fixed, stained with Coomassie Brilliant Blue G 250, and destained by the procedure of Neuhoff *et al.* (1985).

Cherry fruit inoculations and assays for β-galactosidase activity. Immature sweet cherry fruits (*P. avium* cv. Bing) were collected 6–8 wk after set and stored at 4° C for pathogenicity tests. Maximum storage time for fruits was no longer than 4 wk. Surface-sterilized fruits were inoculated with single bacterial strains grown to late exponential phase in NBY broth and then washed and suspended in sterile deionized water as described previously (Xu and Gross 1988a). Each cherry fruit was inoculated at three sites as described earlier (Xu and Gross 1988a) except that fruits were injected with 5×10^4 cells per inoculation site to permit recovery of sufficient numbers of bacterial cells for β-galactosidase assays. Treatments consisted of inoculations (~100 fruits per treatment) with the parental strains B301D-R and B3A-R and the *syrB*::*lacZ* mutants BR132, BR253, and B3AR132 and injections of sterile deionized water (5 μl per site). Each treatment was replicated three times. Fruits were incubated at 25° C at a high relative humidity for up to 5 days.

To recover the bacteria from inoculated cherry fruits, the infected tissues were sliced with a razor blade into sections <1-mm-thick and then soaked in sterile deionized water (10–15 ml per replicate) for 30 min at room temperature. Ten fruits per replicate were sampled on day 1 to ensure recovery of sufficient numbers of bacterial cells for measuring β-galactosidase activity. Thereafter, three fruits per replicate were sampled each day to yield satisfactory numbers of bacteria for β-galactosidase assays. The bacteria released by streaming from the infected tissue were separated from the tissue sections by passage through six layers of MicroWipes (Scott Paper Co., Philadelphia, PA). The bacterial cells were recovered by centrifugation, washed once in Z-buffer (5 ml), and suspended in Z-buffer (1.5 ml) before determining cell density at 600 nm. Plant pigments were not visible after washing cells in Z-buffer. Cells were lysed, and units of β-galactosidase activity were determined by the procedure of Stachel *et al.* (1985). Tissue sections from fruits (10 fruits per replicate) injected only with sterile deionized water served as an internal check on methodology and did not yield detectable β-galactosidase activity. Total populations of bacteria per fruit were determined at 1-day intervals by a previous method (Xu and Gross 1988b) to verify similarities to the report of Xu and Gross (1988b) in growth kinetics and populations among strains with or without transposon insertions within *syrB*. The above experiment was performed on three occasions, and average β-galactosidase activities for the three trials were calculated.

Preparation of cherry leaf extracts. Mature cherry leaves (cv. Bing) were collected in July and prepared for long-term storage by lightly rinsing in deionized water, and then blotting and air-drying on paper towels to remove surface moisture. Leaves were frozen in liquid nitrogen and stored at −80° C. An extract was prepared from 100 g (fresh weight) of leaves homogenized in a Waring blender with 300 ml of deionized water for ~15 min. The homogenate was centrifuged at 10,000 × *g* for 10 min, and the supernatant was collected and filter-sterilized. Extracts were frozen and stored at −20° C until use; no detectable loss in ability to induce β-galactosidase activity in strain B3AR132 was observed after storage of extracts for at least 3 months.

Cherry leaf extracts were assayed for effects on expression of the *syrB*::*lacZ* fusion in B3A-R by adding 100 μl of extract to 4.9 ml of SRM medium containing 10^6 cfu/ml of *P. s.* pv. *syringae* prepared as described above. Duplicate SRM cultures were prepared for each day of the 5-day study for the following treatments: 1) parental strain B3A-R with 100 μl of leaf extract or sterile deionized water; 2) B3AR132 with 100 μl of leaf extract or sterile deionized water; and 3) SRM liquid medium containing 100 μl of leaf extract but no bacterial cells. The experiment was repeated on four occasions to yield average values. Cultures were incubated and assayed for β-galactosidase activity as described above.

RESULTS

Isolation and characterization of Tn*3*HoHo1 insertions in the *syrB* gene. The plasmid pYM1, which contains a 16-kb *Hin*dIII fragment of genomic DNA from strain B301D-R cloned into pGS72, was subjected to Tn*3*HoHo1 mutagenesis in *E. coli* HB101 to generate transcriptional fusions of a promoterless *lacZ* gene with *syrB*. Tn*3*HoHo1 insertions into the *syrB* gene were identified by the inability to complement strain W4S770, containing a Tn*5* insert within the *syrB* gene, for syringomycin production on PDA. Approximately 80 transconjugants were obtained that failed to complement strain W4S770 to yield a Tox^+ phenotype, and these insertions were mapped within a 3.1-kb region of pYM1 corresponding to *syrB* and extending from insert 136 to insert 143 (Fig. 1). Each *syrB*::*lacZ* fusion arose from the insertion of only one copy of Tn*3*HoHo1. Thirty-one of the inserts within *syrB* were mapped at distinct locations (22 of the inserts in *syrB* are shown in Fig. 1), relative to the location of known sites for restriction endonucleases. A 0.9-kb region contained 17 of the 31 insertions mapped within *syrB*. Twenty-five of the Tn*3*HoHo1 insertions in *syrB* transferred to *P. s.* pv. *syringae* W4S770 formed blue colonies on PDA containing X-gal due to β-galactosidase activity, indicating that the direction of transcription was from left to right according to the map of pYM1. Three of the Tn*3*HoHo1 insertions (132, 257, and 329) within *syrB* expressed high β-galactosidase activity as observed by the formation of dark blue colonies on PDA containing X-gal; all other positive colonies produced only faint blue colonies due to low β-galactosidase activity. Those insertions directly downstream of *syrB* partially complemented W4S770 for syringomycin production as evidenced by the lack of growth of *G. candidum* on the surface of the bacterial colonies.

The three *syrB*::*lacZ* fusions expressing high β-galactosidase activity were marker exchanged into the genome of strain B301D-R to yield strains BR132, BR257, and BR329. The mutants were cultured on PDA for 5 days at conditions normally permitting syringomycin production to measure β-galactosidase activity. All three of the *syrB*::*lacZ* fusions expressed similar levels of β-galactosidase activity of approximately 1,300 units. In contrast, the weakly active fusion 146 (Fig. 1), after marker exchange

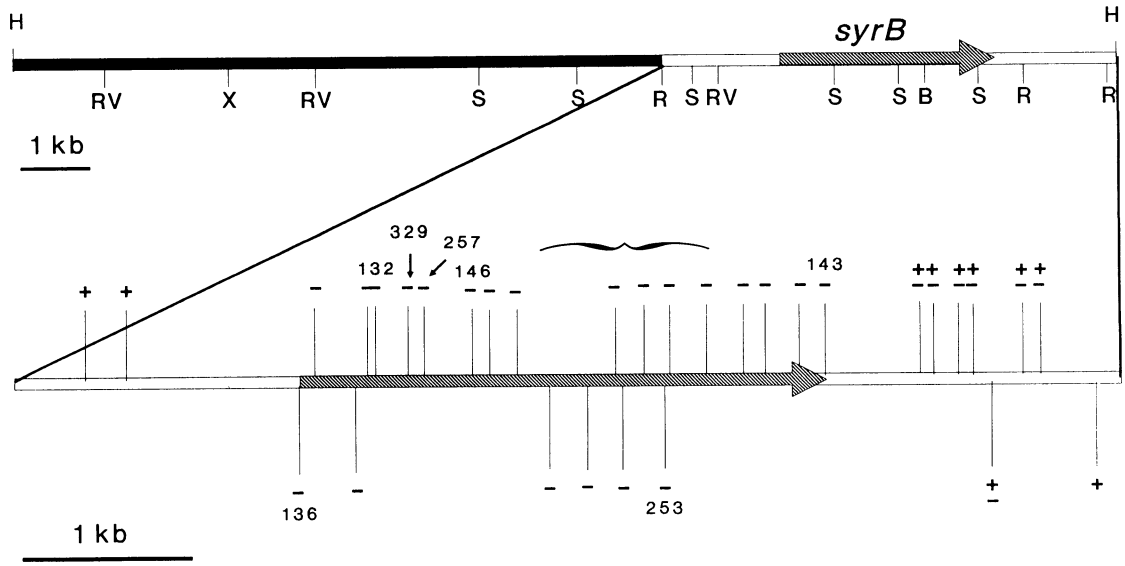

Fig. 1. Tn*3*HoHo1 map of the *syrB* gene required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. The *syrB* gene contained within a 16-kilobase (kb) *Hin*dIII fragment of genomic DNA from strain B301D was cloned into the broad host range vector pGS72 (Selvaraj and Iyer 1985) to yield pYM1 (top map). The restriction sites shown are as follows: H = *Hin*dIII; R = *Eco*RI; S = *Sal*I; RV = *Eco*RV; X = *Xho*I; and B = *Bam*HI. The location of *syrB*, which is approximately 3.1 kb, and its direction of transcription are indicated by the arrow. The bottom map shows the positions of Tn*3*HoHo1 inserts that either expressed (those identified above the line) or did not express (those identified below the line) β-galactosidase activity. The three Tn*3*HoHo1 inserts in *syrB* yielding high β-galactosidase activity were 132, 329, and 257. Inserts that did (+) or did not (−) fully complement strain W4S770 for syringomycin production are identified below the Tn*3*HoHo1 map; seven inserts only partially (±) complemented strain W4S770 (inhibition to *Geotrichum candidum* occurred directly on the colony surface only). Of the 41 Tn*3*HoHo1 inserts mapped in the vicinity of *syrB*, 17 were identified in a 0.9-kb region within *syrB* (eight of the 17 inserts are shown beneath the bracket).

to yield strain BR146, expressed only 17 units of β-galactosidase activity. Strain BR253, containing the promoterless *lacZ* gene oriented opposite to the direction of transcription of *syrB* at insert position 253, expressed only background levels of 7–8 units after incubation for 5 days on PDA.

Strain effects on *in vitro* expression of a *syrB*::*lacZ* fusion with high β-galactosidase activity. Tn*3*HoHo1 insert 132 also was recombined into the genome of strain B3A-R to yield strain B3AR132. Strain B3A previously was shown (Gross 1985) not to produce syringomycin in SRM medium, a defined medium permitting syringomycin production by strain B301D. Consequently, the induction of the *syrB*::*lacZ* fusion in B3AR132 was compared to that of BR132 after culture in SRM liquid medium to determine its relationship to syringomycin production by the parental strains (Fig. 2). For strain BR132, an initial activity of ~200 units of β-galactosidase activity was observed after a 1-day incubation, and a peak activity of more than 2,000 units was observed after a 4-day incubation (Fig. 2A). Thereafter, β-galactosidase activity gradually decreased. In contrast, no β-galactosidase activity was expressed by B3AR132 over the 5-day culture period despite growth comparable to strain BR132. Expression of β-galactosidase activity in SRM medium was in turn compared to that in PDB, a complex medium permitting syringomycin production by both strains B301D and B3A (Gross 1985). Both BR132 and B3AR132 expressed β-galactosidase activity in PDB, although strain BR132 yielded approximately five times more activity after 4 days as compared to strain B3AR132. Peak activity for strain BR132 in SRM occurred at day 4 and was ~800 units higher than that observed in PDB.

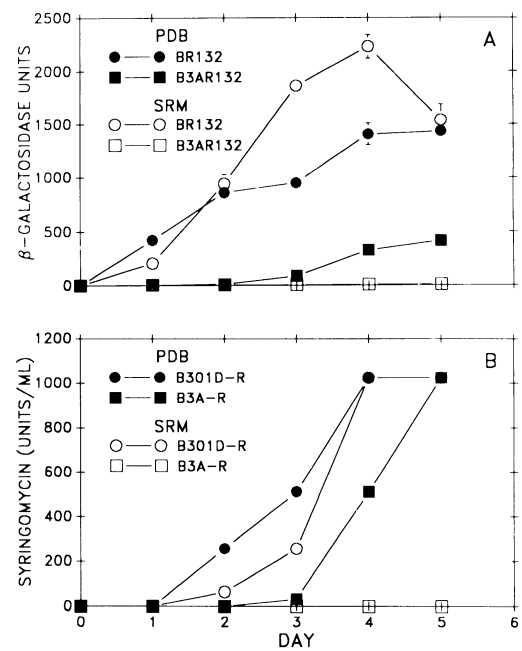

Fig. 2. The effects of growth medium on (**A**) the expression of a *syrB*::*lacZ* fusion as compared to (**B**) syringomycin production itself by two strains of *Pseudomonas syringae* pv. *syringae*. Strains BR132 and B3AR132 were respectively derived by marker exchange of Tn*3*HoHo1 insert 132 into parental strains B301D-R and B3A-R. Expression of β-galactosidase units is shown over a 5-day growth period at 25° C in SRM liquid medium (Gross 1985) and potato-dextrose broth (PDB). Units of syringomycin produced per milliliter were measured for duplicate 50-ml cultures of strains B301D-R and B3A-R incubated at conditions described in **A**. Vertical bars in **A** indicate standard error of the mean activity for triplicate cultures; the standard error was negligible for all points in **B**.

Comparisons of the temporal expression of the *syrB*::*lacZ* fusion in strains BR132 and B3AR132 grown in either SRM medium or PDB (Fig. 2A) to that of syringomycin production by the parental strains (Fig. 2B) showed that the *syrB* gene was expressed about 24 hr before the initial detection of the toxin. Although little or no toxin was produced within the first 48 hr of incubation in either PDB or SRM medium, B301D-R yielded more than 1,000 units per milliliter of syringomycin in both media by day 4. Strain B3A-R had a profile for syringomycin production in PDB similar to that of strain B301D-R, yielding more than 1,000 units per milliliter by day 5, but it did not produce the toxin in SRM medium.

Because iron concentration regulates syringomycin production in a positive manner (Gross 1985), expression of the *syrB*::*lacZ* fusion may likewise be regulated by iron if the *syrB* gene is required for syringomycin synthesis. Incubation of strains BR132 and B3AR132 for 5 days in deferrated PDB supplemented with incremental concentrations of $FeCl_3$ showed the regulatory effect of iron on the expression of β-galactosidase activity (Fig. 3). If no iron was added to deferrated PDB, no β-galactosidase activity was detected. Nevertheless, strain BR132 at concentrations of 1 μM of Fe^{3+} yielded nearly 200 units of activity; further increases in iron concentration up to 5 μM yielded corresponding increases in activity until maximum activities of ~900 units were reached. The effect of iron concentration on expression of the *syrB*::*lacZ* fusion in B3AR132, although kinetically similar to that of BR132, showed a requirement for slightly higher concentrations of iron to achieve similar levels of induction. For example, negligible β-galactosidase activity was expressed at an Fe^{3+} concentration of 2 μM; the highest activity of ~900 units was achieved at an Fe^{3+} concentration of 10 μM.

Effect of the *syrB*::*lacZ* fusions on expression of proteins associated with syringomycin production. SDS-PAGE analysis of cellular proteins isolated from strains BR132 and BR253 containing *syrB*::*lacZ* fusions consistently showed deficiencies in proteins SR4 and SR5 (~350 and ~130 kDa, respectively) relative to parental strain B301D-R (Fig. 4). In addition, the deficiencies in proteins SR4 and SR5 corresponded to those observed for the original *syrB* mutant strain, W4S770, obtained by Tn*5* mutagenesis (Xu and Gross 1988a). Strain BR132 containing a *lacZ* fusion oriented in the direction of transcription also expressed a unique protein of ~120 kDa (not observed in Fig. 4). In contrast, no additional protein bands were observed for strain BR253 containing *lacZ* oriented opposite to the direction of transcription. Visual inspection of gels indicated that proteins SR1 (~470 kDa) and SR2 and SR3 (~400 kDa) were not affected by the Tn*3*HoHo1 insertions in *syrB*.

Expression of a *syrB*::*lacZ* fusion in immature cherry fruits. The *syrB*::*lacZ* fusion in both strains BR132 and B3AR132 was expressed in immature cherry fruits throughout the 3-day period with appreciable activity being observed within the first 24 hr after inoculation (Fig. 5). However, peak activities for induction of the *syrB*::*lacZ* fusion differed between the two strains. Strain BR132 yielded ~100 units of β-galactosidase activity at day 1, and peak activity of ~400 units was not observed until day 3. In contrast, strain B3AR132 expressed peak activity of ~425 units at day 1 from which it decreased to ~175 units by day 3. After 5 days of incubation, strains BR132 and B3AR132 expressed between 100–200 units of β-galac-

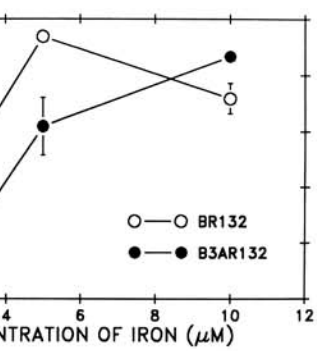

Fig. 3. The effect of iron concentration on the induction (as units of β-galactosidase activity) of the *syrB*::*lacZ* fusion in strains BR132 and B3AR132 of *Pseudomonas syringae* pv. *syringae*. The potato-dextrose broth medium was deferrated as described previously (Gross 1985) before the addition of filter-sterilized $FeCl_3$. Vertical bars indicate the standard error at each iron concentration of the mean β-galactosidase activity for triplicate cultures.

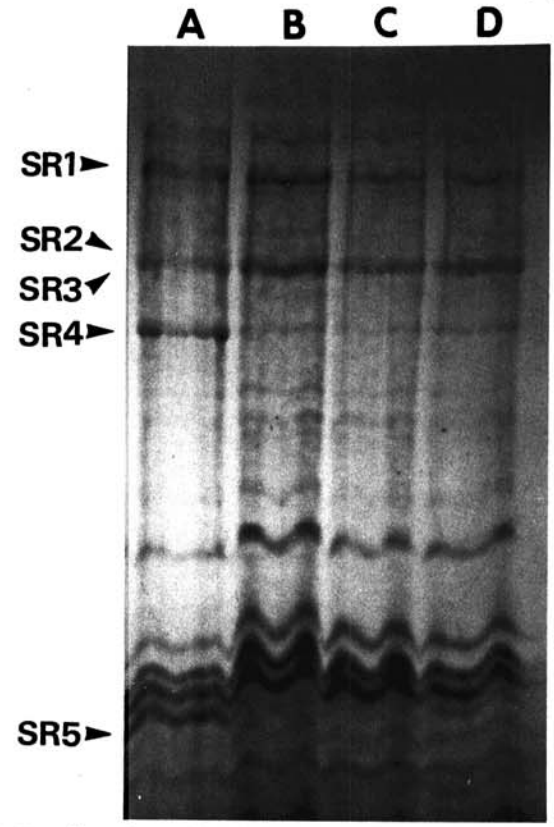

Fig. 4. The effect of *syrB*::*lacZ* fusions on expression of cellular proteins associated with syringomycin synthesis in *Pseudomonas syringae* pv. *syringae*. Proteins isolated from parental strain B301D-R (lane A) and its Tox^- derivatives W4S770 (lane B), BR132 (lane C), and BR253 (lane D) were separated by sodium dodecyl sulfate-PAGE on a 4.5 to 9% polyacrylamide gradient gel. Strains were grown for 5 days in SRM liquid medium (Gross 1985). The iron-regulated proteins associated with syringomycin synthesis are identified as SR1 (~470 kDa), SR2 and SR3 (~400 kDa), SR4 (~350 kDa), and SR5 (~130 kDa). Note that inactivation of *syrB* either by insertion of Tn*5* (i.e., W4S770) or by Tn*3*HoHo1 (i.e., BR132 and BR253) resulted in deficiencies in proteins SR4 and SR5.

tosidase activity in cherry fruits (data not shown); levels of activity were more variable in the disease lesions at day 5, reflecting the senescent state of inoculated fruits. Both *syrB* mutant strains BR132 and B3AR132 were nearly equivalent in virulence, causing necrotic lesions (~2–3 mm in diameter) surrounding the sites of injection by day 3; only slight discoloration was visible at day 1. Populations of the two strains in fruits at each sampling time were not significantly different. Strain BR253, which contains *lacZ* inserted within *syrB* but oriented opposite to the direction of transcription (Fig. 1), did not express β-galactosidase activity even though it was equal to strain BR132 in virulence. Tissues of cherry fruits injected with sterile deionized water neither showed discoloration nor yielded β-galactosidase activity. Strains B301D-R and B3A-R produced lesions approximately 40% larger than their respective *syrB* mutants in cherry fruits, but did not express β-galactosidase activity at any point during the 3-day incubation.

Effect of a cherry leaf extract on induction of a *syrB*::*lacZ* fusion in strain B3A-R. Because the *syrB*::*lacZ* fusion in B3AR132 was expressed *in vitro* in a medium containing an extract from potato and not SRM (Fig. 2A) and was quickly and strongly expressed in inoculated cherry fruits (Fig. 5), we tested the possibility that a plant extract supplied a constituent necessary for induction of the *syrB*::*lacZ* fusion in this strain. Addition of a crude aqueous extract from sweet cherry leaves to SRM liquid medium induced more than 300 units of β-galactosidase activity for strain B3AR132 after 4 days of incubation (Fig. 6). The levels of β-galactosidase activity and the overall induction pattern were comparable to those observed in PDB for strain B3AR132 (Fig. 2A). Furthermore, the levels of β-galactosidase activity that were induced by the cherry extract, as shown in Figure 6, resulted from the addition of an extract from only about 33 mg (fresh weight) of cherry leaf tissue per assay. β-Galactosidase activity was not observed for strain B3AR132 or parental strain B3A-R grown in SRM liquid medium lacking the cherry leaf extract, and activity was not obtained from cultures of strain B3A-R grown in SRM liquid medium containing the cherry extract. Controls of SRM liquid medium supplemented with the cherry extract, but not inoculated with *P. s.* pv. *syringae*, also lacked β-galactosidase activity. No β-galactosidase activity was observed for strain B3AR132 grown in SRM liquid medium adjusted to a final Fe^{3+} concentration of either 20 or 100 μM, eliminating the possibility that iron in the cherry extract was responsible for *syrB*-inducing activity.

DISCUSSION

Syringomycin is a highly active phytotoxin that causes visible necrosis in treated plant tissues after just a few hours of exposure, suggesting that syringomycin is an element of the early stages of disease development caused by *P. s.* pv. *syringae*. Conversely, syringomycin activity is produced only in late exponential to stationary phase cultures of the bacterium (Gross 1985; Gross and DeVay 1977a). Because biologically active syringomycin cannot be recovered from diseased plant tissue (Gross and DeVay 1977b), the question of whether syringomycin is produced in infected tissues was investigated in this study by monitoring expression of a *lacZ* fusion to the *syrB* gene during phytopathogenesis. Results from inoculations of immature cherry fruits showed that transcriptional activation of *syrB* occurred within 24 hr of inoculation in derivatives of both strains B301D-R and B3A-R of *P. s.* pv. *syringae*, which differ in their environmental requirements for syringomycin production *in vitro*. Nevertheless, activities expressed *in vitro* by *syrB*::*lacZ* fusions in these strains showed about a 24-hr lag between initial β-galactosidase activity and detectable syringomycin production. This suggests that *syr* genes are activated shortly after bacterial penetration of host tissues, but that there is a lag period of several hours before the synthesis and cellular release of biologically active syringomycin.

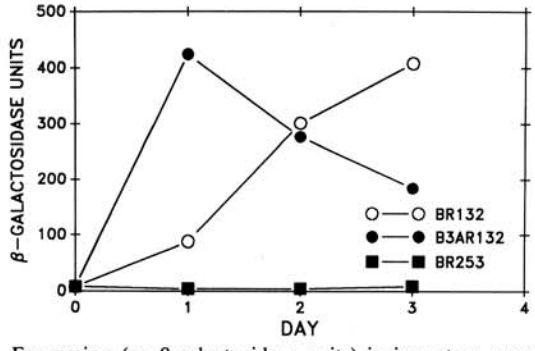

Fig. 5. Expression (as β-galactosidase units) in immature sweet cherry fruits of the *syrB*::*lacZ* fusion in *Pseudomonas syringae* pv. *syringae* over a period of 3 days after inoculation. Fruits were inoculated with 5×10^4 cells per inoculation site, three sites per fruit, and incubated at 25° C. Results are shown for strains BR132, BR253, and B3AR132. In addition to a lack of activity *in situ* for strain BR253, inoculations of B301D-R, B3A-R, or sterile deionized water did not yield β-galactosidase activity. Each value is the mean of three trials conducted on separate occasions; each trial consisted of three replicates. The standard error for mean values ranged from 0 to ±16 β-galactosidase units.

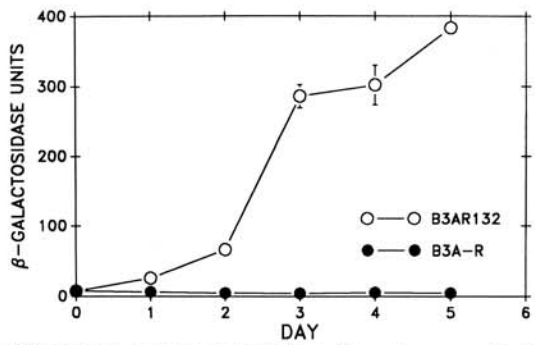

Fig. 6. Effect of an aqueous extract from cherry leaves on the induction of a *syrB*::*lacZ* fusion in *Pseudomonas syringae* pv. *syringae* B3AR132. Extract from approximately 33 mg (fresh weight) of cherry leaf tissue was added per assay after filter sterilization. Assays were conducted over a 5-day period of incubation at 25° C in SRM liquid medium (Gross 1985). Control tests of B3A-R with or without cherry leaf extract, B3AR132 without cherry leaf extract, and no bacterial cells added to the assay mixture yielded no β-galactosidase activity; results for B3A-R with cherry leaf extract are shown above. Vertical bars indicate the standard error for the mean of four trials conducted on separate occasions.

Our analysis of *syrB* expression in cherry fruits used cells precultured in a medium (i.e., NBY) that does not permit syringomycin production and expression of the *syrB*::*lacZ* fusion. Although this permitted evaluations of the time course of *syr* gene induction relative to production of detectable quantities of syringomycin, such a lag period may be nonexistent or abbreviated in nature. *P. s.* pv. *syringae* maintains itself in nature as an epiphytic resident causing disease in a susceptible host when plants are environmentally stressed to favor penetration and subsequent assault on plant tissues by the bacterium. *P. s.* pv. *syringae* attacks cherry trees after penetrating natural openings, such as stomates, or wounds that result from events such as injurious frosts. Even in the epiphytic state, one would expect conditions favorable for at least low-level expression of *syr* genes and production of syringomycin. Certainly the simple nutritional requirements for growth and syringomycin production would be provided along with any necessary plant signal molecules required for activation of *syr* genes. Syringomycin was recently observed by Mott and Takemoto (1989) to close stomata at concentrations as low as 10^{-8} M, presumably due to changes in cellular osmotic pressure of guard cells resulting from a rapid efflux of K^+. Furthermore, the overall stomatal responses as well as the concentrations and rates of activity for syringomycin resembled those of the plant hormone abscisic acid. In contrast to abscisic acid, however, syringomycin also affects plant cells other than guard cells. Consequently, penetration of stomates or other openings breaching the epidermis by epiphytic cells of *P. s.* pv. *syringae* in a natural induced state for syringomycin production could initiate vital physiological effects on the plasmalemma of host cells that favor disease development.

Random Tn*3*HoHo1 mutagenesis of pYM1 generated numerous *lac* fusions to *syrB* that revealed the direction for its transcription and further delineated its length to be ~3.1 kb. Near the 5′ region of *syrB*, three fusions (i.e., inserts 132, 257, and 329) were obtained that were highly inducible, expressing more than 1,000 units of β-galactosidase activity on PDA and permitting quantitative measurements of gene expression in response to various environmental factors. The highly inducible Tn*3*HoHo1 inserts apparently arise due to translational fusions to *syrB*. This is supported in strain BR132 by the expression of a unique chimeric protein of ~120 kDa containing the β-galactosidase protein and the short NH_2-terminal portion of SyrB upstream to insert 132. Western blot analysis using anti-β-galactosidase antibody has tentatively confirmed the ~120-kDa protein to be a translational hybrid (Y.-Y. Mo and D. C. Gross, unpublished). In *Agrobacterium*, those Tn*3*HoHo1 inserts that yield chimeric fusions between β-galactosidase and Vir proteins express the highest activities (Stachel and Nester 1986). After marker exchange into strains B301D-R and B3A-R, the *syrB*::*lacZ* fusion at position 132 expressed sufficient activity to permit measurements of *syrB* expression in immature cherry fruits. Extracts from cherry tissues did not contain substances interfering with β-galactosidase assays of the *syrB*::*lacZ* fusion, because cells were washed extensively in Z-buffer before analysis and no activity was observed for strain BR253 containing *lacZ* fused to *syrB* but oriented in the opposite direction to transcription. Furthermore, cherry fruits inoculated with parental strains B301D-R and B3A-R showed no detectable β-galactosidase after washing in Z-buffer.

The partial complementation of *syrB* mutant strain W4S770 by pYM1 containing Tn*3*HoHo1 inserts downstream of *syrB* was attributed to the presence of a distinct gene called *syrC* (Y.-Y. Mo and D. C. Gross, unpublished). Tn*3*HoHo1 inserts within *syrC* after marker exchange into strain B301D-R resulted in a fully Tox^- phenotype; the 3-kb *Bam*HI-*Hin*dIII fragment from pYM1 subcloned into pLAFR3 in both orientations complemented *syrC* but not *syrB* mutants. Although the relationship between *syrB* and *syrC* cannot be explained at this time, it appears that mutations in *syrB* can exert polar effects on the expression of *syrC*.

A pivotal role is ascribed to the *syrB* gene in syringomycin synthesis, even though the specific function of its gene product remains unknown. First, all Tn*3*HoHo1 insertions within *syrB*, regardless of orientation, resulted in a Tox^- phenotype. Second, two large polypeptides (i.e., SR4 and SR5), which are hypothesized to function as syringomycin synthetases, were deficient in *syrB* mutants obtained by Tn*3*HoHo1 mutagenesis, which is consistent with similar protein deficiencies observed previously in a *syrB* mutant obtained by Tn*5* mutagenesis (Xu and Gross 1988b). Third, factors that regulate expression of syringomycin production similarly controlled expression of a *syrB*::*lacZ* fusion. For example, expression of both syringomycin production and a *syrB*::*lacZ* fusion was similarly regulated in a positive manner by iron; Fe^{3+} concentrations ≥ 2 μM were required for maximum expression, depending on the *P. s.* pv. *syringae* strain (Fig. 3; Gross 1985). High quantities of syringomycin were produced by strain B301D-R in SRM, a nutritionally defined medium, which is in sharp contrast to no production in SRM by strain B3A-R. Correspondingly, strain B3AR132, obtained by marker exchange of *syrB*::*lacZ* insert 132 into B3A-R, expressed no β-galactosidase activity in SRM medium despite measurable activity when grown in PDB. The same *syrB*::*lacZ* fusion recombined into the genome of strain B301D-R (i.e., BR132) expressed β-galactosidase activity in both SRM medium and PDB. Consequently, the use of a *syrB*::*lacZ* fusion appears to generate valid measurements of the effects of environmental factors that impinge on expression of syringomycin synthesis both *in vitro* and *in situ*.

Innate differences in *syrB* expression are observed in B301D-R and B3A-R strain backgrounds that appear to have a bearing on how the two strains respond in the plant-bacteria interaction. The most notable observation is the rapid and strong expression of *syrB* in strain B3AR132 in immature cherry fruits after only 24 hr of incubation. Greater than four times the β-galactosidase activity was expressed by the *syrB*::*lacZ* fusion in strain B3AR132 as compared to the same fusion in strain BR132. Another pertinent observation is the dissimilar expression of the *syrB*::*lacZ* fusion in strains B3AR132 and BR132 grown in SRM medium and PDB. Although strain BR132 expressed β-galactosidase activity in both media, strain B3AR132 was active in PDB and not in SRM despite growth comparable to strain BR132. Therefore, *syrB*

activity in strain B3AR132 was observed only in an environment containing plant constituents, namely a potato extract *in vitro* and cherry fruit tissue *in situ*. This suggested that a plant component or signal molecule was required for expression of the *syrB*::*lacZ* fusion in strain B3AR132. Accordingly, the addition of a water-soluble extract from cherry leaves to SRM converted it into a medium conducive to expression of β-galactosidase activity. We therefore speculate that the cherry extract supplied a nonnutritive constituent(s) that functions as a signal molecule perceived by the bacterium and transduced to the *syrB* transcriptional apparatus. Nevertheless, the requirement for plant signal molecules for *syr* induction is not universal, since strain B301D-R and its derivatives expressed activity in the nutritionally stringent SRM medium. It is unclear why strain B3A-R might require plant signals for *syrB* induction as opposed to strain B301D-R. Perhaps B3A-R responds more strongly to the dynamic plant environmental conditions conducive to infection. Regardless, syringomycin is a key component of virulence, and there appears to be a complex network of communication between *P. s.* pv. *syringae* and plant hosts that can regulate expression of genes required for its production.

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