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Analysis of the *syrB* and *syrC* Genes of *Pseudomonas syringae* pv. syringae Indicates that Syringomycin Is Synthesized by a Thiotemplate Mechanism†

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The *syrB* and *syrC* genes are required for synthesis of syringomycin, a lipodepsipeptide phytotoxin produced by *Pseudomonas syringae* pv. syringae, and are induced by plant-derived signal molecules. A 4,842-bp chromosomal region containing the *syrB* and *syrC* genes of strain B301D was sequenced and characterized. The open reading frame (ORF) of *syrB* was 2,847 bp in length and was predicted to encode an ~105-kDa protein, SyrB, with 949 amino acids. Searches of databases revealed that SyrB shares homology with members of a superfamily of adenylate-forming enzymes involved in peptide antibiotic and siderophore synthesis in a diverse spectrum of microorganisms. SyrB exhibited the highest degree of overall similarity (56.4%) and identity (33.8%) with the first amino acid-activating domain of pyoverdin synthetase, PvdD, of *Pseudomonas aeruginosa*. The N-terminal portion of SyrB contained a domain of ~600 amino acids that resembles the amino acid-activating domains of thiotemplate-employing peptide synthetases. The SyrB domain contained six signature core sequences with the same order and spacing as observed in all known amino acid-activating domains involved in nonribosomal peptide synthesis. Core sequence 6 of SyrB, for example, was similar to the binding site for 4 -phosphopantetheine, a cofactor required for thioester formation. The *syrC* ORF (1,299 bp) was located 175 bp downstream of the *syrB* ORF. Analysis of the transcriptional and translational relationship between the *syrB* and *syrC* genes demonstrated that they are expressed independently. The *syrC* ORF was predicted to encode an ~48-kDa protein product of 433 amino acids which is 42 to 48% similar to a number of thioesterases, including fatty acid thioesterases, haloperoxidases, and acyltransferases, that contain a characteristic GXS(C)XG motif. In addition, a zinc-binding motif was found near the C terminus of SyrC. The data suggest that SyrB and SyrC function as peptide synthetases in a thiotemplate mechanism of syringomycin biosynthesis.

A characteristic trait of phytopathogenic strains of *Pseudomonas syringae* pv. syringae is the production of lipodepsipeptides which cause necrotic symptoms in plants (20, 41). Most strains produce syringomycin (Fig. 1), a cyclic lipodepsinonapeptide composed of a polar peptide head and hydrophobic 3-hydroxy fatty acid tail 10, 12, or 14 carbon units in length (8, 50). The phytotoxic activity of syringomycin is centered around an ability to form transmembrane pores that disturb the dynamic balance of ions across the plasma membrane (19). The most prominent effect of pore formation is a massive cytoplasmic influx of Ca^{2+} which activates events associated with cellular signalling (22). Only nanomolar quantities of toxin are required for pore formation and ultimate cell death (19). Consequently, syringomycin contributes significantly to the virulence of the phytopathogenic bacterium. Quantitative evaluations of syringomycin-negative mutants of strain B301D harboring transposon insertions in either the *syrB* or *syrC* genes indicated that the toxin nearly doubled the virulence of *P. syringae* pv. syringae (41, 62).

Vital roles in syringomycin production have been attributed to three *syr* genes within a region spanning ~7 kb of the chromosome of strain B301D (Fig. 2) (35, 41). Transposon mutagenesis of any one of these genes results in the loss of syringomycin production and deficiencies in one or more large proteins predicted to function as part of the syringomycin synthetase complex (41). The formation of two large proteins, SR4 (~350 kDa) and SR5 (~130 kDa), is associated with the *syrB* locus, and it was postulated that *syrB* encodes a syringomycin synthetase or a subunit of a multimeric synthetase. The *syrC* gene maps immediately downstream of the *syrB* gene. A mutant of *syrC* (e.g., BR334) phenotypically resembles a *syrB* mutant (e.g., BR132) because the two mutants exhibit similar reductions in virulence and show the same deficiencies in large proteins associated with syringomycin production (41), suggesting that these genes may be functionally linked in toxigenesis. In addition, previous evidence indicated that the *syrB* and *syrC* genes may be transcriptionally and translationally linked, on the basis of the failure of pYM1.143 (see Table 1 and Fig. 2) to complement either *syrB* or *syrC* mutants (35). The *syrD* gene, however, is transcribed in the opposite direction to *syrB* and *syrC* and encodes a protein that shares a high degree of similarity to the ATP-binding cassette transporter proteins involved in target-specific secretion (41). Consequently, it was proposed that syringomycin is transported across the cytoplasmic membrane by SyrD, which functions as an ATP-driven efflux pump. No *syr* genes have been mapped to lie downstream of *syrD* (36), although additional *syr* synthetase genes were clustered downstream of *syrC* (18). Quigley and Gross (40) demonstrated recently that the *syrB* and *syrD* genes are conserved as single loci within a 15-kb DNA region among diverse strains of *P. syringae* pv. syringae, including strains that produce amino acid analogs of syringomycin (e.g., syringotoxin

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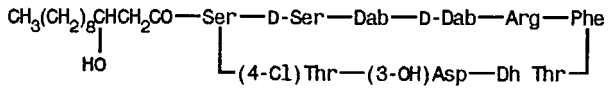

FIG. 1. Lipodepsinonapeptide structure of syringomycin. Syringomycin form SRE, containing a 12-carbon 3-hydroxy fatty acid, is the predominant form produced by strain B301D of *P. syringae* pv. syringae. Amino acid abbreviations: Arg, arginine; (3-OH)Asp, 3-hydroxyaspartic acid; Dab, 2,4-diaminobutyric acid; D-Dab, the D-isomer of Dab; Phe, phenylalanine; Ser, serine; D-Ser, the D-isomer of Ser; (4-Cl)Thr, 4-chlorothreonine; Dh Thr, dehydrothreonine.

and syringostatin). Other related pathovars of *P. syringae* do not produce the toxin and lack homologous *syr* sequences, which emphasizes the importance of these genes in toxigenesis by *P. syringae* pv. syringae.

Several lines of recent evidence indicate that syringomycin biosynthesis can be induced synergistically by two specific classes of plant-derived signal molecules (34, 36). A *syrB-lacZ* reporter gene fusion has been used to demonstrate that certain phenolic glycosides such as arbutin serve as the primary signal molecules and that specific sugars such as fructose substantially increase the sensitivity of *syrB* expression to the phenolic signal. Accordingly, plants such as sweet cherry (*Prunus avium* L.), which are susceptible to infection by *P. syringae* pv. syringae, contain large amounts of *syrB*-inducing signal compounds. The leaves of sweet cherry contain two flavonol triglycosides (quercetin 3-rutinosyl-4′-glucoside and kaempferol 3-rutinosyl-4′-glucoside) and a flavanone glycoside (dihydrowogonin 7-glucoside) with *syrB*-inducing activity. The leaves also are rich in fructose, which causes about a 10-fold stimulation of signal activity when the flavonoid glycosides occur at low concentrations. Furthermore, the syringomycin biosynthetic mechanism is activated by plant signal molecules in a diverse spectrum of *P. syringae* pv. syringae strains, with some strains producing toxin in vitro only in the presence of signal molecules (40).

Syringomycin biosynthesis is predicted to occur on a multifunctional complex of enzymes by the thiotemplate mechanism, as originally described for medically important peptide antibiotics produced by *Streptomyces* and *Bacillus* spp. (23, 55). Peptide synthetases contain multifunctional polypeptide chains, sometimes exceeding 500 kDa in size (49), that serve as templates for the sequential addition of amino acids to peptides. Recent genetic advances in gramicidin S synthesis in *Bacillus brevis* (30, 52, 55) and in surfactin synthesis by *Bacillus subtilis* (3) have provided valuable models of gene organization and function in nonribosomal peptide synthesis. There are two important features of the genetic organization of all thiotemplate synthetase systems, which are analogous to the synthesis of long-chain fatty acids (30, 52). First, the multienzyme complex is composed of amino acid-activating domains that catalyze the adenylation of the constituent amino acids and thioester formation. Each amino acid-activating domain is composed of a region of about 600 residues with between 30 and 80% identity with other domains, and successive domains are separated by a nonhomologous region of about 500 amino acids. Second, the organization of the domains is colinear with the sequence of the cognate amino acids in the peptide chain. The growing peptide chain is transferred sequentially from one amino acid-activating domain to the next, where another amino acid is added by formation of a peptide bond. Analysis of the syringomycin structure (Fig. 1) suggests the occurrence of enzymes that promote transthiolation or modify constituent amino acids, including synthetases with thioesterase, acyltransferase, racemase, and chloroperoxidase activities (13, 23).

To further define the genetic organization for syringomycin biosynthesis in *P. syringae* pv. syringae, the *syrB* and *syrC* genes were sequenced and characterized. We report that the *syrB* gene encodes a protein that exhibits significant homology to a family of adenylate-forming enzymes which activate and bind amino acids. In contrast, *syrC* encodes a protein that contains a signature enzymatic motif for thioesterase activity and resembles proteins that function as chloroperoxidases. The transcriptional and translational relationship between the *syrB* and *syrC* genes also is reported. These results are discussed in relation to a thiotemplate mechanism of synthesis for syringomycin.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* DH5α (15), used as a host in subcloning and sequencing of the *syrB* and *syrC* genes, was grown at 37°C in Luria-Bertani broth or on Luria-Bertani agar (45). The Top10 strain of *E. coli* (10), used as a host for overexpression of the truncated SyrB protein, was purchased from Invitrogen (San Diego, Calif.). Top10 was cultured in SOB or SOC medium (45) according to the procedure provided with the Xpress System

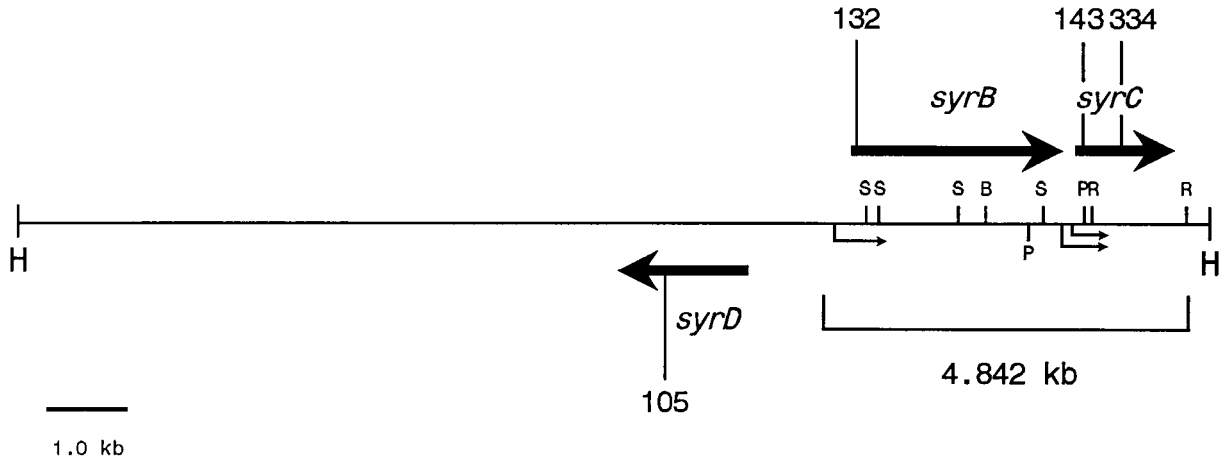

FIG. 2. Map of the *syrB*, *syrC*, and *syrD* gene cluster of *P. syringae* pv. syringae B301D on pYM101. The relative positions, orientations, and sizes of the ORFs (thick horizontal arrows), key Tn*3*HoHo1 inserts in the three *syr* genes (numbered vertical lines), and the transcription start site(s) for *syrB* and *syrC* (thin horizontal arrows) are indicated. The locations of only those restriction sites used for subcloning and sequencing in this study are indicated as follows: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; and S, *Sal*I. The bracketed region indicates the 4,842-bp DNA fragment whose sequence is displayed in Fig. 3.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	15
Top10	*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara*, *leu*)*7697 galU galKI-rpsL endA1 nupG*	10
P. syringae pv. syringae		
B301D	Wild type from pear	2
B301D-R	Spontaneous Rif^r derivative of B301D	62
BR132	*syrB*::Tn*3*HoHo1 derivative of B301D-R; Pp^r Rif^r	35
BR334	*syrC*::Tn*3*HoHo1 derivative of B301D-R; Pp^r Rif^r	41
BR143	*syrC*::Tn*3*HoHo1 derivative of B301D-R; Pp^r Rif^r	35
W4S770	*syrB*::Tn*5* derivative of B301D-R; Km^r	62
pUC18, pUC19	High-copy-number cloning vector; Ap^r	63
pPR510	Ultra-high-copy-number derivative of pUC19; Cm^r	42
pYM1	pGS72 carrying a 16-kb *Hin*dIII fragment from strain B301D; Tc^r	35
pYM1.132	pYM1(*syrB*::Tn*3*HoHo1 insert 132); Pp^r Tc^r	35
pYM1.143	pYM1(*syrC*::Tn*3*HoHo1 insert 143); Pp^r Tc^r	35
pYM1.334	pYM1(*syrC*::Tn*3*HoHo1 insert 334); Pp^r Tc^r	35
pRK2013	Broad-host-range mobilization helper; Km^r	5
pYM101	pUC19 carrying the 16-kb *Hin*dIII DNA fragment from pYM1 containing the *syrB*, *syrC*, and *syrD* gene cluster; Ap^r	41
pTrcHisC	pUC-derived protein fusion expression vector bearing an IPTG-inducible *Trc* promoter and the His-Tag metal-binding motif upstream of the multiple cloning site which is translated through ORF C; Ap^r	Invitrogen
pTrcHisACAT	Same as pTrcHisC, except that it carries the chloramphenicol acetyltransferase gene and is translated through ORF A; Ap^r	Invitrogen
pTHBC11	pTrcHisC carrying the 1.44-kb *Bam*HI-*Eco*RI DNA insert from pYM101 containing 1,082 bp of the 3′ end of the *syrB* ORF and 184 bp of the 5′ end of the *syrC* ORF; Ap^r	This study
pTHBC21	pTrcHisC carrying the 2.71-kb *Bam*HI-*Hin*dIII DNA insert from pYM101 which included the same 3′-end region of *syrB* as pTHBC11 as well as the complete *syrC* ORF; Ap^r	This study

[a] Ap^r, Cm^r, Km^r, Pp^r, and Rif^r, resistance to ampicillin, chloramphenicol, kanamycin, piperacillin, and rifampin, respectively.

(Invitrogen). For isolation of total cellular proteins, *P. syringae* pv. syringae B301D and its *syrB* (BR132) or *syrC* (BR334) mutant derivatives were grown as still cultures for 5 days in potato dextrose broth (50 ml) at 25°C (35, 41). Antibiotics (Sigma Chemical Co., St. Louis, Mo.) were added to media at the following final concentrations: ampicillin, 100 μg/ml; chloramphenicol, 200 μg/ml; kanamycin, 50 μg/ml; and piperacillin, 25 μg/ml.

The complementation of the *syrB* mutant W4S770 by pYM1.143 was performed as described previously (35). Complementation tests included pYM1 and pYM1.132 as positive and negative controls, respectively.

DNA manipulations and sequencing. Routine procedures (41) were used to isolate, subclone, and sequence a 4,842-bp DNA region of pYM101 containing the *syrB* and *syrC* genes (Fig. 2). Three cloning vectors, pPR510, pUC19, and pUC18 (Table 1), were used to make subclones of the DNA region with the desired sizes and orientations for nucleotide sequencing. Nested deletions of subclones were generated by using exonuclease III (Boehringer Mannheim, Indianapolis, Ind.). Sequencing reactions were performed with a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). Both strands were sequenced by using the M13 −40 primer and the M13 −48 reverse primer (supplied by U.S. Biochemical Corp.); custom-synthesized oligonucleotides complementary to specific internal regions of cloned fragments were used to resolve mobility compressions. In general, mobility compressions were resolved by using either the dITP method (included in the Sequenase 2.0 kit) or the terminal deoxynucleotidyl transferase method (Gibco BRL Life Technologies Inc., Grand Island, N.Y.). The Tn*3*HoHo1 insertion sites in *syrB* of pYM1.132 and *syrC* of pYM1.143 and pYM1.334 were subcloned and sequenced by using the Tn*3*-specific primer and procedure described by Quigley et al. (41).

Computer analysis of the *syrB* and *syrC* nucleotide sequences. Sequence data were entered into a computer with a GP-7 digitizer (Science Accessories Corp., Bainbridge Island, Wash.) and analyzed initially with the GenePro software (Riverside Scientific Enterprises Inc., Bainbridge Island, Wash.). The nucleotide and deduced amino acid sequences were then analyzed by using the Wisconsin Sequence Analysis programs of the Genetics Computer Group (release beta 8.0) (4). The following Genetics Computer Group programs were used: GAP, for comparisons of two sequences; TERMINATOR, for identification of rho-independent transcription terminators; MOTIFS, for identification of known protein sequence patterns; PILEUP, for analysis of similarities among multiple sequences; PEPLOT, for hydropathy analysis; and ISOELECTRIC, for prediction of the protein charge. PRETTYBOX (59) was used to align multiple sequences. Database searches for genes and proteins that are homologous with the *syrB* and *syrC* open reading frames (ORFs) were performed by using BLAST (1). The significance of protein sequence similarity was evaluated by sequence randomization and calculation of *Z* scores using GAP.

The predicted SyrB protein sequence was analyzed as described by Cosmina et al. (3) for a possible region involved in substrate binding. The sequence of SyrB and those of approximately 30 adenylate-forming enzymes obtained from GenBank were aligned by using PILEUP. A region of about 36 amino acids within the putative substrate-binding domain of SyrB (residues 301 through 336; see Fig. 3) was compared with the corresponding regions of the adenylate-forming enzymes. PILEUP was also used to construct a dendrogram representing the evolutionary distances of the sequences compared among the adenylate-forming enzymes.

Codon usage analysis of the *syrB* and *syrC* genes was performed as described by Quigley et al. (41), except that the sequence of *syrD* was included in the database.

Isolation of RNA and primer extension analysis. High-quality total RNA was prepared from *P. syringae* pv. syringae B301D by a modification of the method of Ladin et al. (26). All buffers and supplies used for RNA isolation were pretreated with 0.1% diethyl pyrocarbonate (DEPC) (Sigma) as described by Sambrook et al. (45). Because syringomycin synthesis occurs primarily in stationary phase (12), strain B301D was grown in potato dextrose broth still cultures (50 ml; 250-ml flask) for 3 to 4 days. The bacterial cells were pelleted by centrifugation at 3,000 × *g* (5 min, 4°C) and suspended in 10 ml of solution D (4 M guanidium isothiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, and 0.1 M β-mercaptoethanol), and then the RNA was isolated by the method of Ladin et al. (26). The RNA-containing pellet was further purified by chromatography using a QIAGEN-tip 100 column (QIAGEN Inc., Chatsworth, Calif.). Finally, the purified RNA was dissolved in 200 μl of sterile distilled DEPC-treated water, diluted with 600 μl of RNase-free absolute ethanol, and stored at −80°C.

The transcription initiation sites of the *syrB* and *syrC* genes were defined by the modified primer extension procedure provided with the Superscript RNase H$^-$ enzyme by Gibco BRL. The custom-synthesized oligonucleotide primers used were specific for either *syrB* or *syrC* and are shown in Fig. 3. Purified total RNA (15 to 20 μg) was dissolved in 8 μl of DEPC-treated water. The primer (2 μl of a 5 μM stock) was added to the RNA solution, and the mixture was incubated in

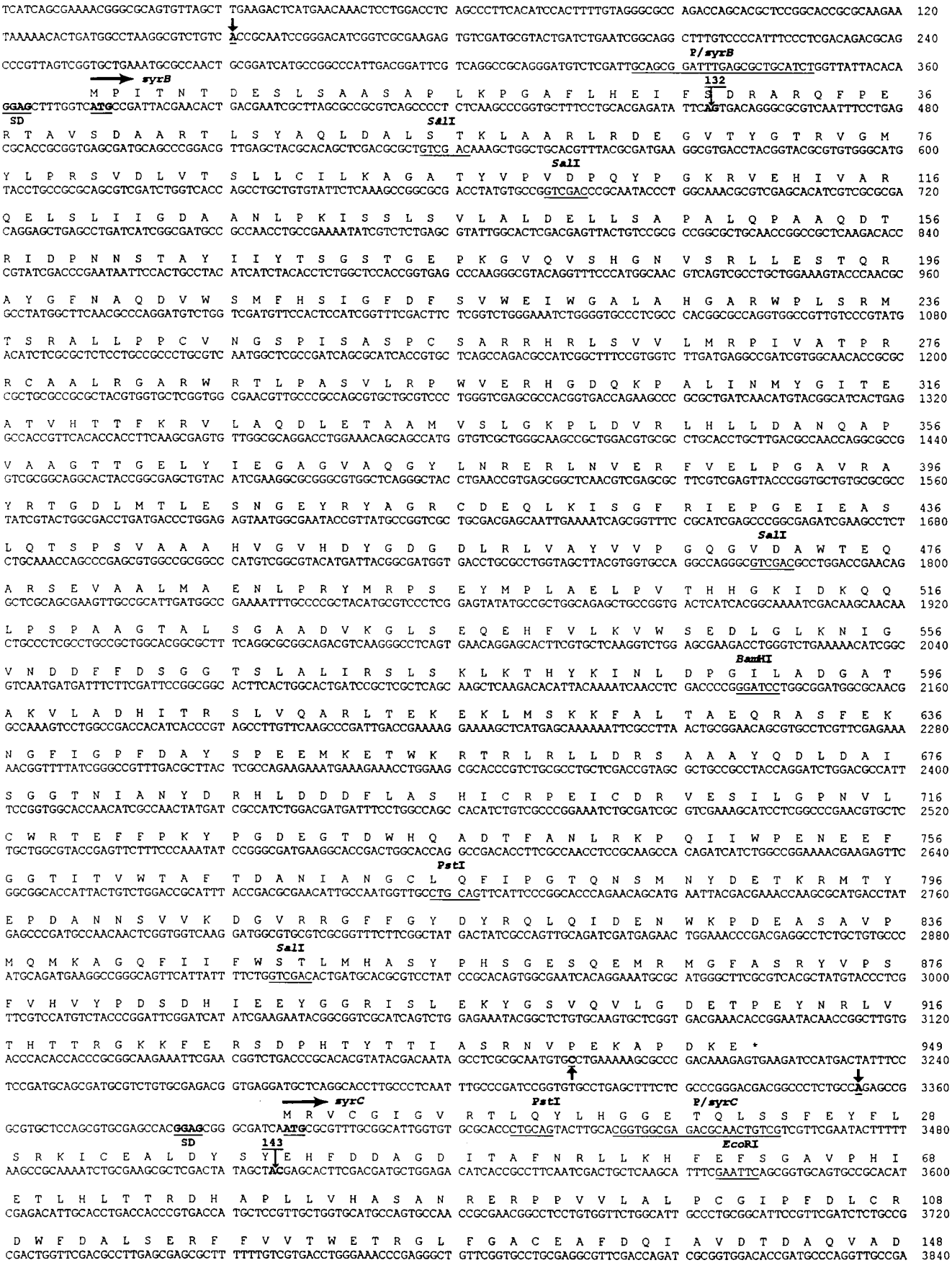
P/syrB
syrB
132
SD
SalI
SalI
SalI
BamHI
PstI
SalI
syrC
PstI
P/syrC
SD
143
EcoRI

FIG. 3. Nucleotide sequence of a 4,842-bp DNA region from strain B301D of *P. syringae* pv. syringae containing the *syrB* and *syrC* ORFs and some upstream and downstream DNA (see Fig. 2 for the map position of the sequenced region). The putative ORFs of *syrB* and *syrC* are indicated (horizontal arrows). The amino acid sequences of the ORFs are presented in the standard one-letter code above the first base of the corresponding codon. The start codons and the potential ribosome-binding sites (SD) of the ORFs (underlined boldface letters), the stop codons (asterisks), the transcription start sites for $mRNA_{syrB}$ and $mRNA_{syrC}$ (underlined boldface with vertical arrows), and a potential rho-independent transcription terminator downstream of *syrC* (underlined italic boldface) are indicated. The Tn*3*HoHo1 insertions in *syrB* (insert 132 [in frame]) and in *syrC* (inserts 143 [out of frame] and 334 [in frame]) are indicated (underlined numbers with vertical arrows) (see Fig. 2 for map positions). The restriction sites for *Sal*I, *Bam*HI, *Pst*I, and *Hin*dIII are underlined. The homologs of the oligonucleotides used for primer extension studies (Fig. 4) (P/*syrB* [the primer for *syrB*] and P/*syrC* [the primer for *syrC*] are underlined.

a 75°C water bath for 10 min and then chilled on ice. The mixture was centrifuged for 10 s, and then 9 μl of the reverse transcription solution (4 μl of 5× reverse transcription buffer [Gibco BRL], 2 μl of 0.1 M dithiothreitol [Sigma], 1 μl of deoxynucleoside triphosphates [10 μM each; Sigma], 1 μl of [α-^{35}S]dCTP [12.5 μCi/μl; DuPont, Wilmington, Del.], 1 μl of RNase inhibitor [Boehringer Mannheim]) was added; this mixture was incubated for 2 min in a 45°C water bath. Superscript RNase H$^-$ (1 μl; 200 U) was then added, and the incubation was continued for 1 h. The reaction was stopped by addition of sodium hydroxide (5 μl of a 0.5 M stock) followed by heating at 90°C for 3 min and addition of 95 μl of a stop solution (50 mM Tris [Sigma] [pH 7.4] and 25 mM HCl). Reverse transcriptase was removed by extraction with 120 μl of phenol-chloroform-isoamyl alcohol (25:24:1). Reverse transcription products were precipitated with 12 μl of sodium acetate (3 M; pH 7.0) and 340 μl of absolute ethanol, centrifuged for 15 min at 12,000 × *g*, and washed with 200 μl of 70% ethanol. Prior to being run on sequencing gels, the dried pellet was dissolved in 6 μl of TE buffer (10 mM Tris, 1 mM Na_2EDTA [pH 8.0]) and mixed with 4 μl of sequencing stop solution (U.S. Biochemical Corp.).

SDS-PAGE and Western blot analysis. The total proteins from strain B301D of *P. syringae* pv. syringae and its Tn*3*HoHo1 in-frame *syrB* (BR132) and *syrC* (BR334) insertion mutants were compared by Western blot (immunoblot) analysis to identify the SyrB-LacZ and SyrC-LacZ fusion proteins. The method of Xu and Gross (62), as modified by Mo and Gross (35), was used to isolate high-molecular-weight proteins associated with syringomycin production, except that Laemmli sample buffer (27) was used for cell lysis. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% acrylamide gels (5 to 6 h at 30 mA constant current) (27) and stained with Brilliant Blue G colloidal concentrate (Sigma) according to the manufacturer's instructions.

For Western blot analysis, the proteins separated by SDS-PAGE were electroblotted (0.5 A, 2 to 3 h) onto a BA-85 nitrocellulose membrane (S&S NC; Schleicher & Schuell, Inc., Keene, N.H.). The membrane was air dried and then incubated with agitation for 1 h at room temperature in blocking solution (5% [wt/vol] nonfat dry milk dissolved in phosphate-buffered saline [PBS]) (16). The blocking solution was replaced with inoculation solution (blocking solution with 0.3% [vol/vol] Tween 20 [Bio-Rad]) containing anti-β-galactosidase monoclonal antibody (Sigma) diluted 1:2,000. After incubation for 1 h, the blot was washed twice (10 min each) at room temperature in PBS containing 0.3% Tween 20. The membrane was then incubated at room temperature for 1 h with the second antibody (alkaline phosphatase-labeled goat-anti-mouse immunoglobulin G included in the Bio-Rad Immuno-Blot assay kit) diluted 1:3,000 in the inoculation solution. Finally, the membrane was washed twice as described above. The alkaline phosphatase color assay was performed according to the instructions included in the Immuno-Blot assay kit. The sizes of the proteins in SDS-polyacrylamide gels and Western blots were estimated by using protein standards (29 to 205 kDa; Sigma) which included the 116-kDa β-galactosidase protein.

Overexpression of the truncated SyrB protein in *E. coli*. The 3′ end of *syrB* was cloned into the pTrcHisC expression vector (Table 1) and overexpressed in *E. coli* Top10 (Xpress System from Invitrogen). Two constructs which both contained 1,082 bp of the 3′ end of *syrB* (Fig. 2) were made in pTrcHisC: pTHBC11 contained a 1.44-kb *Bam*HI-*Eco*RI insert with only 183 bp of the 5′ end of the *syrC* ORF, and pTHBC21 contained a 2.71-kb *Bam*HI-*Hin*dIII DNA insert with the complete *syrC* ORF. After transformation into *E. coli* Top10, the *syrB* gene was overexpressed according to Xpress protocol. The overexpressed SyrB fusion proteins were prepared by the same method used to isolate protein from *P. syringae* pv. syringae (described above). Proteins were separated by SDS-PAGE and visualized as described above on 12% mini-gels run for 45 to 50 min at constant voltage (200 V). As negative controls, proteins were isolated from uninduced Top10(pTHBC11) and Top10(pTHBC21) strains and from strains Top10 and Top10(pTrcHisC). As a positive control, proteins were isolated from strain Top10(pTrcHisACAT).

Nucleotide sequence accession number. The nucleotide sequence of the 4,842-bp DNA region (Fig. 3) containing the *syrB* and *syrC* genes is available in the GenBank database under accession no. U25130.

RESULTS

The nucleotide sequence of the *syrB* and *syrC* genes. A 4,842-bp DNA region (Fig. 2) containing the *syrB* and *syrC* genes was sequenced and analyzed. Two prominent ORFs which were 175 bp apart and translated in the same direction were identified (Fig. 3). The upstream *syrB* ORF (2,847 bp) and the downstream *syrC* ORF (1,299 bp) were confirmed by sequencing DNA flanking the Tn*3*HoHo1 insertions on pYM1.132, pYM1.334, and pYM1.143 (Table 1). Insert 132, which formed an in-frame *lacZ* fusion with *syrB* and expressed a high level of β-galactosidase activity (35), was located between 82 and 83 bp downstream of the start codon of *syrB*. Insert 334, which formed an in-frame *lacZ* fusion with *syrC* and expressed β-galactosidase activity comparable to that of insert 132 in *syrB* (41), was located between 475 and 476 bp downstream of the start codon of *syrC*. In an earlier report (35), insert 143 was mapped by restriction analysis to lie in the *syrB* ORF. However, sequence analysis has now established that insert 143 formed an out-of-frame *syrC-lacZ* fusion with Tn*3*HoHo1 inserted between residues 119 and 120 downstream of the start codon of *syrC*. The positions of these in-

sertions in the DNA segment sequenced are indicated in Fig. 3. We have established that *syrB* and *syrC* are transcribed independently by demonstrating that the *syrB*::Tn*5* mutant W4S770 can be complemented in *trans* by pYM1.143 and that strain W4S770 produces similar quantities of syringomycin after complementation in *trans* by pYM1.143 and pYM1 (data not shown).

The *syrB* and *syrC* genes are 59.6 and 60.5% G+C, respectively, which is typical of other sequenced genes of *P. syringae*, including *syrD* (41). Codon usage analyses of the *syrB* and *syrC* genes indicated that both genes contain a high frequency of methionine codons, as previously reported for other *P. syringae* genes and for *syrD* (41).

The proposed translation start sites of *syrB* and *syrC* are preceded by a consensus ribosomal binding site (GGAG) 8 bp upstream of the *syrB* ORF and 10 bp upstream of the *syrC* ORF (Fig. 3). A typical rho-independent terminator (Fig. 3) was found 4 bp downstream of the *syrC* ORF stop codon by the TERMINATOR program. No consensus rho-independent terminator sequence was found downstream of the *syrB* ORF.

Primer extension analysis of *syrB* and *syrC* transcription. A 24-mer oligonucleotide (P/*syrB*) complementary to a region 25 to 48 bp upstream of the 5′ end of the *syrB* ORF was used as the first reverse transcriptase primer (Fig. 3). Primer extension analysis indicated that *syrB* transcription initiated at an adenine residue 222 bp upstream of the start codon (Fig. 4A). The second primer was a 22-mer (P/*syrC*) complementary to a region 45 to 66 bp downstream of the 5′ end of the *syrC* ORF (Fig. 3). The *syrC* primer produced two primer extension products, indicating a prominent transcription start site at a cytosine residue 202 bp upstream of the *syrC* start codon (Fig. 4B) and a relatively minor start site at an adenine residue 44 bp upstream of the *syrC* start codon (Fig. 4C).

The SyrB protein is homologous to adenylate-forming enzymes employed in peptide synthesis. The SyrB protein is predicted to contain 949 amino acids and to be ~105 kDa in size. Searches of the databases with both the *syrB* gene and its putative protein product revealed homology with a superfamily of adenylate-forming enzymes involved in carboxyl group activation. In particular, SyrB displayed the greatest similarity with the thiotemplate-employing peptide synthetases involved in peptide antibiotic and siderophore synthesis in a variety of microorganisms (Fig. 5). All of the thiotemplate-employing peptide synthetases that are homologous to SyrB activate specific substrate amino acids by simultaneous hydrolysis of ATP (23, 30). The adenylate-forming peptide synthetases which had the greatest overall similarity to SyrB are identified in Table 2. The first amino acid-activating domain of a pyoverdin synthetase, PvdD (32), of *Pseudomonas aeruginosa* exhibited the greatest overall similarity (56.4%) and identity (33.8%) to SyrB. In addition, SyrB exhibited significant levels of similarity to many other peptide synthetases, including SrfAP2-2 (50%), which is encoded by the second ORF of the *srfA* operon involved in surfactin synthesis of *B. subtilis* (3); GrsA (46.8%), the gramicidin S synthetase 1 of *B. brevis* (17, 24); EntF (47.7%), the enterobactin synthetase of *E. coli* (44); the fourth amino acid-activating domain of HTS-4 (47.6%), the HC-toxin synthetase of *Cochliobolus carbonum* (49); and CmaA (51.2%), the putative amino acid activation domain involved in coronamic acid synthesis by *P. syringae* pv. glycinea (56). The significance of the homology of SyrB with these peptide synthetases was validated by *Z* scores which were all >18 (Table 2). The Kyte-Doolittle (25) hydropathy profile of SyrB was most similar to those of GrsA, TycA, and EntF (data not shown).

The alignment of SyrB with six representatives of thiotemplate-employing peptide synthetases (PvdD-1, SrfAP2-2, GrsA, ACVS-1, EntF, and HTS-4) identified a highly conserved region (identities and similarities ranging from 28.4 to 40.3% and 49.9 to 60.0%, respectively) of approximately 600 amino acids in the N-terminal portion of SyrB (Fig. 5). This conserved region of SyrB closely resembles the amino acid-activating domains of thiotemplate-employing peptide synthetases (30, 52). Six signature core sequences, which are characteristic of amino acid-activating domains in peptide synthetases (55), were observed in the SyrB domain at the characteristic positions and in the defined order (Fig. 5). Core sequence 2 of SyrB, TSGSTGEPKG (residues 170 to 179), resembles the known loop-forming ATP-binding motif (9, 46). Correspondingly, core sequence 4 contains the motif TGD (residues 399 to 401), which is invariably found at the active site of a large family of cation ATPases (9). Core

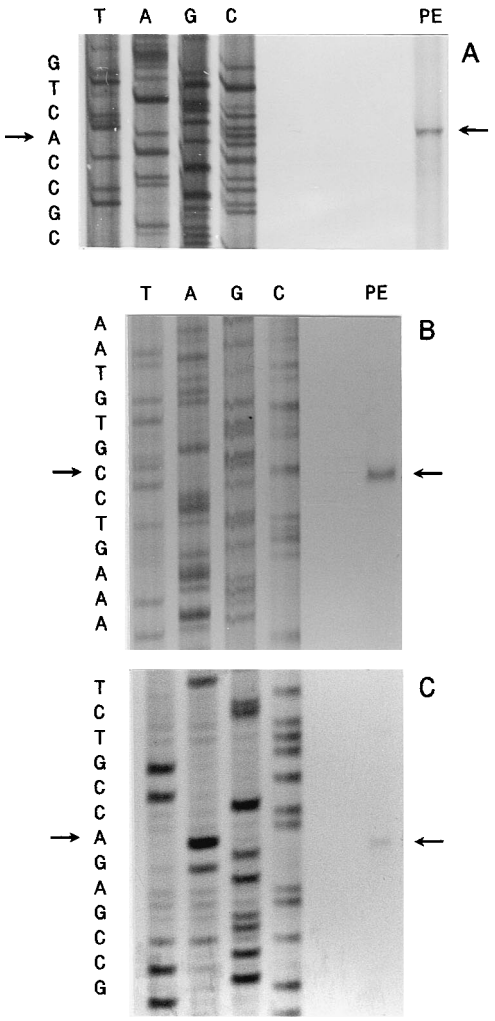

FIG. 4. Determination of the transcription initiation sites for $mRNA_{syrB}$ (A) and $mRNA_{syrC}$ (B and C) by primer extension analysis. A single transcription initiation site for *syrB* which was 222 bases upstream of its translation start codon was mapped (A), and two transcription sites which were 202 (B) and 44 (C) bases upstream of the translation start codon were mapped for *syrC*. The *syrB*- and *syrC*-specific oligonucleotides (the sequences and positions of their homologs are indicated in Fig. 3) were used both for mapping the 5′ end of the mRNAs of B301D in primer extension (PE) reactions and for sequencing the corresponding regions of plasmid pYM101. Transcription initiation sites predicted by the mobility of the PE products (arrows on the right) are indicated on the left (arrows). The nucleotide sequences flanking the transcription initiation sites of the coding strand also are shown.

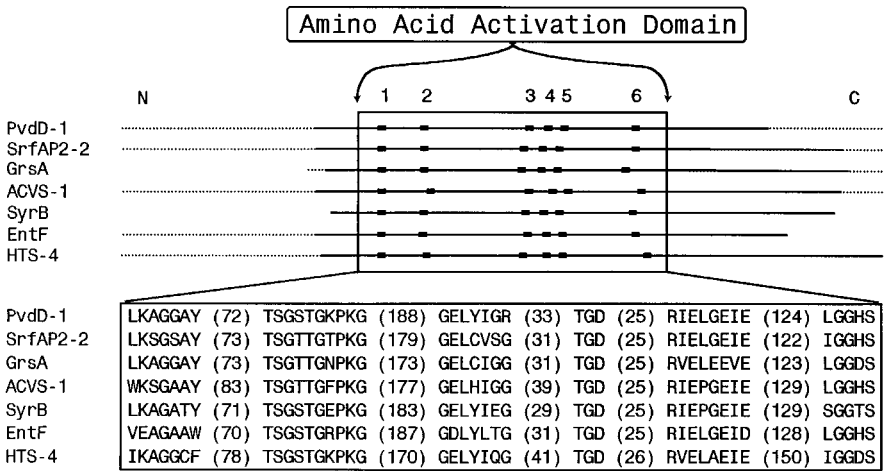

FIG. 5. Diagrammatic alignment of the SyrB protein with known peptide antibiotic synthetases. The putative amino acid activation domain of SyrB is aligned with those of six peptide antibiotic synthetases from the databases of SWISS-PROT (GrsA, ACVS-1, and EntF), PIR (HTS-4), and GENEPEPT (PvdD-1 and SrfAP2-2) by PILEUP. The relative position, order, and spacing of the six predicted core sequences of each domain of the aligned synthetases are indicated (numbered solid boxes). The numbers of amino acids between the conserved core sequences are listed in parentheses. The aligned proteins are identified in Table 2 along with their relative similarity and identity to SyrB. The N-terminal (N) and C-terminal (C) regions of the aligned proteins are indicated.

sequence 6, DDFFDSGGTSL (residues 559 to 569), resembles a sequence containing a motif necessary for binding the 4′-phosphopantetheine cofactor involved in thioester formation (6, 9, 30, 52). In addition, SyrB contained another sequence, NMYGITEATVHTTFKRVLAQDLET (residues 310 to 333), between core sequences 2 and 3 that fell within a region speculated by Cosmina et al. (3) to be responsible for binding the substrate amino acid. This sequence contained amino acid residues that were conserved only among proteins activating the same substrate amino acids, which indicated that the sequence may be involved in substrate specificity. Analysis of the putative substrate-specific binding region of adenylate-forming enzymes determined that SyrB was most similar to EntF (Fig. 6), which binds and activates serine in enterobactin synthesis (44).

SyrC exhibits similarity to thioesterases. Database searches with the predicted 48-kDa SyrC protein consisting of 433 amino acids (Fig. 3) revealed that it is similar to a family of thioesterases which contain the conserved motif GXC(S)XG (Fig. 7). Analyses of proteins containing this thioesterase motif identified several enzymes (Table 3) with a high degree of overall similarity (42 to 48%) to SyrC. These proteins include the following: fatty acid thioesterase II from rats (28, 43) and ducks (39); the haloperoxidases BPO-A2, a bromoperoxidase of *Streptomyces aureofaciens* involved in 7-chlorotetracycline synthesis (38), and CPO, a chloroperoxidase of *Pseudomonas pyrrocinia* involved in pyrrolnitrin synthesis (61); an acyltransferase (*actI*-ORF1) of *Streptomyces coelicolor* involved in actinorhodin synthesis (7); and two proteins that are predicted to function as thioesterases in nonribosomal peptide antibiotic synthesis (GrsT, involved in gramicidin S synthesis in *B. brevis* [24], and *srfA*-ORF4, involved in surfactin synthesis in *B. subtilis* [3]). Except for *actI*-ORF1, SyrC was considerably larger (by about 160 amino acids) than the other proteins containing the GXC(S)XG motif (Fig. 7). The conserved GXCXG motif of SyrC was located at residues 165 to 169. In addition, a zinc-binding motif (HTGAAITNLPF) characteristic of carboxypeptidases (37, 54) was identified near the C terminus of SyrC (Fig. 7, residues 382 to 392).

Among the proteins analyzed for similarity to SyrC, the *Z* scores for sequence comparisons between only SyrC and either BPO-A2 or CPO are >3 (Table 3), indicating that the similarities between SyrC and these two haloperoxidases may be significant. An interesting observation from comparisons of the SyrC sequence with those of CPO and BPO-A2 is the presence of a region containing 26 amino acids (residues 225 to 250 of SyrC) that is lacking in the thioesterases, GrsT and *srfA*-ORF4 (Fig. 7). We also observed other areas with a high level of similarity between SyrC and the haloperoxidases.

The *syrB* and *syrC* genes are translated separately. The translational relationship between the *syrB* and *syrC* genes was investigated by Western analysis of strains BR132 and BR334,

TABLE 2. Sequence similarities among amino acid activation proteins homologous to SyrB

Protein[a]	Description	Similarity (%)	Identity (%)	*Z* score	Reference
PvdD-1	Domain 1 of pyoverdin synthetase D	56.4	33.8	28.9	32
GrsA	Gramicidin S synthetase 1	46.8	24.2	26.8	24
CmaA	Coronamic acid synthetase	51.2	29.4	24.3	56
EntF	Enterobactin synthetase	47.7	27.6	22.4	44
TycA	Tyrocidine synthetase I	49.7	29.3	21.9	33
HTS-4	Domain 4 of the HC-toxin synthetase	47.6	25.4	21.6	49
SrfAP2-2	Domain 2 of surfactin synthetase (ORF2)	50.0	26.2	20.1	3
ACVS-1	Domain 1 of δ-(L-α-aminoadipyl)-L-Cys-D-Val synthetase	46.1	25.4	18.8	51

[a] Source organisms: PvdD-1, *P. aeruginosa*; GrsA, *B. brevis*; CmaA, *P. syringae* pv. glycinea; EntF, *E. coli*; TycA, *B. brevis*; HTS-4, *C. carbonum*; SrfAP2-2, *B. subtilis*; ACVS-1, *Penicillium chrysogenum.*

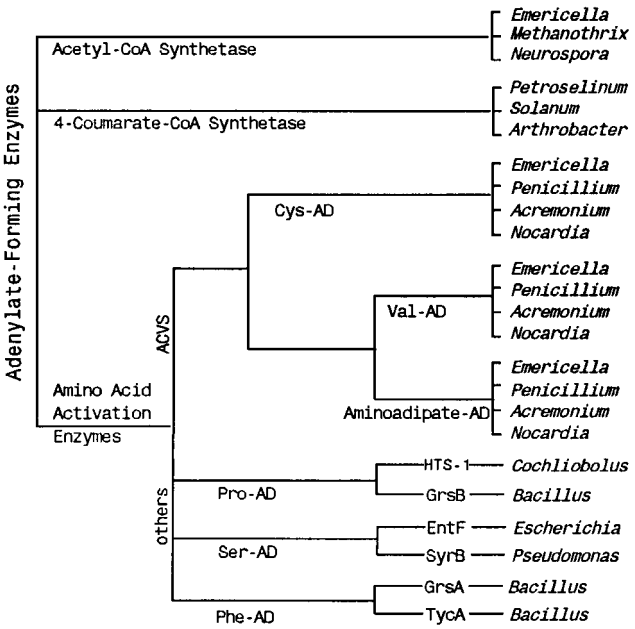

FIG. 6. Dendrogram depicting the phylogenetic relationship of the hypothesized substrate-specific binding site of adenylate-forming enzymes from various microorganisms. The predicted substrate-specific binding site of SyrB (residues 301 to 336) was aligned with the corresponding sequences of about 30 known adenylate-forming enzymes by PILEUP as described in Materials and Methods. The clustering of representative synthetases with defined substrate specificities is shown. ACVS, δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (51); Cys-AD, Val-AD, and aminoadipate-AD, the Cys, Val, and aminoadipate activation domains of ACVS, respectively; HTS-1, the first amino acid activation domain of the HC-toxin synthetase of *C. carbonum* (49); GrsA, gramicidin S synthetase I of *B. brevis* (17, 24); GrsB, the first amino acid activation domain of gramicidin S synthetase II of *B. brevis* (55); EntF, enterobactin synthetase component F of *E. coli* (44); TycA, tyrocidine synthetase I of *B. brevis* (33); CoA, coenzyme A.

which form SyrB-LacZ and SyrC-LacZ fusion proteins, respectively. Using anti-β-galactosidase as the primary antibody, we observed an approximately 125-kDa protein in total protein preparations from strain BR132 (Fig. 8, lane 2). On the basis of the map position of the Tn*3*HoHo1 insert in BR132 (Fig. 3), the SyrB-LacZ fusion protein contained the N-terminal 28 amino acids of SyrB and the entire 116-kDa β-galactosidase protein. Western analysis of BR334 identified a single polypeptide band of approximately 160 kDa (Fig. 8, lane 3). On the basis of the map position of the Tn*3*HoHo1 insert in BR334 (Fig. 3), the SyrC-LacZ fusion protein contained the N-terminal 159 amino acids of SyrC fused to the β-galactosidase protein. The predicted size of the SyrC-LacZ fusion protein was 141 kDa, which was smaller than the SyrC fusion protein observed in gels (Fig. 8). Western analysis showed no evidence for the formation of a SyrB-SyrC fusion protein in strain BR334, and strain B301D contained no protein which reacted with the anti-β-galactosidase antibody (Fig. 8, lane 1).

The translational relationship between the *syrB* and *syrC* genes was analyzed further by overexpression of the C-terminal portion of the SyrB protein in *E. coli* Top10 using two plasmid constructs, pTHBC11 and pTHBC21. Plasmid pTHBC11 contained 1,082 bp of the 3′ end of *syrB* and 183 bp of the 5′ end of *syrC*, whereas pTHBC21 contained the same 3′ end of *syrB* and the complete *syrC* gene (Fig. 2). The inserts were confirmed by restriction mapping and sequencing of the junction region between the 5′ end of each insert and the vector. A protein band of approximately 37 kDa (not including the His-Tag fusion protein of the vector) was overexpressed after IPTG (isopropyl-β-D-thiogalactopyranoside) induction of *E. coli* harboring either pTHBC11 or pTHBC21, which corresponded to the expected size of the protein product of the cloned 3′ region of *syrB*. Therefore, the pTHBC21 expression vector did not yield a SyrB-SyrC fusion protein. In addition, these results confirmed the putative termination site of the *syrB* ORF. Overexpression of a 1.68-kb *Pst*I-*Hin*dIII fragment carrying all but the first 30 bases of the *syrC* ORF yielded a fusion protein of the expected size (46 kDa; data not shown). This result confirmed the size of the predicted *syrC* ORF (Fig. 3).

DISCUSSION

Analysis of the *syrB* and *syrC* genes suggests that they encode syringomycin synthetases that participate in a thiotemplate multienzymatic mechanism of peptide synthesis (23, 31). The predicted SyrB protein contains 949 amino acids and is homologous to adenylate-forming enzymes of diverse origins that activate and bind amino acids in the synthesis of peptide antibiotics and siderophores (30, 44, 52, 55). In particular, SyrB exhibits striking similarity to multifunctional proteins involved in gramicidin S (17, 24, 55), surfactin (3), penicillin (51), and pyoverdin (32) synthesis. For example, GrsA, which binds and activates phenylalanine as the first amino acid to be incorporated into gramicidin S (17, 24), is similar in size to SyrB (GrsA contains 1,089 amino acids) and contains extensive similarity (50.6%) over an N-terminal region of about 600 amino acids. The predicted SyrC protein contains a distinct thioesterase motif, GXS(C)XG, characteristic of medium-chain fatty acyl thioesterases of rats and ducks (28, 39, 43) and thioesterases employed in synthesis of antibiotics such as gramicidin S (24) and surfactin (3). In addition, SyrC shares about 45% similarity with the nonheme haloperoxidases of *P. pyrrocinia* (61) and *S. aureofaciens* (38) involved in the chlorination of pyrrolnitrin and 7-chlorotetracycline, respectively. Analysis of the haloperoxidase amino acid sequences revealed that both proteins contain a thioesterase motif near the center, as observed for SyrC.

A typical amino acid-activating domain was illustrated recently by Marahiel (30) to contain six core sequences with the

TABLE 3. Similarity of SyrC to proteins containing a thioesterase motif[a]

Protein[b]	Description	Similarity (%)	Identity (%)	*Z* score	Reference
BPO-A2	7-Chlorotetracycline bromoperoxidase	42.2	21.5	7.3	38
CPO	Pyrrolnitrin chloroperoxidase	45.6	20.5	5.1	61
GrsT	Gramicidin S thioesterase	45.9	18.9	2.6	24
srfA-ORF4	Surfactin synthetase	47.0	17.8	2.0	3
CmaT	Coronamic acid thioesterase	48.4	21.4	0.6	56
actI-ORF1	Actinorhodin acyltransferase	42.9	20.0	0.1	7

[a] A region of SyrC between residues 65 and 346 was used in all sequence comparisons.
[b] Source organisms: BPO-A2, *S. aureofaciens*; CPO, *P. pyrrocinia*; *srfA*-ORF4, *B. subtilis*; CmaT, *P. syringae* pv. glycinea; GrsT, *B. brevis*; *actI*-ORF1, *S. coelicolor.*

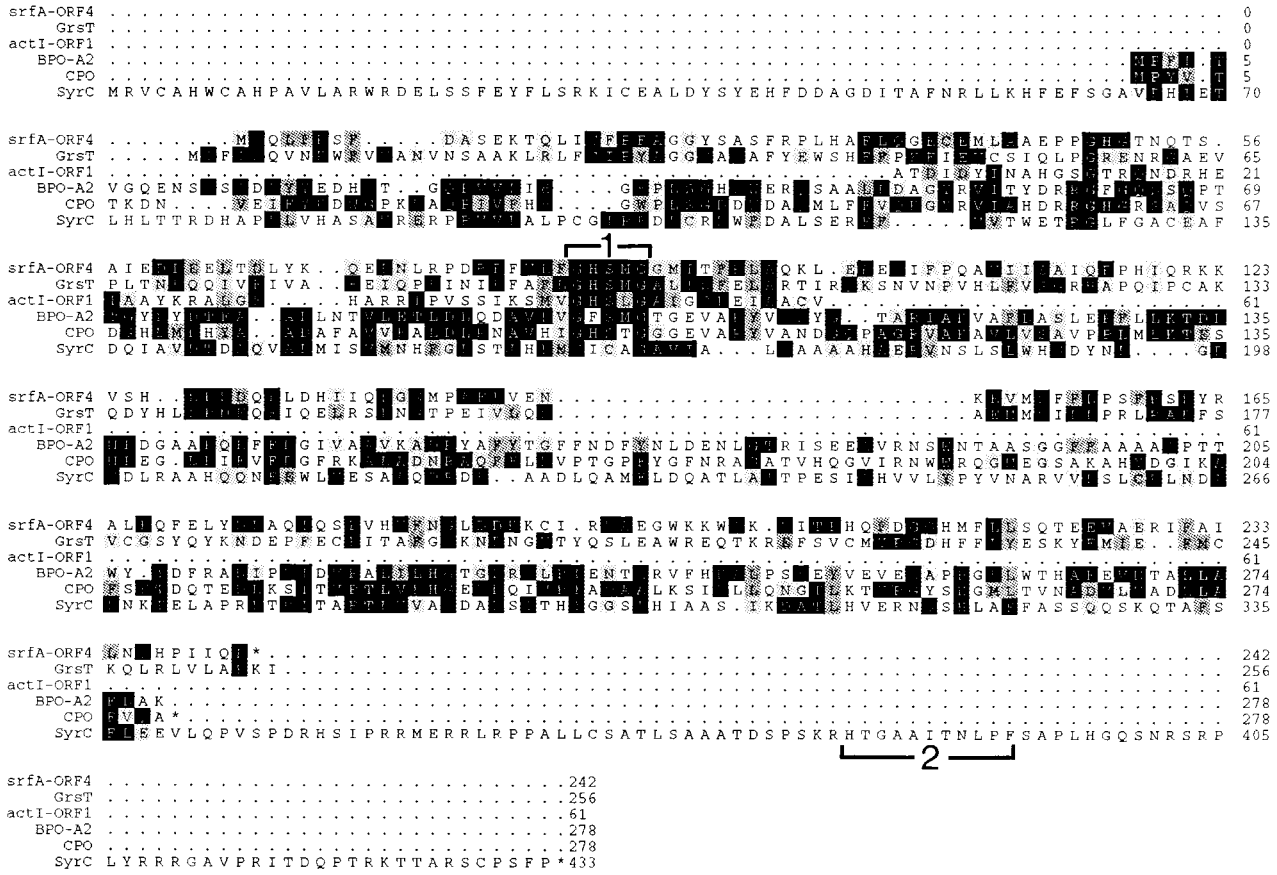

FIG. 7. Alignment of the deduced amino acid sequence of SyrC with those of an acyltransferase (*actI*-ORF1), two haloperoxidases (BPO-A2 and CPO), and representative thioesterases (*srfA*-ORF4 and GrsT) by PILEUP. The degrees of similarity among residues in the aligned proteins are displayed by PRETTYBOX as black boxes (the consensus residues) and regions with dark, light, or no shading (close, more distant, and no similarity of the aligned residues to the consensus residues at each position, respectively). A highly conserved motif, GXS(C)XG (bracket 1), was found in all six proteins, including SyrC. For the *actI*-ORF1 protein of *S. coelicolor* (7), only the sequence flanking the GXS(C)XG motif was aligned. A potential zinc-binding motif (bracket 2) close to the carboxyl terminus of SyrC was found by MOTIFS.

same order and spacing as observed for thioester-forming domains involved in the synthesis of gramicidin S and other peptide antibiotics (3, 17, 24, 55). Core sequences 2 and 4 are reported (9, 30, 52) to contain motifs involved in ATP binding and hydrolysis, respectively, whereas core sequence 6 contains the site of thioester formation (6, 30, 52). For example, within the core sequence 6 homolog of SyrB, we identified an SGGTS sequence, which resembles the pantetheine-binding site of acyl carrier proteins in fatty acid and polyketide synthetases (6, 48, 53). The Ser residue at the end of the motif in SyrB is especially important because a serine at this position has been shown in studies of gramicidin S (48, 53) and surfactin (6, 57) synthesis to bind the 4′-phosphopantetheine cofactor which provides the thiol group participating in thioester formation. Consequently, Ser-568 of core 6 in the SyrB sequence is predicted to be the site for covalent attachment of the substrate amino acid to SyrB by a carboxyl thioester.

Although analysis of the SyrB protein sequence is consistent with the presence of a domain that activates and binds one of the component amino acids of syringomycin, the substrate amino acid of SyrB remains to be identified. Cosmina et al. (3) recently identified a region associated with substrate recognition between core sequences 2 and 4 of individual domains involved in surfactin synthesis. They found a pattern of conservation for amino acid sequences near the ATP-binding motif of peptide synthetase domains that recognize and aminoadenylate similar amino acids as substrates. Accordingly, we aligned the putative substrate-specific binding regions of about 30 synthetases for which the amino acid substrates and protein sequences are known and observed a clustering of domains which activate the same substrate amino acids (Fig. 6). For example, the domains that activate proline from GrsB of *B. brevis* (55) and HTS-1 of *C. carbonum* (49) exhibit significant sequence conservation in a region containing about 30 residues and are clustered in a dendrogram. The analogous region of SyrB (i.e., residues 301 to 336) was found to be most similar to the corresponding domain from EntF, which functions as a serine-activating enzyme in enterobactin synthesis in *E. coli* (44). This suggests that serine, or the structurally related amino acid threonine, may be the specific substrate amino acid activated by SyrB. The syringomycin structure contains two serine residues at the N terminus and one threonine residue at the C terminus, although threonine is modified to form 4-chlorothreonine. Thus, SyrB is predicted to be either the first or the last synthetase participating in a thiotemplate mechanism of syringomycin synthesis.

The similarity of SyrC to nonheme haloperoxidases from *P. pyrrocinia* (61) and *S. aureofaciens* (38) is suggestive of a catalytic role for SyrC in chlorinating L-threonine, the last amino acid incorporated into syringomycin. Unfortunately, little is

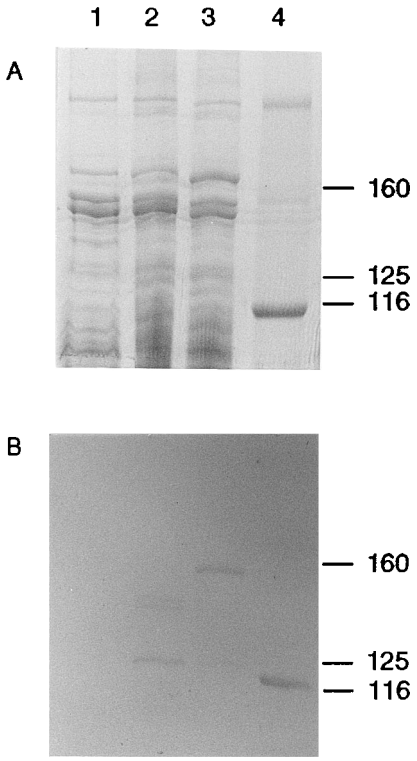

FIG. 8. SDS-PAGE (A) and Western blot (B) analyses of total protein preparations from Tn*3*HoHo1 insertion mutants with in-frame translational fusions with the *syrB* and *syrC* genes of *P. syringae* pv. syringae B301D. The proteins (separated on 7% acrylamide gels) in panel A were stained with Brilliant Blue G and in panel B were electroblotted onto a nitrocellulose membrane and probed with anti-β-galactosidase as the primary antibody. Protein preparations resolved in lanes 1 to 3 are from parental strain B301D, *syrB* mutant BR132, and *syrC* mutant BR334, respectively. The SyrB-LacZ and SyrC-LacZ fusion proteins have apparent sizes of 125 (lane 2) and 160 (lane 3) kDa, respectively. Lane 4 contains molecular size markers (29 to 205 kDa), including the 116-kDa β-galactosidase protein which reacts with the antibody in panel B.

known about the active site of nonheme haloperoxidases. Alignment of SyrC with known haloperoxidase sequences identified conserved regions that may facilitate the identification of motifs involved in halogenation. A thioesterase motif is present in SyrC and is the only discernible motif common to all available sequences of nonheme haloperoxidases. The SyrC thioesterase motif, GXCXG, is especially interesting because it contains a Cys in place of Ser at the proposed active site. Witkowski et al. (60) demonstrated recently that replacement of Ser with Cys in thioesterase II did not significantly affect catalytic activity. Nevertheless, SyrC may function as an acyltransferase, an enzyme which is predicted to modify the first amino acid, L-serine, in syringomycin synthesis to form a 3-hydroxydodecanoly-L-serine conjugate. For example, the thioesterase motif is conserved in *actI*-ORF1, which functions as an acyltransferase during actinorhodin biosynthesis in *S. coelicolor* (7). *srfA*-ORF4, which is involved in surfactin synthesis (3), exhibits levels of sequence similarity to SyrC similar to those of the nonheme haloperoxidases. Cosmina et al. (3) speculated that *srfA*-ORF4 could be associated with the transfer of an acyl group, but its biochemical function remains to be determined. SyrC is larger (at 433 amino acids) than all the proteins with which it has significant similarity (Fig. 7) and contains a zinc-binding motif, HTGAAITNLPF, near its C terminus. This motif, which is commonly found in zinc carboxypeptidases (37, 54), contains a His residue that serves as a zinc ligand (47). The significance of the zinc-binding motif in syringomycin biosynthesis is unclear, but of potential relevance are nonheme haloperoxidases of *Curvularia inaequalis* (29) and *Pseudomonas putida* (21) containing zinc, which may serve as a cofactor in haloperoxidation. Finally, it is possible that SyrC is multifunctional, perhaps possessing both acyltransferase and chloroperoxidase activities.

Analysis of the transcriptional and translational relationship between the *syrB* and *syrC* genes demonstrated that they are expressed independently, although their gene products may be functionally linked. First, sequence analysis confirmed the report of Mo and Gross (35) that the *syrB* and *syrC* genes map close to one another and are transcribed in the same direction. Nevertheless, primer extension analysis identified one transcription initiation site 222 bp upstream of the translation start codon of *syrB*, whereas the transcription of *syrC* initiated at two sites 44 and 202 bp upstream of the *syrC* start codon (Fig. 3). Second, Western analyses of proteins from *P. syringae* pv. syringae strains carrying Tn*3*HoHo1 insertions in the *syrB* (strain BR132) and the *syrC* (strain BR334) genes confirmed the expression of unique chimeric proteins containing the N-terminal regions of SyrB or SyrC fused to β-galactosidase (Fig. 8). Third, complementation analysis showed that pYM1.143, which carries a Tn*3*HoHo1 insertion within the 5′ end of *syrC*, restored full syringomycin production by a *syrB*::Tn*5* mutant strain (W4S770) when introduced by conjugation, indicating that *syrC* was expressed normally in a *syrB* mutant. Thus, a transposon insertion at this site does not disrupt the activity of both the *syrB* and the *syrC* genes as previously reported (13, 35). Finally, overexpression in *E. coli* of a DNA region containing the 3′ end of the *syrB* gene and the complete downstream *syrC* gene yielded a single protein of approximately 37 kDa, which was the size expected for the truncated SyrB protein only. Thus, we found no evidence for the formation of a SyrB-SyrC fusion protein or for *syrC* expression to be dependent on *syrB* expression. We conclude that the ~350-kDa SR4 and ~130-kDa SR5 proteins associated with syringomycin synthesis are not translational products of either the *syrB* or the *syrC* gene. Consequently, the effect of mutation of either *syrB* or *syrC* on formation of SR4 and SR5 remains to be resolved. It is important to emphasize, however, that both the *syrB* and the *syrC* genes are expressed strongly under the same environmental conditions and are responsive to plant signal molecules (34, 36, 41).

Many of the phytotoxins produced by plant pathogenic bacteria and fungi contain one or more amino acids (13, 14, 58). Consequently, the synthesis of many phytotoxins may employ peptide synthetases which resemble SyrB and SyrC. Unfortunately, prior genetic studies of the biosynthetic mechanisms for peptide-containing phytotoxins are limited to those of HC-toxin by the fungus *C. carbonum* (49) and coronatine by *P. syringae* pv. glycinea (56). HC-toxin is a cyclic tetrapeptide synthesized by a multifunctional peptide synthetase of ~574 kDa encoded by the 15.7-kb *HTS1* gene. The HTS protein contains four domains that catalyze the adenylation of amino acids and thioester formation, and each of the four domains exhibits significant similarity to SyrB (approximately 50%). For coronatine synthesis, Ullrich and Bender (56) described two genes involved in the synthesis of coronamic acid, an ethylcyclopropyl amino acid derived from isoleucine. Analysis of the *cmaA* gene sequence determined that it encodes a typical adenylate-forming enzyme that presumably binds and activates isoleucine, whereas the *cmaT* gene product contains a thioesterase motif. We found the predicted CmaA and CmaT protein sequences to be similar to those of SyrB and SyrC, respec-

tively (Tables 2 and 3). Nevertheless, scores for similarity (51%) and identity (29%) between SyrB and CmaA were lower than those for SyrB and the pyoverdin synthetase PvdD-1 of *P. aeruginosa* (56 and 34%). Likewise, SyrC exhibited slightly greater overall similarity to the haloperoxidases CPO and BPO-A2 than to CmaT. This is not particularly surprising, because syringomycin and coronatine represent different classes of toxins synthesized by mechanisms that only partially resemble one another (13).

Characterization of *syrB* and *syrC* provides a valuable foundation for defining the organization and function of *syr* genes encoding a multienzyme system for syringomycin biosynthesis. A thiotemplate mechanism of peptide synthesis would require individual amino acid-activating domains, which physically and functionally resemble SyrB, for all nine constituent amino acids. We therefore predict a relatively large DNA region of approximately 30 kb to be dedicated to syringomycin biosynthesis in *P. syringae* pv. syringae. The inducibility of *syrB* and syringomycin production by specific plant signal molecules (34, 36, 40) adds an exciting dimension to the regulation of phytotoxin synthesis in the plant environment and represents the first example of activation of a nonribosomal peptide synthetase by specific host factors. It now appears that most, if not all, fluorescent pseudomonads possess a multienzymatic complex for synthesizing peptide-containing metabolites as exemplified by pyoverdins (32), which underscores the importance of the thiotemplate biosynthetic mechanism in nature. Although biochemical studies are needed to define the specific functions of the *syrB* and *syrC* genes in toxin synthesis, it appears that they encode either the first or the last enzymes involved in syringomycin synthesis. In addition to sequence analysis suggesting that SyrB and SyrC participate in the formation of either 3-hydroxydodecanoyl-L-serine or 4-chlorothreonine, preliminary evidence indicates that the SyrC protein has thioesterase activity in vitro (11). The relationship of *syrB* and *syrC* expression may reflect an interdependent function in activating and modifying either the N- or the C-terminal amino acid prior to export of the fully synthesized lipopeptide by SyrD.

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