Analysis of the syrP Gene, Which Regulates Syringomycin Synthesis by Pseudomonas syringae pv. syringae†

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Received 6 February 1997/Accepted 11 May 1997

Syringomycin is a lipodepsinonapeptide phytotoxin synthesized by Pseudomonas syringae pv. syringae on multi-enzymatic peptide synthetases. Sequence analysis of the interval between the syrB and syrD genes of P. syringae pv. syringae strain B301D revealed a 1,059-bp open reading frame (ORF), designated syrP. The predicted product of this ORF was a 39.6-kDa protein consisting of 353 amino acid residues. Searches of protein sequence databases demonstrated that SyrP was most similar to histidine kinases such as the CheA regulatory protein of Escherichia coli. The predicted SyrP sequence was aligned with the N terminus of CheA, a region corresponding to the phosphotransfer and acceptor domains of CheA. The SyrP region that aligns with the phosphotransfer domain of CheA contained a His at position 101 which is flanked by a weak consensus sequence of the unorthodox sensory kinase subfamily of two-component regulatory systems. Strain B301D-31, obtained by site-directed insertional mutagenesis of the syrP gene, exhibited an unusual pleiotropic phenotype including a failure to produce syringomycin in liquid media in contrast to production of elevated levels of the toxin on agar media. The syrP mutant was relieved of the suppression of toxin production that accompanies inorganic phosphate concentrations of >1 mM on agar media. Nevertheless, the syrP mutant was substantially less virulent than the wild-type strain in pathogenicity assays in cherry fruits. These results suggest that the syrP gene encodes a regulatory protein that participates in a phosphorylation cascade controlling syringomycin production and virulence in P. syringae pv. syringae.

Most strains of Pseudomonas syringae pv. syringae produce syringomycin, a cyclic lipodepsinonapeptide toxin that causes necrotic symptoms in host plants (8). Syringomycin is cytotoxic due to formation of pores in the plasma membrane which are necrotic symptoms in host plants (8). Syringomycin is cytotoxic to many plant cell types (9). The capacity of diverse environmental factors to influence the expression of genes dedicated to syringomycin synthesis indicates the presence of a complex system of signal transduction which monitors a dynamic environment.

Production of toxins and other secondary metabolites by bacteria is controlled by diverse and often complex systems of regulation (7, 20, 21). Bacillus species produce a variety of peptide antibiotics including gramicidin S, tyrocidine, and surfactin (20, 21). Synthesis of these antibiotics is governed by sporulation genes, which monitor and respond to environmental changes through a multistep phosphorelay signal transduction pathway (9). The phosphorelay pathway is composed of sensor kinases (e.g., KinA) and response regulators through which a phosphoryl group is transferred. The Spo0A transcriptional regulator is activated ultimately and either positively or negatively controls genes dedicated to sporulation and production of antibiotics (9, 26). Recently, the Ypd1 protein of Saccharomyces cerevisiae was identified as a member of a multistep phosphorelay mechanism (27). Phosphorelays appear to be a universal signal transduction apparatus in both prokaroytes and eukaryotes (1).

Phosphate and nitrate are nutritional factors known to control the expression of a variety of genes in Escherichia coli through phosphotransfer systems, including chemotaxis (3, 4, 30, 35, 37). For example, CheA is a cytoplasmic sensor protein that senses chemical signals in the environment through transducers, such as the Tsr and Tar proteins, and then transfers the signals to corresponding response regulators through phosphotransfer (3, 35). It has been demonstrated that a sensor protein may have one or more phosphate receivers that act as intermediate phosphate transmitters. These phosphate transmitters serve as phosphate pools through which the expression of various genes can be cross-regulated (1, 3, 35).
The regulatory mechanism responsible for expression of syringomycin production by *P. syringae* pv. *syringae* is unknown, although it appears to be strongly influenced by environmental factors such as phosphate and plant signal molecules. In previous genetic studies of syringomycin production by *P. syringae* pv. *syringae* strain B301D, three *syr* genes were identified in a 6.9-kb chromosomal region involved in either toxin biosynthesis or secretion (Fig. 1) (29). The *syrB* gene encodes a syringomycin synthetase which binds and activates one of nine structural amino acids of syringomycin (40). The *syrC* gene product, SyrC, shows thioesterase activity (39). The *syrD* gene, which is transcribed in the opposite direction to *syrB* and *syrC*, encodes a protein which resembles members of the ABC (ATP-binding cassette) superfamily of membrane-associated transporter proteins involved in the secretion of antibiotics (29). Thus, it is predicted that syringomycin is synthesized by the thioesterase mechanism (21, 40) on a peptide synthetase complex which includes both the *syrB* and *syrC* proteins, and that the SyrD protein exports the toxin across the cytoplasmic membrane (29). In this study, we identified an open reading frame (ORF), called *syrP*, in the 1.5-kb region between the *syrB* and *syrD* ORFs (Fig. 1). The *syrP* protein exhibits similarity to the phosphotransfer regions of histidine kinases such as CheA (16) and Ypd1 (27). It is proposed that *syrP* participates in a phosphorelay mechanism of signal transduction which controls syringomycin synthesis and influences the virulence of *P. syringae* pv. *syringae*.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** The bacterial strains and plasmids used in this study are described in Table 1. All *E. coli* strains were grown at 37°C either in LB broth or on LB agar (31). Strains of *P. syringae* pv. *syringae* were grown at 25°C by using the following media: King’s medium B (KB) (15) was used for routine culture, potato-dextrose agar (PDA) (7) and potato-dextrose broth (PDB) (7) were used in routine assays for syringomycin production, syringomycin minimal (SRM) agar and liquid media were used for assays of syringomycin production under defined conditions (7), and nutrient broth-yeast extract (NBY) (36) was used for assays of syringomycin production by *syrP* mutants. When needed, the following antibiotics were added to media at final concentrations as indicated: ampicillin, 100 μg/ml; chloramphenicol, 200 μg/ml; and piperacillin, 100 μg/ml. Piperacillin was used to select for the ampicillin resistance phenotype in recombinant *Pseudomonas* strains.

**DNA manipulations and sequence analysis.** Plasmid DNA was isolated routinely by an alkaline lysis miniprep procedure (31, 40). Extraction of DNA fragments from agarose gels was done by using a QIAquick gel extraction kit (Qiagen Inc., Chatsworth, Calif.). The subcloning and sequencing of DNA were performed with a Sequenase version 2.0 kit (U.S. Biochemical Corp., Cleveland, Ohio). The DNA sequence of the *syrP* gene was first recorded with a GP-7 digitizer (Science Accessories Corp., Bainbridge Island, Wash.) and analyzed by GenPro software (Riverside Scientific Enterprises Inc., Bainbridge Island, Wash.). More sophisticated analyses of sequence data were performed with the University of Wisconsin Genetics Computer Group sequence analysis programs (release 8.0) (6). These analyses included database searches, evaluation of the significance of protein sequence similarities (with the GAP program), and multiple sequence alignments (with the PILEUP program). Codon usage and multiple sequence alignments (with the PILEUP program) were calculated using the *University of Wisconsin* Genetics Computer Group sequence analysis programs (release 8.0) (6). These analyses included database searches, evaluation of the significance of protein sequence similarities (with the GAP program), and multiple sequence alignments (with the PILEUP program). Codon usage

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristic(s)*</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169 (φ80lacZ ΔM15)</td>
<td>31</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>hsdS gal (λΔIns857 ind1 Sam7 minF lacU5-T7 gene1)</td>
<td>This study</td>
</tr>
<tr>
<td>Ec181</td>
<td>BL21(DE3) carrying pNQ180</td>
<td>31</td>
</tr>
<tr>
<td><strong>P. syringae pv. syringae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B301D</td>
<td>Wild type from pear</td>
<td>5</td>
</tr>
<tr>
<td>B301D-31</td>
<td>syrP-cat derivative of B301D; Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>B301D-R</td>
<td>Spontaneous Rif' derivative of B301D</td>
<td>38</td>
</tr>
<tr>
<td>B301DR-6</td>
<td>syrP-cat derivative of B301D-R; Rif' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>BR105</td>
<td>syrD::Tn3HoHo1 derivative of B301D-R; Pp' Rif'</td>
<td>29</td>
</tr>
<tr>
<td>BR105-31</td>
<td>syrP-cat derivative of BR105; Pp' Rif' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pYM1</td>
<td>pGS72 carrying a 16-kb <em>HindIII</em> fragment from strain B301D; Tc'</td>
<td>23</td>
</tr>
<tr>
<td>pYM101</td>
<td>pUC19 carrying the 16-kb <em>HindIII</em> DNA fragment from pYM1 containing the <em>syrB</em>, <em>syrC</em>, <em>syrD</em>, and <em>syrP</em> gene cluster; Ap'</td>
<td>24</td>
</tr>
<tr>
<td>pJZ514</td>
<td>pUC18 carrying the 5.25-kb <em>EcoRI</em> fragment from pYM101 containing the complete <em>syrB</em> and <em>syrP</em> genes, 177 bp of the 5' end of <em>syrC</em>, and 576 bp of the 5' end of <em>syrD</em>; Ap'</td>
<td>This study</td>
</tr>
<tr>
<td>pJZ514CM</td>
<td>pJZ514 carrying the 1.58-kb <em>BamHI</em> fragment from pUC18CMR containing the <em>cat</em> gene inserted in the <em>syrP</em> gene; Ap' Cm'</td>
<td>This study</td>
</tr>
<tr>
<td>pUC18</td>
<td>High-copy-number cloning vector; Ap'</td>
<td>31</td>
</tr>
<tr>
<td>pUC18CMR</td>
<td>pUC18 containing the 1.58-kb DNA fragment from pBR325 containing the <em>cat</em> gene; Cm'</td>
<td>32</td>
</tr>
<tr>
<td>pET30a(+)</td>
<td>Protein fusion expression vector carrying an IPTG-inducible T7 promoter and the DNA sequence encoding the S·Tag peptide upstream of the multiple cloning site; Km'</td>
<td>Novagen</td>
</tr>
<tr>
<td>pNQ180</td>
<td>pET30a(+) carrying the 1.2-kb <em>EcoRI</em>-<em>EcoRI</em> DNA fragment from pYM1, which contains the 3' end of <em>syrP</em> (576 bp) and 5' end of <em>syrD</em> (576 bp); Km'</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Abbreviations used: Ap', Cm', Km', Pp', Rif', and Tc' correspond to resistance to ampicillin, chloramphenicol, kanamycin, piperacillin, rifampin, and tetracycline, respectively.
analysis was conducted by comparisons of the syrP gene with a codon usage table (40) constructed from P. syringae pv. syringae gene sequences available in the database.

Site-directed in situ mutagenesis. The syrP gene from pYM101 was subcloned into a 5.25-kb EcoRI fragment of pUC18 to generate plasmid pJZ134. The 1.5-kb BamHI fragment of pUC18CMR (32) containing the cat gene for chloramphenicol resistance was subcloned by blunt-end ligation into the unique EcoRI restriction site in syrP in the pJZ514 clone. After ligation, the DNA was extracted once with phenol and once with chloroform-isomyl alcohol (24:1), precipitated with 2.5 volumes of absolute ethanol, and redissolved in 10 μl of sterile distilled water. Electroporation was used to introduce the DNA (2 to 3 μl) into DH5a cells (20 μl); a Cell Porator/E. coli colt Pulsar ( Gibco BRL Life Technology, Inc., Grand Island, N.Y.) was adjusted to a medium voltage setting. Competent DH5a cells were prepared for electroporation essentially according to the method accompanying the electroporator (Gibco BRL). Transformant colonies, selected on LB containing both ampicillin and chloramphenicol, were screened by restriction analysis for the presence of pJZ514 derivatives containing the cat gene (i.e., pJZ14CM).

Introduction of the mutated syrP gene into P. syringae pv. syringae by marker exchange mutagenesis. pJZ314CM was used to transform competent cells of P. syringae pv. syringae strains (i.e., B301D, B301D-R, and BR105) prepared and electroporated as described above for E. coli. After electroporation, the bacteria were grown at 25°C for 2 to 4 h and then pelleted by centrifugation (5,000 rpm, 5 min; Sorvall SA 600 rotor [DuPont Instruments, Woodland Hills, Calif.]). The pelleted bacterial concentrate in 100 μl of KB broth was spread plated on KB selective medium containing chloramphenicol. To exclude mutants that result from a single cross-over event, 100 colonies were transferred onto KB agar plates containing either piperacillin or chloramphenicol. Those fluorescent colonies which grew in the presence of chloramphenicol and not piperacillin were selected as potential syr-cat mutants.

Complementation of syrP mutant B301D-31. The broad-host plasmid pYM1 (23), which contains the intact syrP gene, was introduced by electroporation into syrP mutant B301D-31. The transformed syrP mutant was tested for production of syringomycin in PDB (7).

Analysis of syrP mutants for syringomycin production. The syrP mutants were tested for syringomycin production by standard bioassays using the fungus Geosmithyna candidus (7, 28). Syringomycin was extracted from liquid media and tested for biological activity as described previously (7). The inhibition of G. candidus by P. syringae pv. syringae in bioassays is due solely to syringomycin and is a reliable measure of syringomycin production.

The effect of phosphate concentration on syringomycin production by syrP mutants was determined by using SRM agar medium adjusted with potassium phosphate (pH 7.0) to a final P0.7 concentration between 0.1 and 52.1 mM. The effect of plant signal molecules on induction of syringomycin production by syrP mutants was determined by addition of atrazine (100 μM) and ∆-fructose (0.1%) to SRM agar medium (i.e., SRMAF) (24). Bacteria were grown overnight in SRM liquid medium and then adjusted to 2 × 10^8 CFU/ml before spotting (0.5 μl) in the center of the petri dish containing SRM agar medium adjusted to a specific phosphate concentration or modified by addition of plant signal molecules. Checks consisted of standard SRM agar medium. Three plates were prepared for each strain. Plates were incubated at 25°C for 4 days and then bioassayed for toxin production. Zones of inhibition of G. candidus were measured by the micro-droplet method as described for the parental (22). All assays for syringomycin production were repeated on three separate occasions.

Pathogenicity tests of syrP mutants in cherry fruits. The virulence of syrP mutants was tested in immature sweet cherry fruits essentially as described by Quigley et al. (29). Each fruit was inoculated at three sites with a 5-μl droplet containing ~5 × 10^7 CFU of bacteria. Inoculated fruits were incubated for 3 days under high humidity at 20°C. The diameters of the necrotic lesions formed at each inoculation site were used to evaluate virulence. Parental strain B301D and syrP mutant BR105 were used as control strains. The populations in cherry fruits of syrP mutant B301D-31 and parental strain B301D were determined after 3 days of incubation as described by Mo and Gross (23). Each treatment was repeated three times, and three fruits were used per treatment.

Expression of syrP in E. coli. The EcoRv-EcoRf fragment of pYMY101 (Fig. 1) carrying the 3′ region of syrP (576 bp) and the 5′ end of syrP (576 bp) was subcloned into pET30a (Novagen, Madison, Wisc.), resulting in plasmid pNQ180. The following procedure was used to overexpress the 3′ end of the syrP gene on plasmid pNQ180 in E. coli strain BL21(DE3) (Novagen). Strain Ec181 (i.e. strain BL21(DE3) carrying pET30a) was grown in 20 ml of LB broth in a 250-ml flask with shaking. Expression of the gene fusion was induced at mid-log phase (~5 × 10^7 CFU/ml) by addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 1 mM. Samples (1 ml) were removed at 0, 0.5, 1.0, 2.0, and 4.0 h. Cells were pelleted and resuspended in approximately 200 μl of potassium phosphate buffer (20 mM, pH 7.0) and stored at ~80°C. The actual volumes used to resuspend the cells were adjusted so that each suspension had approximately the same cell density determined on the basis of measurement of the optical density at 600 nm of the expression culture at each sampling time.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis. The S · Tag-syrP gene fusion protein was resolved in sodium dodecyl sulfate (12%) polyacrylamide (linear) gels using methods described previously (40). All samples were boiled for 5 min in 1 × loading buffer (18) before being loaded onto the gels. For S · Tag Western blotting, the gels were electroblotted onto Optiprin membranes (Schleicher & Schuell, Inc., Keene, N.H.). The S · Tag-syrP fusion protein was detected with alkaline phosphatase-conjugated S protein as directed in the S · Tag Western blotting kit protocol (Novagen).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been deposited in the GenBank database under accession no. U88574.

RESULTS

Sequence and analysis of the syrP gene. The nucleotide sequence of a 1,473-bp DNA region located between the syrB and syrD genes (Fig. 1 and 2) was determined. ORF analysis revealed a 1,059-bp ORF (i.e., syrP) that was transcribed in the same direction as the syrD gene and terminated 80 bp upstream of the syrD ORF. The G+C content (61.9%) and the codon usage frequency of the syrP gene are characteristic of P. syringae genes (29). As described for the syrB, syrC, and syrD genes (29, 40), a high frequency of methionine codons was found in syrP. A conserved ribosomal-binding site was identified at 8 to 11 bp upstream of the start codon of the syrP ORF. No rho-dependent transcriptional terminator was observed at the 3′ end of the syrP gene. An unique EcoRV restriction site was identified, which is consistent with the physical map of this DNA region (Fig. 2). To confirm the syrP ORF, an EcoRV-EcoRI DNA fragment (~1.2 kb) that contains the syrP gene (but lacking 483 bp at its 5′ terminus) and the 5′-terminal portion of the syrD gene (576 bp) was subcloned into expression vector pET30a. The in-frame fusion between the 5′ end of the S · Tag ORF (138 bp) on the vector and the syrP gene was confirmed by sequencing (data not shown). The SyrP protein was overexpressed in E. coli Ec181 and analyzed by Western blot analysis (Fig. 3). The overexpressed fusion protein was readily observed following induction as a 24-kDa band (Fig. 3). The size of the band corresponded to that predicted by the S · Tag-SyrP fusion sequence (24.7 kDa).

The SyrP protein shares similarities with histidine kinases of two-component systems. The syrP gene is predicted to encode a protein product, SyrP, of 353 amino acids and with a molecular mass of 39.6 kDa. Searches of databases revealed a number of histidine kinases involved in signal transduction pathways that exhibit similarity to the SyrP protein. SyrP was most similar to the CheA (50.2%), NarQ (53.3%), and UhpB (50.4%) proteins involved, respectively, in chemotaxis, nitrate assimilation, and phosphate assimilation in E. coli (14, 16, 30). In addition, SyrP was similar to KinA (49.3%), a sensor protein of Bacillus subtilis which participates in the initiation of sporulation (19, 26). The overall similarity among known histidine kinases ranged from about 40 to 53%. The amino acid sequence identity of SyrP was highest for CheA (23.1%) and NarO (22.9%) and was consistent with the overall level of identity among a selection of known histidine kinases, which ranges from 17 to 31%.

Because SyrP (353 amino acids) is much smaller than CheA (654 amino acids), only portions of the conserved histidine kinase domain of CheA were aligned with SyrP. In particular, SyrP corresponded to the phosphotransfer and phosphoacceptor-binding domains P1 and P2, respectively, of CheA (amino acids 1 to 233) (Fig. 4) (22). SyrP lacked the ATP-binding and other functional domains reported for CheA. The Syr region that aligns with the phosphotransfer domain of CheA (Fig. 4) contained a position at 101 which is flanked by a weak consensus sequence (Table 2) of the unorthodox sensory kinase subfamily (12, 27). A Kyte-Doolittle hydropathy profile (17) of SyrP showed no significant hydrophobic transmembrane regions at the N terminus of SyrP (data not shown), and thus that protein sequence resembles the N-terminal portions.
of cytoplasmic histidine kinases such as CheA, Ypd1, and KinA (9, 22, 27, 33).

Site-directed insertional mutagenesis of the \textit{syrP} gene and its effect on syringomycin production. The \textit{syrP} gene was mutated at the unique \textit{Eco} RV restriction site by insertion of the \textit{cat} gene, which encodes chloramphenicol resistance. The resultant plasmid, pJZ514CM, was used to create \textit{syrP}-\textit{cat} mutants of \textit{P. syringae} strains by marker exchange (Table 1). Insertional inactivation of \textit{syrP} at the \textit{Eco} RV site was confirmed by mapping the location of the \textit{cat} gene in pJZ514CM. The \textit{syrP} mutant B301D-31 differed markedly from parental strain B301D in the ability to produce syringomycin. The most striking phenotype of the \textit{syrP} mutant was that it did not produce syringomycin in the liquid medium PDB, whereas the parental strain produced over 4,000 U/ml in this medium. The \textit{syrP} mutant B301D-31 was complemented in trans by pYM1 based on restoration of syringomycin production to wild-type levels in liquid PDB cultures.

FIG. 2. Nucleotide sequence of a 1,473-bp chromosomal region of \textit{P. syringae} pv. syringae strain B301D. This region is located between the \textit{syrB} and \textit{syrD} genes and contains a \textit{syrP} ORF which is transcribed in the same direction as the \textit{syrD} gene (Fig. 1). The \textit{syrP} ORF is indicated by horizontal arrows. The derived amino acid sequence of \textit{syrP} is presented in the standard one-letter code below the first base of each corresponding codon. The predicted ribosomal-binding site is in boldface type. The location of the \textit{syrB} and \textit{syrD} genes and their direction of transcription are indicated by labeled arrows. The \textit{Eco} RV restriction site that was used for site-directed insertional mutagenesis is in underlined boldface type. The consensus sequence that contains a putative phosphorylation site at His-101 is underlined.

FIG. 3. S \textit{\cdot} Tag Western blot of total cellular proteins from Ec181, which overexpresses a truncated SyrP protein fused to the S \textit{\cdot} Tag leader peptide of the expression vector. The overexpressed SyrP fusion protein is 24 kDa. Lanes 1 to 5 represent protein preparations from cells harvested at 0, 0.5, 1.0, 2.0, and 4.0 h, respectively, after induction by IPTG. Lane 6 contains Novagen Perfect Protein markers. Molecular masses (in kilodaltons) are shown to the right of the blot.

FIG. 4. Alignment of the deduced amino acid sequence of SyrP (black box) with that of CheA, a histidine kinase of \textit{E. coli} involved in chemotaxis (22). The degrees of similarity and identity between the two proteins are described in the text. The labeled boxes indicate the functional domains of CheA. The protein region of CheA that aligns with the entire SyrP sequence (353 amino acids) spans amino acids 1 to 233 and contains domains for phosphotransfer and phosphoacceptor binding. The phosphorylation site of CheA at amino acid H-48 in the phosphotransfer domain is indicated.
tion on solid media, however, B301D-31 produced substantially more syringomycin on PDA and SRM agar media than the parental strain B301D. For example, strain B301D-31 formed 8-mm zones of inhibition of *G. candidum*, compared to only 2-mm zones for B301D on PDA. Similar results were observed on SRM agar developed specifically for the production of syringomycin under defined culture conditions; B301D-31 formed 6.2-mm zones of antifungal activity, compared to 1.7-mm zones for B301D (Fig. 5). Furthermore, B301D-31 differed from the parental strain by production of a small zone (1.5 mm) of antifungal activity on NBY agar. Neither strain produced zones of antifungal activity on KB and LB agar media.

**TABLE 2. Alignment of the amino acid sequences flanking the putative histidine phosphorylation site His-101 of SyrP with known or predicted phosphorylation sites of several histidine kinases**

<table>
<thead>
<tr>
<th>Proteina</th>
<th>Organism</th>
<th>Sequenceb</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>SyrP</td>
<td><em>Pseudomonas syringae</em> pv. <em>syringae</em></td>
<td>FAEALS [DPLHGTYG]DLPKKEGGRNYRS</td>
<td>93–120</td>
</tr>
<tr>
<td>BarA</td>
<td><em>Escherichia coli</em></td>
<td>NEPEGLVDLITHKLGISGYSGVRPRWNLCL</td>
<td>822–849</td>
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<tr>
<td>CheA</td>
<td><em>Escherichia coli</em></td>
<td>QLNAIFRAAHSIKGAGTTGFSLVQETT</td>
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<tr>
<td>CheA</td>
<td><em>Thermotoga maritima</em></td>
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<td>36–63</td>
</tr>
<tr>
<td>ArcB</td>
<td><em>Escherichia coli</em></td>
<td>DKKGIVERDKINGAAGSVGHLHLQQGL</td>
<td>708–715</td>
</tr>
<tr>
<td>Ypd1</td>
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<td>NLELOEMLHPKGGSSAALGQLR1AWVC</td>
<td>55–82</td>
</tr>
<tr>
<td>BvgS</td>
<td><em>Bordetella pertussis</em></td>
<td>DWKVDQDMHRLRAGERVVDUKAMIDTV</td>
<td>1163–1190</td>
</tr>
</tbody>
</table>

a For protein sequences, see references as follows: BarA (12), CheA from *E. coli* (16), CheA from *T. maritima* (34), ArcB (13), Ypd1 (27), and BvgS (2).

b Boldface letters indicate those amino acids in the SyrP sequence which are identical to amino acids in one or more of the sequences being compared.

**Effects of phosphate concentration and plant signal molecules on syringomycin production by syrP mutants.** Because the *syrP* mutant B301D-31 produced syringomycin on NBY agar, which contains high levels of phosphate, the effect of phosphate concentration (0 to 51.2 mM) on toxin production by B301D-31 was determined by using SRM agar medium. Syringomycin production by the *syrP* mutant was relatively insensitive to high inorganic phosphate concentrations compared to parental strain B301D (Fig. 6). For example, strain B301D-31 showed little change in toxin production with increasing phosphate concentration and produced a 7-mm zone of antifungal activity at the highest phosphate concentration tested (51.2 mM). In contrast, syringomycin production by parental strain B301D was strongly inhibited by phosphate concentrations of 0.8 mM and higher, and no zone of antifungal activity was observed at a phosphate concentration of 51.2 mM.

The production of syringomycin by both B301D and B301D-31 was significantly higher in the presence of plant signal molecules, arbutin and D-fructose, on SRM agar medium (i.e., SRM<sub>AF</sub>) (Fig. 5). On SRM<sub>AF</sub> agar medium, strain B301D produced zones of antifungal activity whose diameters were almost three times those produced in the absence of the plant signal molecules. Syringomycin production by the *syrP* mutant was relatively insensitive to high inorganic phosphate concentrations compared to parental strain B301D (Fig. 6). For example, strain B301D-31 showed little change in toxin production with increasing phosphate concentration and produced a 7-mm zone of antifungal activity at the highest phosphate concentration tested (51.2 mM). In contrast, syringomycin production by parental strain B301D was strongly inhibited by phosphate concentrations of 0.8 mM and higher, and no zone of antifungal activity was observed at a phosphate concentration of 51.2 mM.
SyrP was most similar to the N-terminal region of CheA, a component of two-component regulatory systems in bacteria and yeasts. Sequence analysis indicated that SyrP is in-pleiotropic phenotype affecting both syringomycin production and virulence. Sequence analysis indicated that SyrP is in-

 mutant, B301D-31, likewise was induced by the plant signal molecules, based on the formation of large antifungal zones with an average size of 8.5 mm. A double mutant, BR105-31, carrying the syrP mutation together with a syrD mutation also was evaluated for syringomycin production in the presence of plant signal molecules. Whereas this double mutant produced negligible inhibition zones in the absence of plant signal molecules, in the presence of these signals, antifungal zones measuring approximately 2.5 mm were recorded.

The syrP gene mutants are reduced in virulence. Pathogenicity tests of syrP mutant strain B301D-31 in immature cherry fruits showed a significant reduction in virulence of about 60% compared to parental strain B301D (Fig. 7). After 3 days of incubation, the syrP mutant formed only small necrotic lesions with an average diameter of 3 mm, which were comparable in size to lesions formed by the syrD mutant, BR105 (29). BR105 was about 60% less virulent than its parental strain, the rifampin-resistant B301D-R. The virulence of the syrP-syrD double mutant, BR105-31, was not significantly different from that of either the syrP or syrD mutants. The average population of B301D-31 after 3 days of incubation was $1.4 \times 10^9$ CFU/fruit, which was about 10-fold lower than the population of parental strain B301D ($\sim 1.5 \times 10^9$ CFU/fruit).

**DISCUSSION**

The syr gene cluster of *P. syringae* pv. syringae encodes syringomycin synthetas that participate in a thiotemplate multienzymatic mechanism of peptide synthesis (40). The syrP gene represents the first regulatory gene identified within the syr cluster. Mutation in the syrP gene results in a complex pleiotropic phenotype affecting both syringomycin production and virulence. Sequence analysis indicated that SyrP is involved in phosphotransfer in regulating syringomycin production based on similarity to related regions of histidine kinases of two-component regulatory systems in bacteria and yeasts. SyrP was most similar to the N-terminal region of CheA, a cytoplasmic protein of *E. coli* containing five characteristic functional domains (22) (Fig. 4). The predicted SyrP protein overlapped with both the phosphotransfer and phosphoacceptor-binding domains of CheA (22, 33); the N-terminal phosphotransfer domain of CheA is conserved among all histidine kinases (3, 22, 27). Thus, the syrP gene may encode a member of a multicomponent signalling pathway which controls syringomycin production.

Gene expression in bacteria is frequently controlled by environmental signals mediated by signal transduction pathways, as exemplified by those involved in antibiotic synthesis and sporulation in *B. subtilis* (2, 3, 9), and by those involved in phosphate metabolism (3, 37), nitrate metabolism (4), and chemotaxis in *E. coli* (35). The common feature in phosphorelay signal transduction is the occurrence of one or more transmitter proteins which relay a signal between sensors and response regulators, characteristic of two-component regulatory systems (1, 9). Environmental signals are perceived by a sensor protein which in turn becomes phosphorylated. The phosphate is transferred to one or more response regulators directly or through intermediate sensory transmitters such as KinA of *B. subtilis* (9, 19) and CheA of *E. coli* (3, 33, 35). Although the SyrP protein exhibits similarity to the phosphotransfer domains of KinA and CheA, SyrP lacks the domains associated with autophosphorylation activity. Thus, it is hypothesized that SyrP functions in a phosphorelay system as an intermediate between a sensor protein and a response regulator. The likely site of phosphorylation of SyrP is His-101 which is flanked by a sequence (Table 2) resembling the weak consensus, described by Ishige et al. (12), of unorthodox histidine kinases including CheA and Ypd1 (1, 27). In particular, the Ypd1p protein of *S. cerevisiae* is a small cytoplasmic protein, similar in size to SyrP, with a histidine phosphorylation site that receives a phosphate from an aspartate in the Snl1p receiver domain and then transfers the phosphate to the aspartate in Ssk1p (27). Posas et al. (27) demonstrated that the Ypd1p His-64 was phosphorylated in the presence of the plant signal molecules in the plant-pathogen interaction. Therefore, it is suggested that SyrP serves a similar function in a phosphorelay mechanism. The other regulatory syr genes participating with syrP in controlling syringomycin production remain to be identified, but they do not occur adjacent to syrP.

The importance of the syrP gene in the plant-pathogen interaction is emphasized by a 60% reduction in virulence of syrP mutants in pathogenicity tests using immature sweet cherry fruits. A comparable reduction in virulence is observed for syrD mutants which produce little or no toxin in vitro (29). In contrast to syrP, the syrD gene appears to encode an ABC transporter protein involved in toxin secretion and does not directly regulate toxin production (29). Thus, a strong effect of a syrP mutation on virulence may reflect a failure to produce toxin in infected plant tissues. This would be consistent with the failure of the syrP mutant B301D-31 to produce syringomycin in PDB and SRM liquid media. Accordingly, these results indicate that the SyrP protein primarily functions as a positive regulator of toxin production with a corresponding effect on virulence.

SyrP also appears to function as a negative regulator of syringomycin production under certain environmental conditions. Although syringomycin production is normally suppressed by high concentrations of phosphate (7), toxin production by syrP mutant B301D-31 was not suppressed at high ambient phosphate concentrations in agar media. This was especially striking on NBY agar medium, which is rich in phosphate and suppressive to syringomycin production by all wild-type strains of *P. syringae* pv. syringae (7). In addition, hyper-
production of syringomycin occurred for B301D-31 on PDA and SRM agar media, which represent media conducive to toxin production by wild-type strains of *P. syringae* pv. syringae. The dichotomy in phenotypic effects of *syrP* mutation on syringomycin production in liquid versus semisolid agar media is unclear, but it does indicate a dual regulatory function for *SyrP* that depends on the environmental conditions. In *E. coli*, the inorganic phosphate (P) concentration in the environment is monitored through a two-component regulatory system that requires the participation of a sensor (PhoR), a response regulator (PhoB), a P$_7$-specific transporter (Pst), and an accessory protein (PhoU) (37). During *P*$_7$ limitation, PhoR activates genes of the phosphate regulon by phosphorylating PhoB, which in turn initiates transcription of target genes. When *P*$_7$ is in excess, PhoR, Pst, and PhoU together repress the phosphate regulon by dephosphorylating PhoB. Therefore, the phosphate in the environment may be sensed in *P. syringae* pv. syringae by a PhoR-like protein which interacts with SyrP either directly or through an intermediate signal transmitter. Because the SyrP protein is predicted to contain only the histidine phosphorylation site, a protein with phosphotransferase activity will be required to transfer the phosphate from SyrP to a succeeding protein in a phosphorelay system.

The regulatory mechanism for syringomycin is complex, with both nutritional and plant signal molecules modulating production by *P. syringae* pv. syringae. Nevertheless, the *syrP* gene does not appear to directly affect signal transduction mediated by plant signal molecules. Although *syrP* mutants produce more syringomycin in the presence of plant signal molecules on SRM agar medium, the relative levels of toxin production by the *syrP* mutants mirror those observed for parental strain B301D (Fig. 5). Thus, SyrP appears to indirectly affect the efficiency of induction by the plant signal transduction pathway. It also was observed that syringomycin production by B301D was substantially higher in the presence of plant signal molecules in tests conducted on SRM agar media (Fig. 5). In earlier studies (24, 28), strain B301D was found to produce equivalent amounts of syringomycin in SRM liquid medium regardless of whether or not the medium was supplemented with plant signal molecules. This was in sharp contrast to more than 90% of toxigenic *P. syringae* pv. syringae strains which produced larger quantities of toxin in SRM$_{AF}$ liquid medium (28). We now conclude that B301D likewise responds to plant signal molecules.

Since the discovery of the first phosphorelay system controlling sporulation in *Bacillus subtilis* (28), we now conclude that B301D likewise responds to plant signal molecules. Although it does not appear to directly affect signal transduction mediated by plant signal molecules, *syrP* may indirectly affect the virulence of *P. syringae* pv. syringae strains which have commitments in microorganisms (1, 3). An important advantage of phosphorelay systems is that they allow integration of multiple signals at intermediate steps in the regulatory network (1). In *P. syringae* pv. syringae, the expression of syringomycin production is subject to diverse environmental signals. How SyrP contributes to this important regulatory network will remain unclear until other members of the apparent phosphorelay mechanism are characterized. Currently, we are sequencing the entire *syr* gene cluster, and this 30-kb DNA region of the *P. syringae* pv. syringae genome may harbor other regulatory elements dedicated to toxigenesis. We predict that other members of the predicted phosphorelay mechanism exert similar regulatory effects to SyrP on the virulence of *P. syringae* pv. syringae.

**ACKNOWLEDGMENTS**

We thank Steve Thompson of the VADMS Center for assistance with the Genetics Computer Group program package. This work was supported in part by grant 92-37303-7732 from the National Research Initiative Competitive Grants Program of the U.S. Department of Agriculture, Science and Education Administration (to D.C.G.), and by grant MCB 9219200 from the National Science Foundation (to N.B.Q.).

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