MOLECULAR ANALYSIS OF SECRETION GENES LOCATED ON THE *SYR-SYP* GENOMIC ISLAND OF *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* STRAIN B301D

A Dissertation

by

HYOJEUNG KANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Plant Pathology

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Approved as to style and content by:

Dennis C. Gross (Chair of Committee)	Carlos F. Gonzalez (Member)
Daniel J. Ebbole (Member)	Deborah A. Siegele (Member)
	Dennis C. Gross (Head of Department)

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ABSTRACT

Molecular Analysis of Secretion Genes Located on the *Syr-Syp* Genomic Island of *Pseudomonas syringae* pv. *syringae* Strain B301D. (December 2004)

Hyojeung Kang,

B.S., Korea University;

M.S., Korea University

Chair of Advisory Committee: Dr. Dennis C. Gross

An RND (resistance-nodulation-cell division) transporter, called the PseC protein, was identified at the left border of the *syr-syp* genomic island of *Pseudomonas syringae* pv. *syringae* strain B301D. The PseC protein exhibited amino acid homology to a putative RND transporter of *Ralstonia solanacearum* with identities of 61% (i.e., PseC). The *pseC* mutant strain showed a larger reduction in syringopeptin secretion (67%) than syringomycin secretion (41%). A β-glucuronidase assay with a *pseA::uidA* reporter construct indicated that the GacS/A two-component system controls expression of the *pseA* gene. Expression of the *sypA* gene by mutant strain B301D-HK4 corresponded to approximately 13% of that by parental strain B301D, whereas the *syrB1* gene expression by mutant strain B301D-HK4 was nearly 61%. Mutant strain B301D-HK4 was reduced in virulence by about 58% as compared to parental strain B301D. A drug-supersensitive *acrB* mutant of *E. coli* showed increased resistance to acriflavine and tetracycline upon heterologous expression of the *pseA*, *pseB*, and *pseC* genes. Thus,

the PseC protein, an RND transporter, has an important role in secretion of syringomycin and syringopeptin.

An ATP-binding cassette (ABC) transporter, called the PseF protein, was identified at the left border of the *syr-syp* genomic island. The PseF protein exhibited amino acid homology to a putative ABC transporter of *E. coli* W3104 with identities of 57.6% (i.e., PseF to MacB). The *pseF* mutant strain showed significant reduction in secretion of syringomycin (74%) and syringopeptin (71%). Expression of the *sypA* gene by mutant strain B301D-HK7 was approximately 6.9% as compared to that of parental strain B301D, while the *syrB1* gene expression by mutant strain B301D-HK7 was nearly 14.6%. Mutant strain B301D-HK7 was less virulent by approximately 67% than parental strain B301D. Expression of the *pseF* gene was induced approximately six times by strain B301D grown on SRM_{AF}, as compared to that of strain B301D grown on SRM. During infection of bean plants by *P. syringae* pv. *syringae* strain B728a, expression of the *pseF* gene increased 3 days after inoculation. Thus, the PseF protein, an ABC transporter, responsible for secretion of syringomycin and syringopeptin is required for full virulence.

DEDICATION

To my wife, Hyosun Cho, and my parents, Mr. Jaehong Kang and Ms. Kisun Kim.

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CHAPTER I

GENERAL INTRODUCTION

Pseudomonas syringae is known to induce a variety of symptoms on monocot and dicot plants, including blights, leaf spots, and galls (10). A distinctive characteristic of *P. syringae* pv. *syringae* is the ability to secrete two different classes of lipopeptide toxins, named syringomycins and syringopeptins (8). The major form of syringomycin, SRE, is a cyclic nonapeptide attached to a 3-hydroxy fatty acid tail. In contrast, the major form of syringopeptin, $SP_{22}A$, contains a cyclic peptide with 22 amino acids attached to a 3-hydroxy fatty acid tail.

Based on the ability of lipopeptide phytotoxins to form pores in artificial planar lipid bilayers at low concentrations, the mode of action for syringomycin and syringopeptin is to insert into the plasma membrane of host cells, form pores, disrupt the electrical potential across the host cell membrane, and ultimately cause lysis (37).

Genes responsible for biosynthesis, regulation, and secretion of the phytotoxins are contained in the *syr-syp* genomic island (31). The genomic island is located in a genomic island spanning approximately a 155-kb DNA region of *P. syringae* pv. *syringae* strain B301D (31). A *syrB1* (syringomycin synthetase gene) mutation and a *sypA* (syringopeptin synthetase gene) mutation result in approximately 40% and 60% reduction, respectively, in virulence compared to the parental strain B301D (81). The

This dissertation follows the style and format of the Journal of Bacteriology.

GacS/A two-component system (GacS for a sensor and GacA for a response regulator) is known to have an important role in syringomycin production, lesion formation, and ecological fitness in *P. syringae* pv. *syringae* (34). A *gacS* mutant is complemented for syringomycin production with expression of the *salA* (syringomycin and lesion formation) gene, which is present in single copy (41, 50). This result suggests that syringomycin production is regulated by the GacS/A system through the SalA protein (41). Mutations in the *salA* gene cause a larger reduction in syringomycin production than mutations in the *gacS* or *gacA* genes (41). The *salA* mutation also affects expression of the *syrF* gene, indicating that the SalA protein controls expression of the *syrF* gene (50). The SyrF protein is involved in regulating syringomycin production (50).

In addition to phytotoxin biosynthesis and its regulation, secretion of lipopeptide phytotoxins is an important activity in the toxigenesis of *P. syringae* pv. *syringae* strain B301D (71). The *syrD* gene is found at the left border of the *syr* gene cluster. The SyrD protein is predicted to be a cytoplasmic membrane protein of an ATP-binding cassette (ABC) transporter system, and contains an ABC transporter family signature and an ATP/GTP-binding site motif A (P-loop) (79). Genetic studies were performed to define the function of the *syrD* gene and its contribution to virulence (71). Syringomycin secretion by strain BR105 (a *syrD* mutant) is reduced by approximately 80% as compared to parental strain B301D. Mutant strain BR105 is approximately 70% less virulent than parental strain B301D. Previous studies (31, 50, 71, 81) present an overall mechanism for production and secretion of syringomycin and syringopeptin

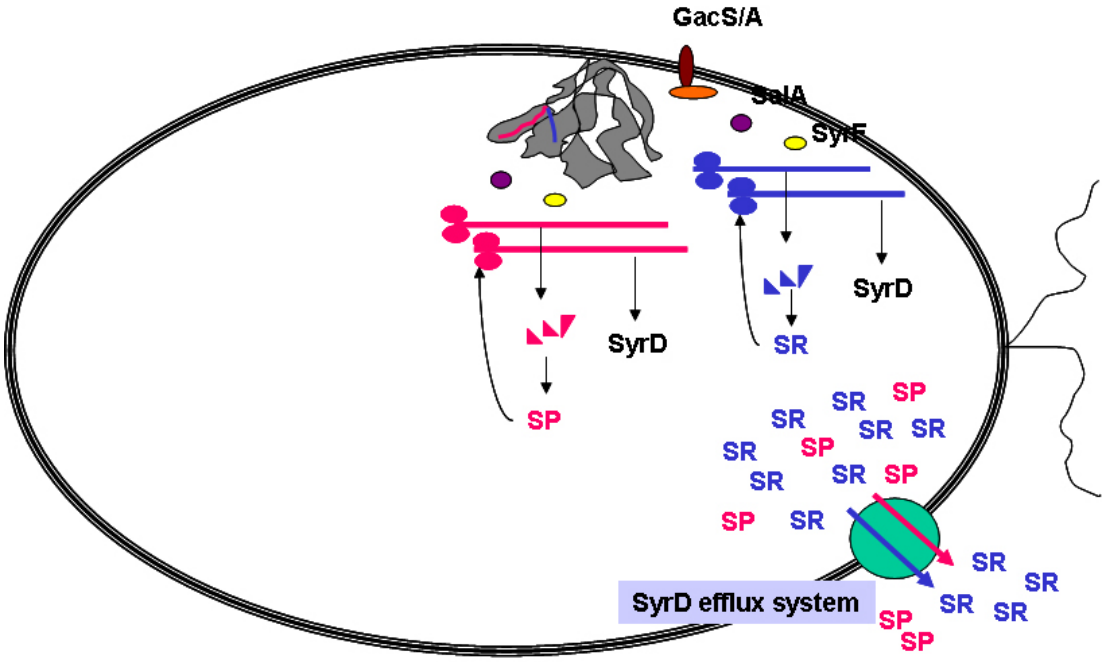

FIG. 1. Proposed overall mechanism for secretion of syringomycin and syringopeptin prior to these studies. The GacS/A two-component system captures an environmental signal and subsequently triggers signal transduction for production of syringomycin and syringopeptin. The signal may be delivered to the SalA and SyrF transcriptional regulators, which induce expression of syringomycin and syringopeptin synthetase genes. Resultant synthetases catalyze syringomycin (SR) and syringopeptin (SP) synthesis. The lipopeptide phytotoxins induce expression of the efflux genes (*syrD* efflux genes). The efflux systems are associated with the cytoplasmic membrane and are involved in the export of lipopeptide phytotoxins. On the basis of functional studies with mutants of the efflux genes, the SyrD efflux systems is proposed to pump out syringomycin and syringopeptin without preference for either substrate.

(Fig. 1). *P. syringae* pv. *syringae* B301D captures an environmental signal through the GacS/A two-component system, which subsequently triggers signal transduction for production of syringomycin and syringopeptin. The signal may be delivered to the SalA and SyrF transcriptional regulators, which induce expression of syringomycin and syringopeptin synthetase genes. The resultant synthetases catalyze syringomycin (SR) and syringopeptin (SP) synthesis. The lipopeptide phytotoxins are exported by the SyrD efflux systems encoded by the *syrD* gene. The SyrD efflux system is the only characterized transporter system that is responsible for secretion of syringomycin and syringopeptin prior to this study.

A distinctive characteristic of Type I secretion systems is to transport their substrates across cellular membranes by an one-step transport process (74). Although the SyrD protein is the only recently characterized Type I secretion system to secrete syringomycin and syringopeptin in *P. syringae* pv. *syringae*, numerous efflux proteins utilizing Type I secretion system are found widely in Gram-negative bacteria, and they are subclassified into the transporter families such as the ABC transporter family and the resistance-nodulation-cell division (RND) transporter family (69).

One of the best-studied RND transporter systems is the *E. coli* AcrAB-TolC system (64). The AcrAB-TolC efflux system plays an important role in resistance of *E. coli* by exporting various compounds such as bile salts and antibiotics. TolC is an outer membrane protein that forms a pore to diffuse antibiotics across the outer membrane (97). The crystal structure of TolC reveals a mainly α-helical trimeric protein that is composed of a β-barrel (spanning the outer membrane) and α-helical barrel (periplasm)

(44). AcrB is an RND transporter predicted to have 12 transmembrane α-helices and two large hydrophilic loops (28, 57). The RND transporter (AcrB) is predicted to be a drug-proton antiport across the inner membrane, and it is energized by the proton motive force for the export of drugs (58). The crystal structure of the RND transporter (AcrB) is organized as a homotrimer and it spans the inner membrane as 12 transmembrane α-helices and the periplasm as two large hydrophilic loops (β-strand and α-helix) (57). AcrA, which belongs to the membrane fusion protein family, is a periplasmic protein anchored to the inner membrane. The membrane fusion protein is predicted to promote the association of an RND transporter (AcrB) and an outer membrane protein (TolC) (102).

The ABC transporter family is one of the largest transport families and is found in almost all the phyla of the three kingdoms, including Gram-negative bacteria (80). Powered by the energy of ATP hydrolysis, several members of this ABC transport family are characterized to be involved in the export of secondary metabolites, such as toxins (76, 79, 93). One of the best-studied ABC transporter systems is the *E. coli* HlyBD-TolC efflux system (90). Pathogenic strains of *E. coli* secrete hemolysin (HlyA), a 107 kDa protein, when they infect the urinary tract and extraintestinal regions of mammals such as pigs, horses, and humans (7). The mode of action of HlyA is to bind to cell surface receptors, such as β_2-integrin in leukocytes or glycophorin in red blood cells, to be inserted into the cell membrane, and to ultimately cause lysis (16). The HlyBD-TolC efflux system secretes hemolysin A across both membranes of *E. coli* (90). The efflux system consists of an outer membrane protein (TolC) forming an outer membrane

spanning channel, a cytoplasmic membrane protein of the ABC transporter system (HlyD) containing a nucleotide binding domain (Walker A and B, ABC signature), and a membrane fusion protein (HlyB) that associates with the HlyD and TolC proteins, (90).

During sequencing of the *syr-syp* genomic island, eight ORFs (open reading frame) are identified that showed significant similarity to type I secretion systems (31). The genomic island is predicted to encode three cytoplasmic membrane efflux proteins (SyrD, PseC, and PseF), three membrane fusion proteins (PseB, PseE and an MtrC homolog), and two outer membrane efflux proteins (PseA and an OprM homolog). Based on the analysis of amino acid sequence similarity and motifs of the cytoplasmic membrane efflux proteins, three different secretion systems are observed, namely one RND-type transporter system (58) and two ABC-type transporter systems (72). In contrast to the SyrD protein, the PseC (an RND-type transporter) protein and the PseF (an ABC transporter) protein are located at the left border of the *syp* gene cluster (31, 83). The existence of the PseC and PseF proteins in the *syr-syp* genomic island led to speculation that the SyrD efflux system is not the sole transporter system responsible for secretion of syringomycin and syringopeptin and the other transporter systems might cooperate with the SyrD efflux system to secrete lipopeptide phytotoxins (Fig. 1). Accordingly, strain BR105 (a *syrD* mutant) (70, 81) was observed to produce small inhibition zones to the indicator organisms (*Geotrichum* and *Bacillus*) due to production of syringomycin and syringopeptin (H. Kang and D. C. Gross, *unpublished data*), although mutations in the *syrD* gene cause a significant reduction in the secretion of lipopeptide phytotoxins. These data support the hypothesis that these are other transport systems, in addition to

the SyrD efflux system, for secretion of lipopeptide phytotoxins. Therefore, it was important to characterize the functional roles of the two additional secretion systems (the RND-type and second ABC-type transporter systems) in secretion of syringomycin and syringopeptin, which would help understand the overall mechanism for secretion of lipopeptide phytotoxins in *P. syringae* pv. *syringae* (Fig. 1).

OBJECTIVES

The goal of my research was to determine the contribution of the transporter systems to secretion of syringomycin and syringopeptin, and to virulence of *P. syringae* pv. *syringae*. The three hypotheses that were tested by these studies are as follows:

1. An RND transporter, called the PseABC efflux system, is a major syringopeptin transporter and is important for virulence.
2. An ABC transporter, called the PseEF efflux system, is one of the major syringomycin and syringopeptin transporters, together with the SyrD protein, and is an important virulence factor.
3. Expression of the bacterial efflux genes (*syrD*, *pseF*, and *pseC*) is induced during infection of *P. syringae* pv. *syringae* strain B728a in bean, a susceptible plant.

CHAPTER II

MOLECULAR ANALYSIS OF AN RND TRANSPORTER SYSTEM IDENTIFIED IN THE *SYR-SYP* GENOMIC ISLAND OF *P. SYRINGAE* PV. *SYRINGAE* B301D

INTRODUCTION

Pseudomonas syringae pv. *syringae* is a common plant bacterial pathogen in nature that causes necrosis in a wide spectrum of monocot and dicot plants (10). A distinctive characteristic of *P. syringae* pv. *syringae* is the secretion of two different classes of lipopeptide phytotoxins, called syringomycins and syringopeptins (8). The major form of syringomycin, SRE, is a cyclic nonapeptide attached to a 3-hydroxy fatty acid tail. In contrast, the major form of syringopeptin, $SP_{22}A$, contains a cyclic peptide with 22 amino acids attached to a 3-hydroxy fatty acid tail. The mode of action for the phytotoxins is to cause cellular lysis by the formation of transmembrane pores in the plasma membranes of host cells that lead to disruption of the membrane electrical potential (37). The phytotoxins are encoded by the *syr-syp* genomic island spanning approximately a 155-kb DNA region, which corresponds to over 2% of the genome of *P. syringae* pv. *syringae* strain B301D (31, 83). The *syr-syp* genomic island consists of genes required for phytotoxin biosynthesis, secretion, and regulation (8, 50, 71, 83).

Type I secretion systems are characterized by an one-step transport process and are ubiquitous among Gram-negative bacteria, including *Pseudomonas* and *Xanthomonas*

(74). For example, *P. syringae* pv. *tomato* DC3000, whose genome sequence was released recently (12), possesses 15 ATP-binding cassette (ABC) transport systems and nine resistance-nodulation-cell division (RND)-type efflux systems (12). These transport systems are predicted to be involved in sugar transport (ABC) and the export of drugs or cations (RND). In addition in *P. syringae*, the type I secretion system is known to be essential for biosynthesis and transport of secondary metabolites in Gram-negative bacteria. *P. aeruginosa* PAO produces pyoverdine, a siderophore whose secretion requires a protein homologous to an ABC transporter called PvdE (54). The *Xanthomonas oryzae* pigment, xanthomonadin, is localized to the outer membrane by a putative RND-type transporter (27). Thus, type I secretion systems are required for the export of a variety of metabolic products.

Certain families of the RND-type transporter superfamily form a functional three-component efflux system with a membrane fusion protein and an outer membrane protein (69, 74, 92). The resultant efflux system is proposed to utilize a dual entrance to pump out hydrophobic and hydrophilic substrates from the cytoplasmic membrane or the periplasm to the external environment (57). The most intensively studied RND-type efflux system is the AcrAB-TolC efflux system found in *E. coli* K-12 (64) and *Salmonella typhimurum* SH5014 (61). The AcrAB-TolC efflux system plays an important role in bacterial resistance by exporting various compounds such as acriflavine, antibiotics, and lipophilic molecules. AcrA is a periplasmic protein anchored to the inner membrane. AcrB is an RND transporter composed of 12 transmembrane α-helices and two large hydrophilic loops. TolC is an outer membrane protein that forms

a channel to allow antibiotics to diffuse across the outer membrane. However, in spite of the prevalence of RND-type efflux systems in Gram-negative bacteria (101), little is known about the RND-type efflux system involved in the secretion of lipopeptide toxins produced by Gram-negative bacteria.

The secretion of lipopeptide toxins is essential for toxigenesis by *P. syringae* pv. *syringae* (71). The *syrD* gene is located between the *syr* and *syp* genomic islands. Based on the analysis of the sequence of the SyrD protein, it is homologous to a cytoplasmic membrane protein of the ABC transporter family (79). Strain BR105 (a *syrD* mutant) is significantly reduced in secretion of syringomycin and syringopeptin (71). Mutant strain BR105 shows a greater reduction (70%) in virulence in immature sweet cherry fruits than strain BR132 (a *syrB1* mutant, 40% less) and strain B301D-208 (a *sypA* mutant, 60% less) as compared to parental strain B301D (81). The reduction in virulence is due to a decrease in secretion of syringomycin and syringopeptin. However, mutation of the *syrD* gene fails to cause a complete loss of secretion of lipopeptide phytotoxins (29), which led to speculation that the SyrD protein is not the sole transport system responsible for secretion of syringomycin and syringopeptin.

In this study, a tripartite RND-type efflux system, called the *Pseudomonas syringae* syringomycin and syringopeptin efflux system (PseABC efflux system), was identified at the left border of the *syr-syp* genomic island of *P. syringae* pv. *syringae* strain B301D by sequencing cosmid JS115. The PseABC efflux system was hypothesized to have a role in secretion of syringomycin and syringopeptin. The objective of this study was to elucidate the function of the putative RND-type efflux system and its contribution to

virulence in *P. syringae* pv. *syringae* strain B301D. I characterized the function of the PseABC efflux system, which provided evidence that disruption of the *pseC* gene caused a large reduction in syringopeptin secretion, a limited reduction in syringomycin secretion, and a substantial reduction in virulence of *P. syringae* pv. *syringae* strain B301D.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH10B (Gibco-BRL) used for DNA manipulations was cultured at 37°C in Luria-Bertani (LB) broth or on LB agar (77). *P. syringae* pv. *syringae* strains were cultured routinely in nutrient-broth yeast extract (NBY) liquid or agar media (94). PDA (potato dextrose agar) supplemented with 0.4% casamino acids and 1.5% glucose (30) was used in bioassays for production or secretion of syringomycin and syringopeptin. When required, antibiotics (Sigma) were added to media at the following final concentrations: 100 μg/ml of ampicillin (*E. coli*), 50 μg/ml of kanamycin (*E. coli* and *Pseudomonas*), 10 μg/ml of gentamicin (*E. coli*), 200 μg/ml of chloramphenicol (*E. coli* and *Pseudomonas*), and 6.25 to 25 μg/ml of tetracycline (*E. coli* and *Pseudomonas*).

DNA manipulations and sequence analysis. Routine procedures (78) were used for plasmid isolation from *E. coli*, restriction endonuclease digestion, and subcloning. An 8.4-kb *Kpn*I fragment from pJS115 containing the *pseABC* efflux genes and a 4.7-kb *Hind*III fragment from pJS091 containing the *pseA* gene were sequenced (82).

TABLE 1. Bacterial strains and plasmids used in chapter II

Strain or plasmid	Relative Characteristics[a]	Reference or source
E. coli strains		
DH10B	F⁻ *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*	Invitrogen
AG100	*ArgE3 thi-1 rpsL xyl mtl galK supE441* Δ*(gal-uvrB)* λ⁻	(64)
AG100A	Kan^r, same as AG100 but Δ*acrAB::kan*	(64)
P. syringae pv. *Syringae*		
B301D	Wild type from pear	(14)
BR132	*syrB1*::Tn3HoHo1 derivative of B301D-R; Pip^r Rif^r	(55)
B301D-208	*sypA*::Tn5 derivative of B301D; Km^r	This study
B301D-HK2	*pseA*::*nptII* derivative of B301D; Km^r	This study
B301D-HK3	*pseB*::*nptII* derivative of B301D; Km^r	This study
B301D-HK4	*pseC*::*nptII* derivative of B301D; Km^r	This study
BR132-HK4	*pseC*::*nptII* derivative of BR132; Pip^r Rif^r Km^r	This study
B301D-HK5	*gacA*::*nptII* derivative of B301D; Km^r	This study
B301D-SL7	*salA*::*nptII* derivative of B301D; Km^r	(50)
P. aeruginosa		
PAO1	Wild type	(40)
Plasmid		
pBSK(+)	Cloning vector; Ap^r	Strategene,
pBR325	Cloning vector; Cm^r Tc^r Ap^r	(68)
pSL02	pBI101 carrying the 0.85-kb *aacCI* gene from pUCGM inserted into the *EcoR*I site downstream of the *uidA* gene; Km^r Gm^r	(50)
pGEMT-Easy	Cloning vector; Ap^r	Promega
pUCP26	Cloning vector; Tc^r	(65)
pBSL15	Kanamycin resistance gene cassette; Km^r	(3)
pJS091	pBSK carrying a 4.7-kb *Hind*III fragment from strain B301D; Ap^r	(87)
pHK091	pUCP26 carrying the 4.7-kb *Hind*III fragment from pJS091, Tc^r	This study
pHK092	pUCP26 carrying the 4.7-kb *Hind*III fragment from pHK091 with the 3.2-kb *uidA-aacCI* fragment from pSL02 inserted into the *Pml*I site in-frame of *pseA* in forward orientation; Tc^r Gm^r	This study
pJS115	pBSK carrying an 8.4-kb *Kpn*I fragment from strain B301D; Ap^r	(83)
pHK115	pUCP26 carrying the 8.4-kb *Kpn*I fragment from pJS115	This study
pHK116	pHK115 carrying site-directed mutagenesis in the *pseC* gene	This study
pHK01	pGEM T-Easy carrying the 5.9 kb *Pvu*II-*Sca*I fragment from pJS115 at the *EcoR*V	This study
pHK21	pJS091 carrying the 1.2-kb *nptII* gene from pBSL15 inserted into the *Pml*I site of *pseA*	This study
pHK22	pBR325 carrying the 3.6-kb *Hind*III-*Stu*I fragment from pHK21 at the *EcoR*V	This study
pHK31	pHK01 carrying the 1.2-kb *nptII* gene of pBSL15 inserted into the *EcoR*V of *pseB*	This study
pHK32	pBR325 carrying the 5.0-kb *Pml*I fragment from pHK31 at the *EcoR*V	This study
pHK41	pHK01 carrying the 1.2-kb *nptII* gene of pBSL15 inserted into the *Age*I of *pseC*	This study
pHK42	pBR325 carrying the 6.4-kb *Apa*I-*Spe*I fragment of pHK31 at the *EcoR*V	This study
pHK51	pGEM T-Easy carrying the 2.4 kb PCR product spanning a *gacA* at the *EcoR*V	This study
pHK52	pHK51 carrying the 1.2-kb *nptII* gene inserted into the *EcoR*V site of *gacA*	This study
pHK53	pBR325 carrying a 3.5-kb *Not*I fragment of pHK52 at the *EcoR*V	This study

[a] Cm^r, Tc^r, Ap^r, Km^r, and Gm^r indicate resistance to chloramphenicol, tetracycline, ampicillin, kanamycin, and gentamicin, respectively.

Sequence data were analyzed using the Wisconsin Sequence Analysis programs of Genetic Computer Group (GCG) package Version 10.0 (17) and Lasergene expert sequence analysis software (Version 5.0; DNASTAR). GCG programs FINDPATTERNS and TERMINATOR were used to identify Shine-Dalgarno sequences and to predict rho-independent transcriptional terminators. Sequence randomization and calculation of Z scores were performed using the GAP program, which evaluates the significance of protein sequence similarity as described previously (82). Protein sequence similarity was considered to be significant and to indicate homology when the Z score value was greater than 6. Database searches for genes and proteins were performed using the BLAST servers of the National Center for Biotechnology Information (http://www.ncbi.nih.gov) and the Transporter Protein Analysis Database server (http://66.93.129.133/transporter /wb/ index2. html) (5). A motif search was performed using the Pfam server (http:// motif.ad.jp/motif-bn/Srch_Motif_Lib) (6). Hydropathy analysis was performed to predict transmembrane segments (TMS) using Protean (Lasergene) and the hydropathy analysis server (http://megaman. ucsd.edu/progs/hydro.php) (45). Multi-alignment of nucleotide or protein sequence was performed using the MegAlignprogram (Lasergene) and the MultiAlign server (http://prodes.toulouse.inra.fr/ multalin/multalin.html) (15).

Mutagenesis. The *pseA*, *pseB* and *pseC* genes were disrupted by insertion of the *nptII* gene (3). A 1.2-kb *nptII* cassette from pBSL15 was inserted into the *Pml*I site of the *pseA* gene in pJS091, into the *Eco*RV site of the *pseB* gene in pHK01, and into the *Age*I site of the *pseC* gene in pHK01. Resultant *pseA*::*nptII*, *pseB*::*nptII*, and

pseC::*nptII* constructs were subcloned into the *Eco*RV site of pBR325, yielding plasmids pHK22, pHK32, and pHK42, respectively. To allow marker exchange mutagenesis to occur, *P. syringae* pv. *syringae* strain B301D was transformed with pHK22, pHK32, and pHK42 by electrophoration using a Gene Pulser II (Bio-Rad Laboratories) as described previously (81). Transformants were selected on NBY agar supplemented with kanamycin. Double crossover mutations were confirmed by Southern analysis and by the polymerase chain reaction (PCR) (Fig. 2). The confirmed *pseA*, *pseB*, and *pseC* mutants (*pseABC* mutants) were labeled as B301D-HK2, B301D-HK3, and B301D-HK4, respectively. A *syrB1* and *pseC* double mutant (BR132-HK4) was generated by marker exchange of *pseC*::*nptII* into the genome of the *syrB1* mutant strain, BR132 (100). Plasmid pHK115, which carries the *pseABC* efflux genes, was introduced into the *pseABC* mutants in order to test for complementation of the *pseA*, *pseB*, and *pseC* mutations.

To generate a *gacA* mutant, the region flanking 1.0 kb to the 5' terminus of *gacA* gene and 0.6 kb to the 3' terminus of *gacA* gene was amplified from genomic DNA of parental strain B301D by PCR using Vent polymerase (New England Biolabs). The amplified *gacA* gene was cloned into pGEM-T easy vector (Promega) to create plasmid pHK51. Plasmid pHK51 was digested with *Eco*RV to insert the *nptII* gene cassette, which resulted in plasmid pHK52. Plasmid pHK52 was digested with *Not*I to release a 3.5-kb fragment. The resultant 3.5-kb *Not*I fragment was polished with T4 DNA polymerase and inserted into the *Eco*RV site of pBR325 to construct plasmid pHK53. The plasmid pHK53 was introduced into the genome of parental strain B301D to

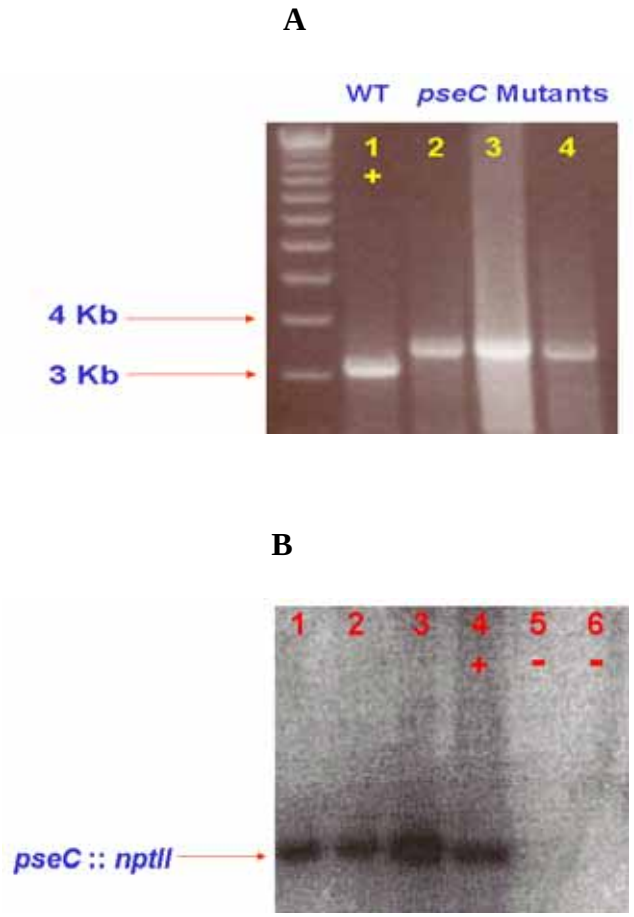

FIG. 2. Confirmation of strain B301D-HK4 (a *pseC* mutant) using PCR and Southern analysis. In panel A (PCR confirmation), the disrupted *pseC* gene amplified by PCR was detected due to its different size (3.3 kb) of PCR products from that of the intact *pseC* gene (3.0 kb). Lane 1 contains the *pseC* gene amplified from parental strain B301D, and the amplified *pseC* gene was approximately 3.0 kb in size. Lanes 2, 3, and 4 show disrupted *pseC* genes by deletion of the 0.9 kb *Age*I fragment of the *pseC* gene and the *nptII* (1.2 kb) insertional mutation. In panel B (Southern analysis), the disrupted *pseC* gene was probed with the *nptII* gene in Southern analysis. Lanes 1, 2, 3, and 4 demonstrated the existence of the *nptII* genes in the genomes of double crossover *pseC* mutants (lane 1 to 3) and in pBR325 containing the *pseC*::*nptII c*onstruct (lane 4). Lanes 5 and 6 verified the absence of the *nptII* gene in the genome of parental strain B301D and the pBR325 vector, respectively.

generate the *gacA* mutant by marker exchange mutagenesis. Site-directed mutagenesis (99) was performed to replace Lys^{906} with Asp in the *pseC* gene of pHK115 (32). The sequence of the mutagenic primer was as follows: K906D, 5'-GGGGATCGTGACCGACAACTCGTACCTGCTG-3'. The primer pair used to introduce the point mutation was anti-parallel and overlapping. PCR products resulting from the mutant strand synthesis reaction were treated with *Dpn*I to digest the *dam*-methylated template plasmid. The resultant unmethylated PCR products were directly transformed into *E. coli*. The correct point mutation was verified by DNA sequence analysis (43). The resultant mutant construct of pHK115 (K906D) was named pHK116.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was used to determine the effect of a *pseC* mutation on expression of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes (25). Bacterial RNAs were extracted using the RNeasy Mini kit (Qiagen) from *P. syringae* pv. *syringae* B301D (a parental strain) and B301D-HK4 (a *pseC* mutant strain) grown on SRM_{AF} medium at 25ºC for 72 h (56). The purified RNA was prepared according to the manufacturer's instructions, which requires DNase digestion using an RNase-Free DNase Set (Qiagen). Oligonucleotide primers were designed by using PrimerSelect software (Version 5.0; DNASTAR).

Reaction components were prepared according to the manufacturer's instructions, except that each reaction was set up in 25 µl with 100 ng of template RNA and 1.25 pmole of each primer. The reverse transcriptase (RT) reaction was performed for 30

min at 94°C, 30 s of primer annealing at 54°C, followed by 45 cycles of 15 s of denaturation at 94°C, 30 s of primer annealing at 54°C, and 30 s of polymerization at 60°C. Primers were evaluated by following the manufacturer's instructions (Qiagen). The fold induction of mRNA was determined from the threshold values (C_T) that were normalized for 16S rDNA expression (endogenous control) and then normalized to the C_T value obtained from parental strain B301D (4).

Before determining expression profiles of the *syrB1* and *sypA* genes, the relative amplification efficiencies of the *syrB1*, *sypA*, and 16S primer pairs were assessed as described in the manufacturer's instructions (Qiagen, 47). Differences in amplification efficiency of the primer pairs were less than 0.1, which indicated that the amplification efficiencies were approximately equal. Quantitative real-time RT-PCR was accomplished by using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and the Smart Cycler (Cepheid). Primers used in quantitative real-time RT-PCR are listed in Table 2.

Screening for syringomycin and syringopeptin secretion by the *pseC* mutant. Strains B301D-HK2 (a *pseA* mutant), B301D-HK3 (a *pseB* mutant), and B301D-HK4 (a *pseC* mutant) were screened for secretion of syringomycin and syringopeptin using standard bioassays as previously reported (81), except that these strains were cultured on PDA (potato dextrose agar) medium plates. To assay syringomycin production, the plates were incubated for 72 h at 25˚C and the indicator fungus *Geotrichum candidum* F-260 was oversprayed and the plates were incubated at 25˚C for another 24 h. To assay syringomycin production, the plates were incubated for 48 h at 25˚C and the indicator bacterium *Bacillus megaterium* Km was oversprayed and the plates were incubated at

TABLE 2. Primer sequences used for quantitative real-time RT-PCR (B301D-HK4)

Genes	Primer[a]
syrB1……..	F-RT-*syrB1*: TTAGCGCCGCGTCAGCCCCT CTCAAG
	R-RT-*syrB1*: GCTCAACGTCCGGGCTGCATCGCTCAC
sypA……..	F-RT-*sypA*: TGCGGGTCGAGGCGTTTTTG
	R-RT-*sypA*: GTTGCCGCGTCCTTGTCTGA
16S……	F-RT-16S: ACACCGCCCGT CACACCA
	R-RT-16S: GTTCCCCTACGGCTACCTT

[a] F, forward ; R, reverse

25˚C for another 24 h. Resultant zones of growth inhibition to *G. candidum* and *B. megaterium* were measured. A low concentration of tetracycline (6.25 μg/ml) was added to the PDA medium in order to maintain pHK115. The PDA plate bioassays were replicated six times.

Virulence assays in immature cherry fruits. Virulence assays of strains B301D-HK2 (a *pseA* mutant) and B301D-HK4 (a *pseC* mutant) were performed in immature cherry fruits as described previously (81). Each wounding site formed on cheery fruits was inoculated with 5 x 10^3 CFU of each strain of *P. syringae* pv. *syringae*. The inoculated fruits were incubated for 4 days at 20°C. Virulence was determined by measuring the diameters of the necrotic lesions formed at each inoculation site. For each experiment, 10 cherry fruits were inoculated per treatment, and the experiment was repeated three times. Parental strain B301D and strain BR132 (a *syrB1* mutant) were used as controls.

Construction of GUS fusions and GUS assay. The *uidA* gene encoding GUS (β-glucuronidase) was inserted into the *pseA* gene in-frame to determine expression of the *pseA* gene in *P. syringae* pv. *syringae* strains (62). Digestion of the plasmid pSL2 (50) with *Hind*III and *Bgl*I was used in cloning the *uidA-aacCI* reporter from pSL2 into JS091 (83). The resultant 3.2-kb *Hind*III-*Bgl*I fragment containing the *uidA-aacCI* reporter was polished with T4 DNA polymerase (New England Biolabs); the polished 3.2-kb *Hind*III-*Bgl*I fragment was inserted in-frame into the *Pml*I site of the *pseA* gene to generate pHK091. The plasmid pHK091 was digested with *Hind*III and *Stu*I to recover a 5.6-kb *Hind*III-*Stu*I fragment. The 5.6-kb fragment was polished with T4 DNA

polymerase and inserted into the *Sma*I site of pUCP26 (65) in reverse orientation to generate pHK092. Sequence analysis of the *pseA*::*uidA* reporter construct verified production of a truncated PseA protein fused with GUS. GUS activity was measured using previously reported methods (85). One unit of activity is defined as cleavage of one pmol of *p*-nitrophenyl β-D-glucuronide (PNPG) per min per bacterium (1).

Minimum inhibitory concentration (MIC) tests. The antimicrobial susceptibilities to acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin and novobiocin (Sigma) were tested using a microtiter broth dilution method (49). The susceptibilities of strain B301D-HK4 (a *pseC* mutant) were compared with those of parental strain B301D and *P. aeruginosa* PAO1 (40). In addition to *Pseudomonas* spp., *E. coli* strains AG100A (an *acrB* mutant) and AG100 ($acrB^+$ parent) were transformed with pHK115, which carries the *pseA, pseB,* and *pseC* genes. Resultant transformed *E. coli* strains were tested for whether the PseABC efflux system enhances the resistance to antibiotics of mutant strain AG100A. Parental strain AG100 was used as a control. Briefly, exponential phase bacterial cells were added to a sterile 96-well microtiter plate containing Mueller-Hinton (MH) broth medium and serial two-fold dilutions of antibiotics (19). Modification of the MICs was observed for mutant strain AG100A when the strain was transformed with pUCP26 (control vector), but the modification of the MICs was eliminated by checking the pH of the MH broth medium and adjusting it to 7.2 after autoclaving the medium. The final cell concentration was adjusted to 5×10^4 CFU/ml per well. The *E. coli* strains were incubated at 37°C for 12 h. The *Pseudomonas* strains were cultured at 25°C for 18 h. The MIC was defined as

the lowest concentration of antibiotic inhibiting visible growth (87), which was confirmed by measuring optical density (600 nm) of cell suspension grown in 96-well plate.

Statistical analysis. Means in each test were compared with one another by using the Tukey's W procedure (66).

RESULTS

Sequence analysis of the *pseA, pseB* and *pseC* genes. Sequencing of p116, pJS091, and pJS115 revealed six open reading frames (ORFs) downstream of the *sypC* gene (83) (Fig. 3). ORF1, ORF2, and ORF3 encoded three components of the PseABC efflux system, which was composed of an outer membrane protein, a periplasmic membrane fusion protein, and a cytoplasmic RND transporter (76). ORF4 encoded a probable class III aminotransferase (38, 83). ORF5 and ORF6 encoded an ABC transporter homolog and a periplasmic membrane fusion protein, respectively (83). The stop codon (TGA) of ORF1 overlapped by 4 bp with the start codon (ATG) of ORF2. Similarly, an overlap of 4 bp was observed between the stop codon of ORF2 (TGA) and the start codon (ATG) of ORF3.

The protein encoded by ORF1, PseA, was 518 amino acids in length and was predicted to encode an outer membrane protein. PseA protein showed 48.2% identity (Z score, 142) to an outer membrane protein of *Ralstonia solanacearum* GMI1000 (77), 32.6% identity (Z score, 54) to the OprM protein of *P. aeruginosa* PAO1 (89), and 23.9% identity (Z score, 11) to the TolC protein of *E. coli* K-12 (9). A probable Shine-

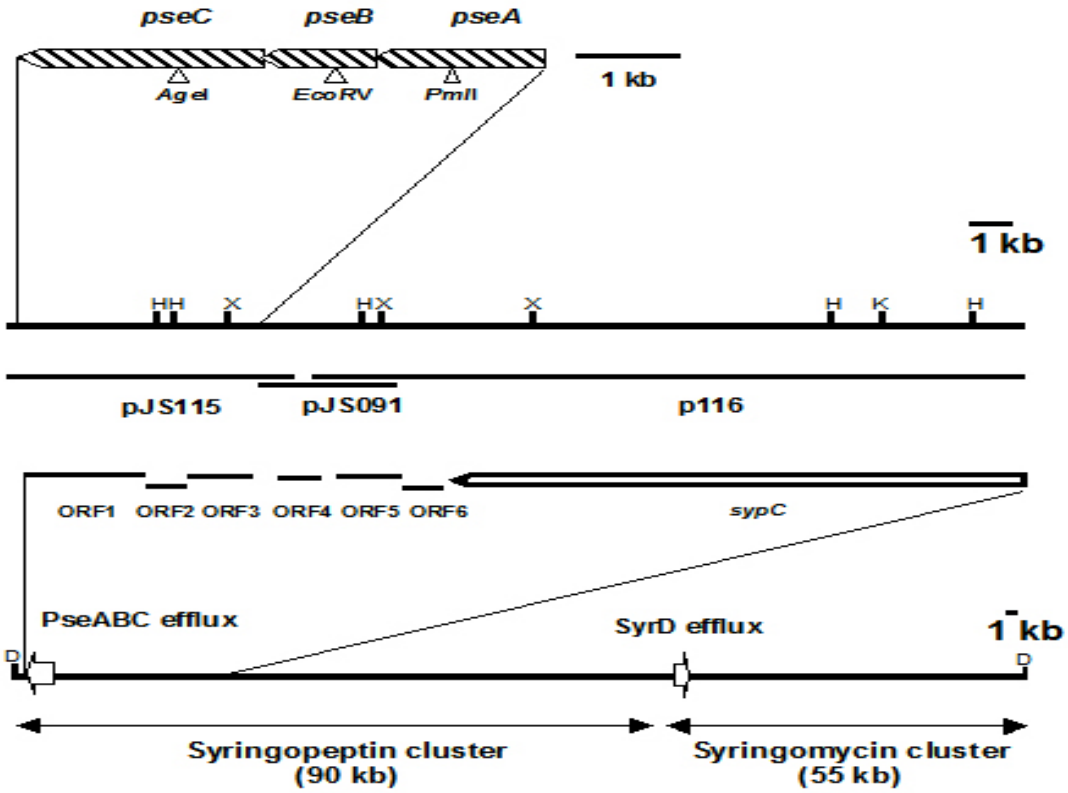

FIG. 3. Diagrammatic representation of the *syr-syp* genomic island on the chromosome of *P. syringae* pv. *syringae* B301D and location of the PseABC efflux system. The approximately 145-kb *Dra*I fragment consists of the syringopeptin (*syp*) gene cluster (90 kb) and the syringomycin (*syr*) gene cluster (55 kb) (83). The left border of the *syp* gene cluster, 51-kb region, is mapped and presented on the *Dra*I fragment. The *pseA* gene (ORF3, 1.5 kb), *pseB* gene (ORF2, 1.1 kb), and *pseC* gene (ORF1, 3.0 kb) are indicated on the map of the 51-kb region. Mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 were generated by disrupting the *pseA* (ORF1), *pseB* (ORF2), and *pseC* (ORF3) genes, respectively, by *nptII* insertional mutagenesis. The triangles identify the restriction sites in which the *nptII* cassette was inserted in the *pseA*, *pseB*, and *pseC* genes. The open arrows on the *syr-syp* genomic island represent the locations of the PseABC and SyrD efflux systems. The restriction enzyme sites are indicated as follows: D=*Dra*I, H= *Hind*III, K=*Kpn*I, and X=*Xho*I.

Dalgarno sequence (AGGCGT) was predicted to be 9 bp upstream of the start codon (ATG) of the *pseA* gene. A rho-independent transcriptional terminator was not found downstream of the stop codon (TGA) of the *pseA* gene. A motif search predicted that the PseA protein contained two motifs characteristic of the outer membrane efflux protein family (39). The first motif corresponded to residues 107 to 292 of the PseA protein (E-value, 1.9e-14), whereas the second motif was located between residues 317 and 498 of the PseA protein (E-value, 2.1e-43). These motifs exhibited heptad repeat patterns that are suggestive of coiled-coil structures (39). Helices of the outer membrane efflux protein family are shown to contain coiled-coil structures, and they are proposed to form a transient complex with periplasmic efflux proteins (membrane fusion protein) for the export of substrate (39). Hydropathy analysis (69) predicted that the N terminus of the PseA protein contained two TMSs (transmembrane segment).

The protein encoded by ORF2, PseB, was 367 amino acids in length and predicted to encode a periplasmic membrane fusion protein. PseB protein showed 51.2% identity (Z score, 87) to a putative membrane fusion protein of *R. solanacearum* GMI1000 (77), 23.9% identity (Z score, 9) to the MexA protein of *P. aeruginosa* PAO1 (89), and 23.4% identity (Z score, 9) to the AcrA protein of *E. coli* K-12 (9). A probable Shine-Dalgarno sequence (GGGCGG) was identified 9 bp upstream of the start codon (ATG) of the *pseB* gene. There was no rho-independent transcriptional terminator identified downstream of the stop codon (TGA) of the *pseB* gene. A motif search predicted that the PseB protein had a hemolysin (Hly) D family secretion protein signature (26). The HlyD protein is a member of the membrane fusion protein family (39). The signature

corresponded to residues 66 to 200 of the PseB protein (E-value, 2.9e-05), and it is suggested to be associated with a periplasmic efflux protein (membrane fusion protein) to make a bridge between an outer membrane protein and a cytoplasmic efflux protein (39). PseB protein was predicted to contain one TMS at the N terminus by hydropathy analysis (69).

The RND-type transporter encoded by ORF3, PseC, was predicted to be 1,009 amino acids in length. PseC protein showed 61.6% identity (Z score, 563) with a probable transporter transmembrane protein of *R. solanacearum* GMI1000 (77), 28.3% identity (Z score, 134) to the MexB protein of *P. aeruginosa* PAO1 (89), and 27.2% identity (Z score, 9) to the AcrB protein of *E. coli* K-12 (9). A probable Shine-Dalgarno sequence (AGGCCC) was located 13 bp upstream of the start codon (ATG) of the *pseC* gene. The primary structure of a rho-independent transcriptional terminator was observed 108 bp downstream of the stop codon (TGA) of the *pseC* gene (primary structure value, 3.52). A motif search predicted that PseC protein contained four AcrB/AcrD/AcrF family motifs (motifs A to D) (69). The AcrB/AcrD/AcrF family (cytoplasmic membrane protein) is one of the most well-studied RND-type transporter families (69). These motifs were dispersed within the amino acid residues 3 to 996 of the PseC protein (E-value, 3e-215). Motif A was located in a loop between TMS1 and TMS2 of the PseC protein and it is predicted to be involved in a reversible conformational change to open and close a transport channel (69). Motif B and motif D were in TMS6 and TMS4, respectively, of the PseC protein and they are proposed to be involved in proton transfer (Fig. 4) (69). Finally, motif C was in TMS11 of the PseC protein and it is

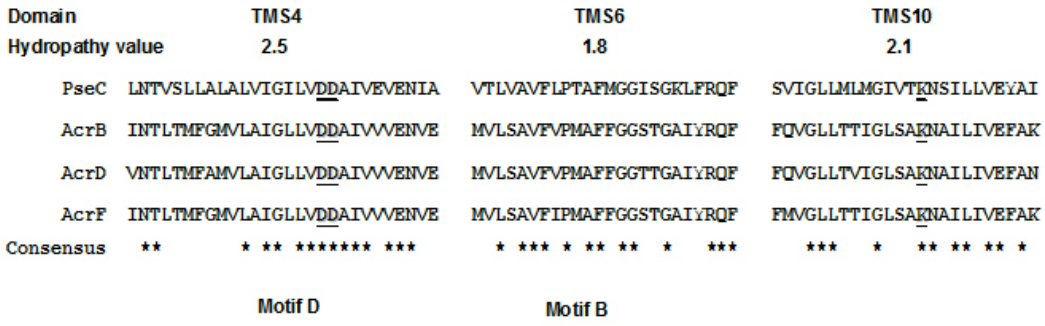

FIG. 4. Amino acid sequence alignment of TMS4, TMS6, and TMS10 of the RND transporters (69). Motifs D and B that are characteristic of the AcrB, AcrD, and AcrF RND transporters were found in TMS4 and TMS6 of the PseC protein, respectively. Hydropathy values of TMS4, TMS6, and TMS7 of the PseC protein are shown above the corresponding amino acid residues. Consensus amino acid residues are marked with asterisks. Amino acid residues underlined in boldface indicate the conserved aspartic acids (D) and lysine (K) residues that are essential residues in TMS4 and TMS10 of the MexB (32) and AcrB (57) proteins. These D and K residues were identified in TMS4 and TMS 10 of the PseC protein.

suggested to dictate the direction of transport (69). Two aspartic acid (D) residues and a lysine (K) residue, possible candidates for proton-translocating pathways (57), were conserved in TMS4 and TMS10, respectively, of the RND transporters including the PseC protein (Fig. 4). Twelve TMSs and two large hydrophilic loops were predicted to be located in the PseC protein, based on hydropathy analysis (69) and multi-alignment analysis with known RND transporters such as the AcrB, AcrD, and AcrF proteins (Fig. 5) (23).

Screening for secretion of syringomycin and syringopeptin by the *pseABC* mutants. Strains B301D-HK2 (a *pseA* mutant), B301D-HK3 (a *pseB* mutant), and B301D-HK4 (a *pseC* mutant) were screened for secretion of syringomycin and syringopeptin on PDA medium using *G. candidum* and *B. megaterium* as indicator microorganisms, respectively (81). All three mutants showed reduced zones of inhibition to *G. candidum* between approximately 40% and 45% (6 mm radius), as compared to parental strain B301D (11 mm radius) (Fig. 6, Table 3); results for mutant strains B301D-HK2 and B301D-HK3 are not shown. Zones of inhibition to *B. megaterium* by mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 appeared to be reduced between approximately 50% and 60% (4 to 5 mm), as compared to parental strain B301D (9 mm radius) (Table 3); results of mutant strains B301D-HK2 and B301D-HK3 are not shown. However, mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 that carried pHK115 produced large zones with the radius of the zone of inhibition to *G. candidum* and *B. megaterium* averaging approximately 9 and 8 mm, respectively (Fig. 6, Table 3). These data indicated complementation of the *pseA, pseB,*

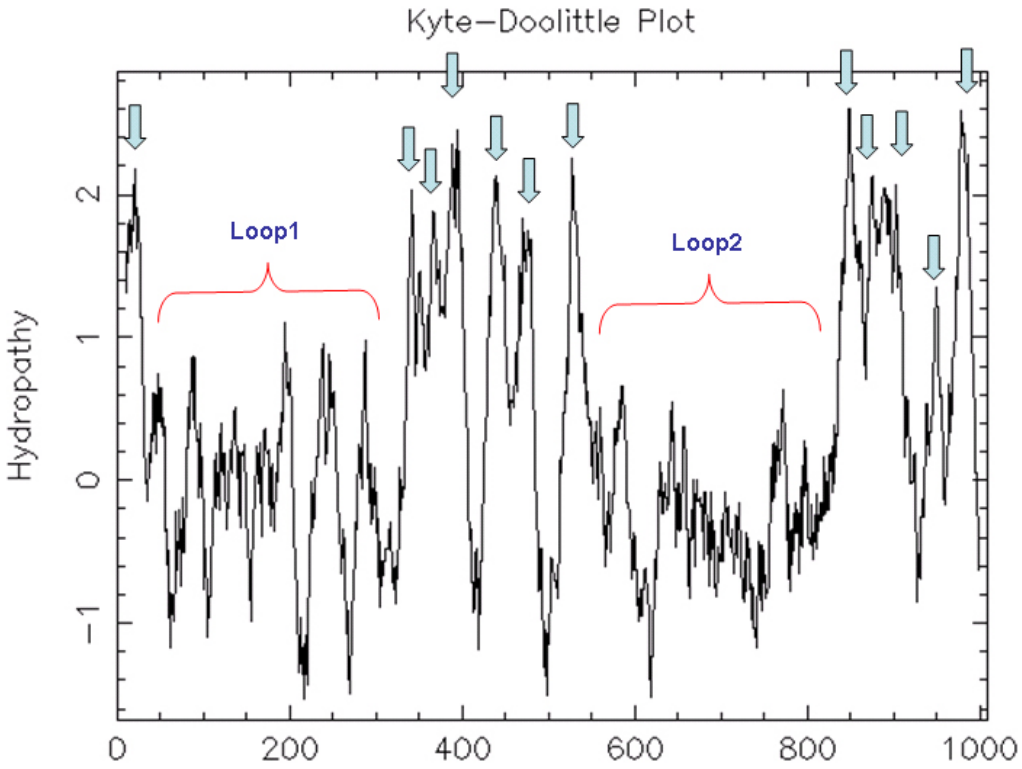

FIG. 5. Hydropathy analysis and prediction of TMSs of the PseC protein (45). The PseC protein was predicted to contain 12 TMSs and two large loops. One loop was located between TMS 1 and TMS 2, and the other loop between TMS 7 and TMS 8. Arrows indicate sites of TMSs within the PseC protein.

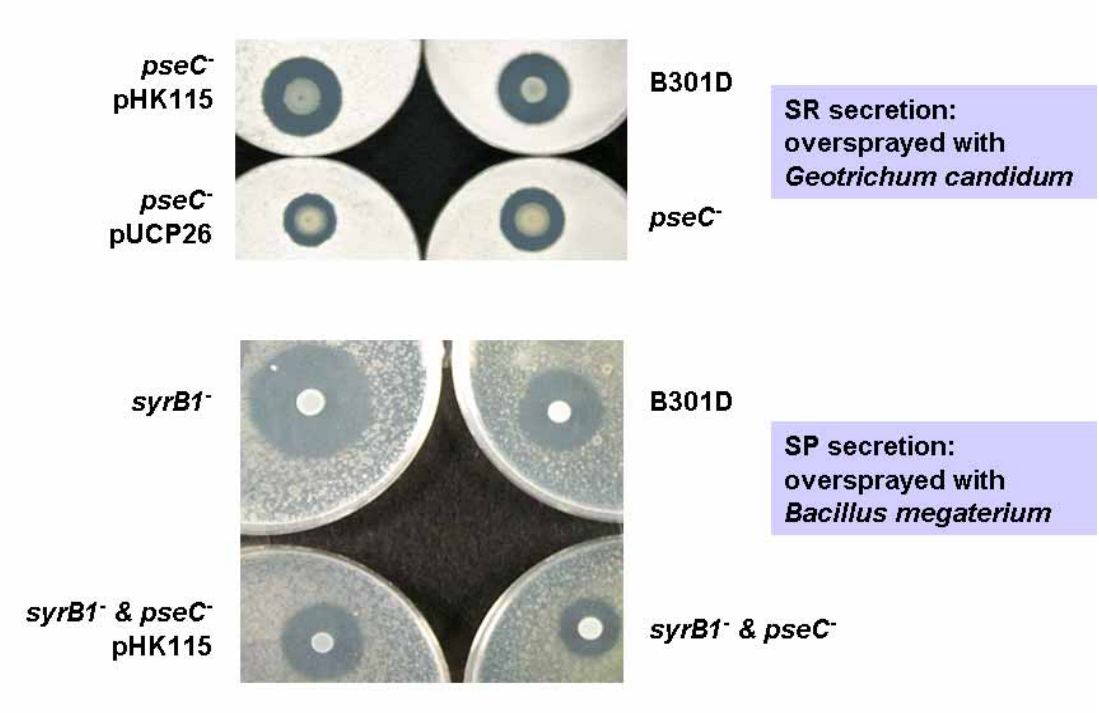

FIG. 6. Inhibition due to secretion of syringomycin and syringopeptin by different *P. syringae* pv. *syringae* mutant strains. A bioassay for syringomycin was performed by incubation of the strains on PDA medium for 3 days and then overspraying with *G. candidum*. To assay syringopeptin, the strains were incubated for 2 days on PDA medium, followed by overspraying with *B. megaterium*. Zones (radii) of inhibition of *G. candidum* and *B. megaterium* were measured from the margins of the bacterial colonies (103). Syringomycin and syringopeptin are abbreviated as SR and SP, respectively. pHK115 is pUCP26 carrying the *pseABC* efflux genes. *syrB1*$^-$, *pseC*$^-$, and *syrB1*$^-$ & *pseC*$^-$ indicate strains BR132 (a *syrB1* mutant), B301D-HK4 (a *pseC* mutant), and BR132-HK4 (a *syrB1* and *pseC* double mutant), respectively.

TABLE 3. Inhibition due to production of syringomycin and syringopeptin by *P. syringae* pv. *syringae* strains B301D and B301D-HK4 (a *pseC* mutant)

Strain	Bioassay on PDA medium	
	Syringomycin	Syringopeptin[a]
B301D	11.2[b] ± 1.2 (mm)[c]	9.2 ± 1.1 (mm)
B301D-HK4 (a *pseC* mutant)	6.6 ± 1.2	3.7 ± 0.7
B301D-HK4 (pHK115)	9.4 ± 0.9	7.7 ± 1.0

[a]Syringomycin and syringopeptin production was assessed by measuring the radius (mm) of inhibition zones to *G. candidum* and *B. megaterium*, respectively, on PDA medium.
[b]Results are represented as an average of six determinations that were acquired from three separate experiments
[c]Standard error of the means.

and *pseC* mutations by pHK115, which carries the *pseABC* efflux genes. Based on a previous study reporting that syringomycin inhibits the growth of *B. megaterium* in PDA plate bioassays (81), strain BR132-HK4 (a *pseC* and *syrB1* double mutant) was generated to exclude the effect of syringomycin on bioassays for syringopeptin secretion. Mutant strain BR132-HK4 was screened for syringopeptin secretion by a PDA plate bioassay with *B. megaterium*, and it was observed to form reduced zones of inhibition by approximately 70% (4 mm radius), as compared to strain BR132 (*syrB1* mutant, 12 mm radius) (Fig. 6 and 7). The conserved D and K residues in the RND transporter family are reported to have an essential role in the export of substrates by functional studies with the AcrB and MexB RND transporters (32). The conserved K residue found in the PseC protein (Fig. 4) was tested to see whether it had a significant role in syringopeptin secretion. The plasmid pHK116 was generated by replacing Lys^{906} with Asp in the *pseC* gene of pHK115, and then it was introduced into mutant strain BR132-HK4 to determine the functional importance of the Lys^{906} residue of PseC protein for syringopeptin secretion (Fig. 7). Mutant strain BR132-HK4 carrying pHK116 produced zones of inhibition to *B. megaterium* with a radius of approximately 5 mm, while mutant strain BR132-HK4 carrying pHK115 exhibited larger zones of inhibition of approximately 8 mm in radius. In comparison, strain BR132 (a *syrB1* mutant) produced zones of inhibition averaging 11.5 mm in radius. Therefore, these data showed that the Lys^{906} residue of PseC protein was functionally important in syringoepeptin secretion.

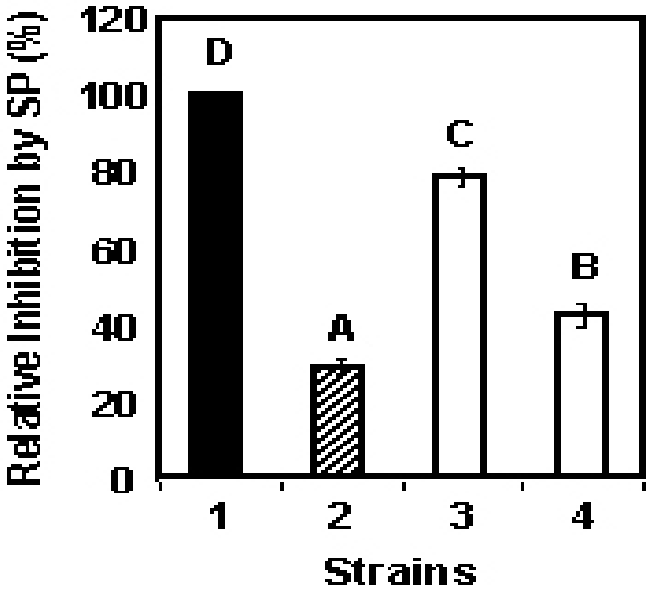

FIG. 7. Relative inhibition due to syringopeptin (SP) secretion by *P. syringae* pv. *syringae* mutant strains. A bioassay for syringopeptin was performed by incubation of the strains on PDA medium containing 0.5 mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) for 2 days, followed by overspraying with *B. megaterium*. Zones of inhibition to *B. megaterium* caused by strain BR132-HK4 (a *syrB1* and *pseC* double mutant) were compared relative to strain BR132 (a *syrB1* mutant) (55). Differences between treatments were determined by Tukey's W procedure ($\alpha = 0.05$) (66). Column 1, mutant strain BR132; 2, BR132-HK4; 3, BR132-HK5 carrying pHK115 (*pseABC* efflux genes); and 4, BR132-HK4 carrying pHK116 (pHK115 carrying site-directed mutation in the *pseC* gene).

Effect of the *pseC* mutation on expression of the syringomycin and syringopeptin synthetase genes. Quantitative real-time RT-PCR was used to determine the effect of the *pseC* mutation on transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes (8). Expression of the synthetase genes was compared between strain B301D-HK4 (a *pseC* mutant) and parental strain B301D, following culture of these strains on SRM_{AF} medium for 72 h (56). Transcript levels of the *syrB1* and *sypA* genes in mutant strain B301D-HK4 corresponded to approximately 61% and 15%, respectively, of those in parental strain B301D (Fig. 8).

Virulence of the *pseC* mutant on cherry fruits. The virulence of strains B301D-HK2 (a *pseA* mutant) and B301D-HK4 (a *pseC* mutant) was determined in immature Bing cherry fruits using methods described previously (71). The lesion diameters were used to quantify relative virulence in the cherry fruits. Mutant strains B301D-HK2 and B301D-HK4 produced lesions nearly 1.8 mm in diameter, which corresponded to approximately 45% and 42% ($P = 0.05$), respectively, of that formed by parental strain B301D (5.0 mm) (Fig. 9). In comparison, the average virulence shown by mutant strain BR132 (3.7 mm) corresponded to approximately 60% of that observed for parental strain B301D.

Control of expression of the *pseABC* efflux genes by the GacS/A two-component system. A translational fusion of the *pseA* gene with the *uidA* gene encoding β-glucuronidase was used as a reporter to determine the regulatory relationship between the GacS/A system and *pseA* expression. Plasmid pHK92, which contained the *pseA*::*uidA* reporter construct, was introduced into parental strain B301D, strain B301D-

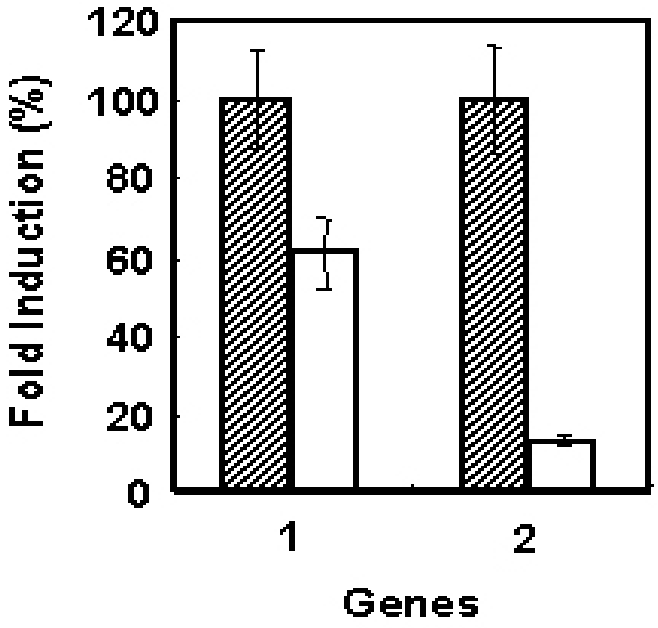

FIG. 8. Effects of the *pseC* mutation on expression of the *syrB1* (55) and *sypA* (81) synthetase genes. Expression of the synthetase genes was compared between strain B301D-HK4 (*pseC* mutant, open bar) and parental strain B301D (hatched bar) using quantitative real-time RT-PCR. Relative differences were measured (4). The relative levels of mRNA was determined from the threshold values (C_T) that were normalized for 16S rDNA expression (endogenous control) and then the WT (parental strain B301D) value was defined as 100%. Error bars represent the standard error of the means. Relative expression of the *syrB1* (column 1) and *sypA* (column 2) genes by mutant strain B301D-HK4 were compared with those of parental strain B301D.

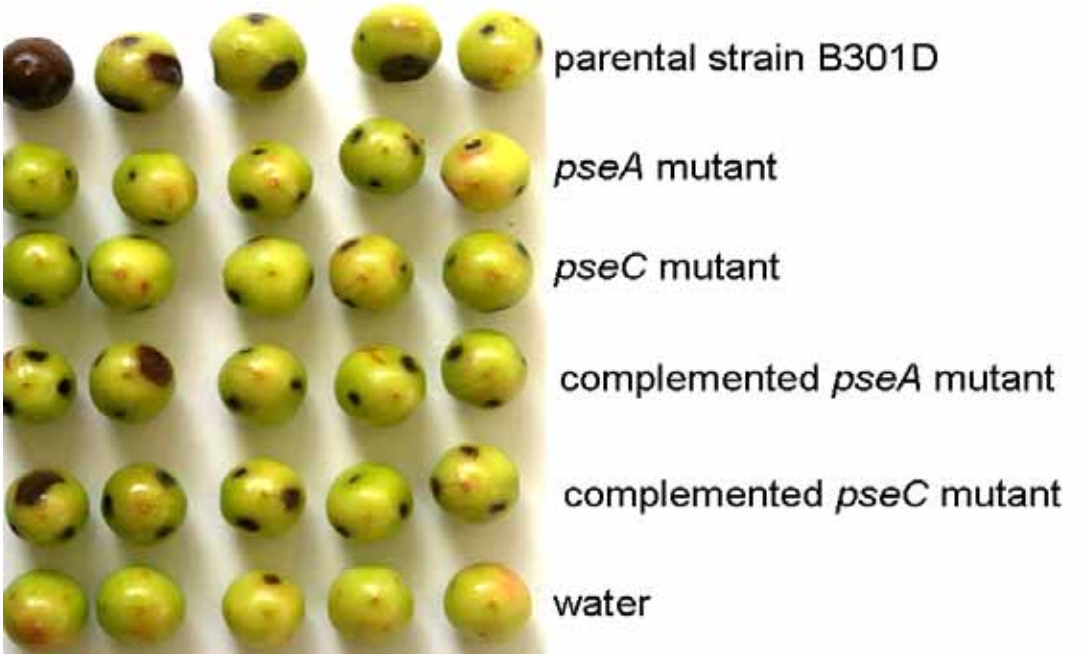

FIG. 9. Virulence of strains B301D-HK2 (a *pseA* mutant) and B301D-HK4 (a *pseC* mutant) compared with parental strain B301D in immature cherry fruits. Each fruit was incubated at three sites with 5 x 10^3 cells per site. Control fruits were injected with sterile water. Symptoms were observed in 4 days after inoculation. For comparison, mutant strains B301D-HK2 and B301D-HK4 were complemented with pHK115 *in trans*. Resultant complemented mutants were inoculated into cherry fruits to evaluate their virulence.

HK5 (a *gacA* mutant), and strain B301D-SL7 (a *salA* mutant). Mutant strains B301D-HK5 and B301D-SL7 carrying pHK92 produced GUS activities of approximately 340 units and 234 units per 10^8 CFU, respectively, which corresponded to approximately 15% of the GUS activity expressed by parental strain B301D (2,742 units per 10^8 CFU) (Fig. 10).

Antibiotic susceptibility of *pseC* mutant. Antimicrobial susceptibility tests were performed to determine whether the PseABC efflux system contributed to antibiotic resistance in *P. syringae* pv. *syringae* strains. Mutant strain B301D-HK4 and parental strain B301D exhibited the same susceptibilities to all the tested antibiotics (Table 4); MICs for acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin and tetracycline were 12.5 μg/ml, 100 μg/ml, 200 μg/ml, 50 μg/ml, 12.5 μg/ml, 6.3 μg/ml and 0.2 μg/ml, respectively. In comparison, *P. aeruginosa* PAO1 exhibited similar MICs reported in the previous studies (2, 52, 59) (Table 4); MICs for acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, and gentamicin were 200 μg/ml, 50 μg/ml, 50 μg/ml, 200 μg/ml, 100 μg/ml, 12.5 μg/ml, and 3.1 μg/ml, respectively.

In order to define the functional relationship of the PseABC efflux system with the *E. coli* AcrAB-TolC efflux system (64), *E. coli* strain AG100A (an *acrB* mutant) was transformed with pHK115 and then assessed for susceptibility to aztreonam, chloramphenicol, acriflavine, erythromycin, gentamicin, novobiocin, and tetracycline (Table 5). Heterologous expression of the *pseABC* efflux genes failed to increase resistance of mutant strain AG100A to the tested antibiotics except for gentamicin and

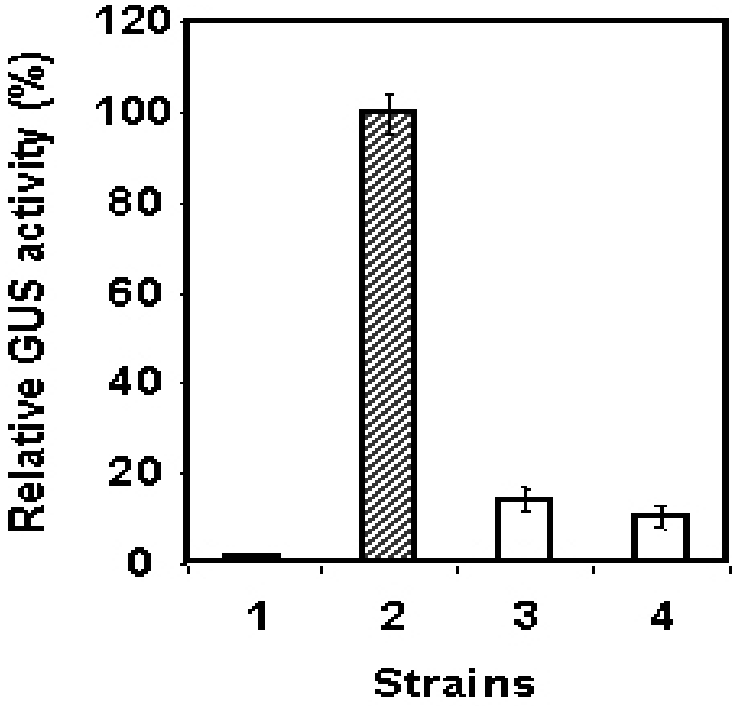

FIG. 10. Effects of the *gacA* and *salA* mutations on expression of the *pseA*::*uidA* reporter construct. The *pseA*::*uidA* reporter construct was inserted into the *Sma*I site of pUCP26 (65) in reverse orientation to the *lacZ* promoter of pUCP26, which generated pHK092. The strains were transformed with pHK092 and incubated on PDA media for 3 days, and then tested for GUS activity. The measured GUS activities of strains B301D-HK5 (a *gacA* mutant) and B301D-SL7 (a *salA* mutant) were compared relative to that of parental strain B301D. Vertical bars indicate standard error of the means. Column 1, B301D with a pUCP26 (vector); 2, B301D with pHK092; 3, B301D-HK5 with pHK092; and 4, B301D-SL7 with pHK092 ($\alpha = 0.05$).

TABLE 4. Antibiotic susceptibility of *P. syringae* pv. *syringae* strains B301D, B301D-HK4, and *P. aeruginosa* strain PAO1

Strains	MIC (μg/ml)[a]						
	AF	AZ	CA	CM	EM	GM	TC
B301D[b]	12.5	100	200	50	12.5	6.25	0.20
B301D-HK4[c]	12.5	100	200	50	12.5	6.25	0.20
PAO1[d]	200	50	50	200	100	12.5	3.13

[a]Abbreviations: AF, acriflavine; AZ, aztreonam; CA, carbenicillin; CM, chloramphenicol; EM, erythromycin; GM, gentamicin; TC, tetracycline.

[b] *P. syringae* pv. *syringae* strain B301D (a parental strain).

[c] *P. syringae* pv. *syringae* mutant strain B301D-HK4 (a *pseC* mutant).

[d] *P. aeruginosa* strain PAO1 (wild type).

tetracycline. Resistance to gentamicin and tetracycline was increased two times, respectively, in mutant strain AG100A expressing the *pseABC* efflux genes carried in a pUCP26 vector, as compared to that in mutant strain AG100A carrying only a pUCP26 vector. Mutant strain AG110A carrying pHK115 showed MICs for gentamicin of 0.4 μg/ml and tetracycline of 50 μg/ml, whereas mutant strain AG100A carrying only a pUCP26 vector exhibited MICs for gentamicin of 0.2 μg/ml and tetracycline of 25 μg/ml. In comparison, *E. coli* parental strain AG100 carrying a pUCP26 vector was used as control, and its MICs for gentamicin and tetracyline were 0.8 μg/ml and 100 μg/ml, respectively.

DISCUSSION

P. syringae pv. *syringae* strain B301D secretes two major phytotoxins, called syringomycin and syringopeptin (8). Previous studies (29, 71) suggest that phytotoxin secretion is greatly facilitated by the SyrD efflux system, although the *syrD* mutation fails to cause a complete loss in the capacity to secrete syringomycin and syringopeptin. However, during further sequencing of the *syp* gene cluster, another transporter system was identified at the left border of the *syp* gene cluster (83), called the PseABC efflux system. The predicted PseA protein was found to be homologous to a probable RND outer membrane protein (*R. solanacearum*) (77), the OprM protein (*P. aeruginosa*) (89), and the TolC protein (*E. coli*) (9). Furthermore, The PseA protein is predicted to contain two TMSs and has motifs characteristic of the outer membrane protein family. The amino acid sequence of the predicted PseB protein was homologous to those of a

TABLE 5. Antibiotic susceptibility of *E. coli* mutant strain and its transformants expressing the *pseABC* efflux genes

Strains (plasmid)	MIC (μg/ml)[a]						
	AF	AZ	CM	EM	GM	NV	TC
AG100[b] (pUCP26)	100	0.10	3.1	25	0.8	100	100
AG100A[c] (pUCP26)[d]	50	0.05	1.6	12.5	0.2	100	25
AG100A (pHK115)[e]	50	0.05	0.8	1.6	0.4	100	50

[a]Abbreviations: AF, acriflavine; AZ, aztreonam; CM, chloramphenicol; EM, erythromycin; GM, gentamicin; NV, novobiocin; and TC, tetracycline.
[b] *E. coli* strain AG100 (a parental strain) (64).
[c] *E. coli* strain AG100A (an *acrB* mutant) (64).
[d] pUCP26 carrying the tetracycline resistance genes (65).
[e] pUCP26 carrying the *pseABC* efflux genes.

probable RND membrane fusion protein (MPF, *R.solanacearum*) (77), the MexA protein (*P. aeruginosa*) (89), and the AcrA protein (*E. coli*) (9). Moreover, the PseB is predicted to have one TMS and contains the HlyD family secretion protein motif that is characteristic of the membrane fusion protein family. The predicted PseC protein was homologous to a putative RND transporters (*R. solanacearum*) (77), the MexB protein (*P. aeruginosa*) (89), and the AcrB protein (*E. coli*) (9). In addition, the PseC protein is predicted to encompass 12 TMSs, two large perplasmic loops, and has four motifs (A, B, C and D) characteristic of the RND-type transporter family. Based on the arrangement of the *pseA, pseB* and *pseF* genes, which are closely spaced, these three genes were predicted to be transcribed as an operon. Strains B301D-HK2 (a *pseA* mutant), B301D-HK3 (a *pseB* mutant), and B301D-HK4 (a *pseC* mutant) were significantly reduced in secretion of syringomycin and syringopeptin. In addition to phytotoxin secretion, functional studies with the mutant strains showed that the PseABC efflux system was required for full virulence of *P. syringae* pv. *syringae* strain B301D to immature cherry fruits. When substrates are transported, an outer membrane protein, a membrane fusion protein, and an RND transporter are formed into a functional three-component complex, such as the AcrAB-TolC and MexAB-OprM efflux systems (102). Similarly, the PseA, PseB, and PseC proteins were predicted to be organized into a functional three-component complex in order to secrete the lipopeptide phytotoxins. In summary, the PseABC efflux system was classified as a member of the RND transporter family and it was critical to lipopeptide phytotoxin secretion and virulence of *P. syringae* pv. *syringae* B301D.

Strain B301D-HK4 (a *pseC* mutant) was as sensitive to a series of antibiotics as parental strain B301D in MIC tests. These data suggested that the PseABC efflux system was not involved in altering resistance of strain B301D of *P. syringae* pv. *syringae* to the tested antibiotics. Thus, it was proposed that the PseC might have several homologs in the B301D genome or another efflux system might be responsible for conferring resistance to antibiotics. Because of the absence of a complete genome sequence of strain B301D, the PseC homologue was searched against the draft genome sequence of the *P. syringae* pv. *syringae* strain B728a (http://genome.jgi-psf.org /draft_microbes/psesy/psesy.home.html). There was only one PseC homolog (96.4% identity; Z score, 207) found in the B728a genome. Nonetheless, it is unknown how many genes encode RND-type transporters in the B728a genome. Thus, the annotated genomes of *P. syringae* pv. *tomato* strain DC3000 (12), *P. putida* strain KT2440 (60), and *P. aeruginosa* strain PAO1 (89) were searched for genes encoding members of the RND-type transporter family. Interestingly, multiple genes encoding RND-type transporters were found in these genomes: nine genes encoding probable RND-type transporters in the DC3000 genome, 19 genes in the KT2440 genome, and 17 genes in the PAO1 genome (74). Furthermore, most of the RND-type transporters were predicted to be multidrug exporters (74). Therefore, it was speculated that, in addition to the *pseC* gene, genes encoding additional RND-type transporters might exist and their products might be responsible for the export of antibiotics and metabolites in strain B301D.

Heterologous expression of the *pseABC* efflux genes in *E. coli* indicated that the

PseABC efflux system might have different patterns of substrate specificity from the AcrAB-TolC efflux system. The expression of *pseABC* efflux genes in the *E. coli* strain AG100A (an *acrB* mutant) was observed to increase resistance to gentamicin and tetracycline by two times, respectively, among the tested antibiotics (Table 5). In order to confirm that the PseABC efflux system increased resistance to gentamicin, *E. coli* strain HNCE1a (an *acrB* and *acrD* double mutant) carrying pHK115 was tested for MIC of gentamicin (20). The AcrD protein is another RND transporter in the genome of *E. coli* K-12 (75). Mutant strain HNCE1a carrying pHK115 (0.4 μg/ml) showed resistance to gentamicin two times higher than that carrying only pUCP26 vector (0.2 μg/ml). This result was consistent with a previous study (20) reporting that expression of the *acrD* gene in mutant strain HNCE1a increases resistance against gentamicin. These results indicated that the PseABC efflux system has substrate specificity for gentamicin, which subsequently indicated that there was a difference in substrate specificity between the PseABC and AcrAB-TolC efflux systems (64). Moreover, a previous study (20) concluded that the AcrB RND transporter is involved in the export of a variety of lipophilic and amphiphilic drugs, dyes, and detergent molecules (including tetracycline, chloramphenicol, erythromycin, and bile acids), whereas the AcrD RND transporter is responsible for the export of a narrow range of substrates such as aminoglycosides (including amikacin, gentamicin, tobramycin, kanamycin, and neomycin). Furthermore, the PseC protein showed higher levels of amino acid sequence identity to the AcrD protein (30.2% identity; Z score, 186) than the AcrB protein (27.2% identity; Z score, 131). Thus, the PseC RND transporter is likely to be

functionally closer to the AcrD RND transporter than the AcrB RND transporter. Interestingly, there were common characteristics found in the structures of gentamicin and syringopeptin. Gentamicin possesses a hydrophilic structure which contributes to the export of gentamicin across the cytoplasmic membrane and subsequently to reduction in the reentry of exported gentamicin into the cytoplasm (75). Syringopeptin is composed of a hydrophobic 3-hydroxy carboxylic acid tail and a cyclized charged (positive) peptide head, which is suggested to contribute to insertion of the phytotoxin into the cytoplasmic membrane and subsequently to formation of transmembrane pores (37). However, similar to gentamicin, the phytotoxin is expected not to enter into the cytoplasm of cells (37). Whenever hydrophilic compounds are pumped out of a bacterial cell, they are predicted to rarely reenter into the cytoplasm due to their hydrophilic structures, although they may accumulate in the periplasmic space. These results indicated that the PseABC efflux system is a specialized RND-type transporter for secretion of syringopeptin and syringomycin.

Expression of the *pseA*::*uidA* reporter was reduced 85% in strains B301D-HK5 (a *gacA* mutant) and B301D-SL7 (a *salA* mutant) as compared to parental strain B301D, indicating that expression of the *pseABC* efflux genes was controlled by a GacS/A two-component system. The sensor kinase GacS and response regulator GacA trigger signal transduction to express genes required for the biosynthesis of secondary metabolites including phytotoxins (34). *P. syringae* pv. *syringae* strain B728a uses the GacS/A two-component system to control production of syringomycin, extracellular polysaccharide, and proteases that are involved in virulence (41). The SalA

transcriptional regulator is essential to syringomycin production and lesion formation by strains B728a and B301D (41, 50). Thus, the GacS/A two-component system is known to control syringomycin production and lesion formation by regulating expression of the *salA* gene (41). However, it was unknown whether either the GacS/A two-component system or the SalA protein controls expression of the *pseABC* efflux genes required for secretion of syringomycin and syringopeptin. Results of this study demonstrated that expression of the *pseA*::*uidA* reporter construct was significantly reduced in both *salA* and *gacA* mutants (Fig. 10). These data indicated that the GacS/A two-component system controls expression of the *pseA* gene through *salA* expression, indicating co-regulation of the phytotoxin synthetase genes and the *pseABC* efflux genes by the GacS/A two-component system. This co-regulation is likely to help cells balance phytotoxin biosynthesis and secretion.

As shown previously, expression of the *syrB1* gene is reduced in strain BR105 (a *syrD* mutant) (71), and a mutation in the *pseC* gene caused a significant reduction in expression of the *syrB1* and *sypA* genes. Expression of the *sypA* gene in mutant strain B301D-HK4 was greatly decreased as compared to parental strain B301D (85% reduction). In addition there was a significant decrease in expression of the *syrB1* gene in mutant strain B301D-HK4 (49% reduction). These results demonstrated that mutations in the *pseC* or *syrD* gene reduce transcript levels of the syringomycin and syringopeptin synthetase genes. Previous studies demonstrate that end-products can regulate secondary metabolite production (95, 96). For example, pathogenic strains of *E. coli* secrete hemolysin (HlyA), a 107 kDa protein, when they infect the urinary tract

and extraintestinal regions in mammals such as pigs, horses, and humans (7). The HlyBD-TolC efflux system secretes hemolysin A across both membranes of *E. coli* (24). The efflux system consists of a cytoplasmic membrane protein of the ABC transporter system (HlyB), a membrane fusion protein (HlyD) and an outer membrane protein (TolC) (24). It is observed that the total intracellular levels of hemolysin are low in *hlyB*, *hlyD*, and *tolC* mutants (97), indicating that a regulatory coupling exists between hemolysin production and toxin secretion (97). Some strains of *Bacillus thuringiensis*, a saprogenic Gram-positive insect pathogen, produce β-exotoxin I, a nonproteinaceous toxin (21). This toxin is thought to inhibit RNA polymerase by acting as an analog of ATP and interfering with the polymerization reaction (86). β-exotoxin is suggested to be translocated through the bacterial cytoplasmic membrane by a putative ABC transporter system encoded by the *berA* and *berB* genes (21). Although all the genes required for the synthesis of β-exotoxin are not yet identified, a genetic study demonstrated that the *berAB* mutants produce small amounts of β-exotoxin as compared to the *B. thuringiensis* parental strain (21). This study suggests that the ABC transporter system encoded by the *berA* and *berB* genes is essential for production of β-exotoxin. Furthermore, a negative feedback mechanism that requires the ABC transporter system is proposed to regulate β-exotoxin production. A similar regulatory coupling was observed in this study. A mutation in the *pseC* gene interfered with secretion of syringomycin and syringopeptin, and also reduced expression of the *syrB1* and *sypA* genes. Accordingly, low production of syringomycin and syringopeptin was attributed to reduced virulence in strain B301D-HK4 (a *pseC* mutant).

This study is the first report that demonstrates involvement of an RND-type efflux system is involved in secretion of phytobacterial toxins. Based on a phylogenetic study (74), it appears to be a unique RND-type transporter system in that it secretes specific lipopeptide phytotoxins that are natural secondary metabolites produced by a Gram-negative bacterium, *P. syringa*e pv. *syringae* (31). However, to support the uniqueness of the PseABC efflux system, it is necessary to determine whether other substrates are secreted by the PseABC efflux system.

The crystal structure of AcrB reveals the presence of a TolC docking domain in the extra-membrane headpiece of the AcrB protein, which indicates a direct interaction between the TolC and AcrB proteins (57). Thus, the AcrAB-TolC efflux system transports substrates through a membrane-spanning conduit formed by this interaction. Correspondingly, the PseC (RND-type transporter) might contain a docking domain for the PseA protein (outer membrane protein). A direct interaction between the PseC and PseA proteins is likely to occur and form a membrane-spanning transit pathway. Thus, the PseABC efflux system is expected to secrete lipopeptide phytotoxins through the transit pathway with preference for syringopeptin. In summary, this study demonstrates that an RND-type transporter system, in addition to the SyrD ABC transporter system, was required for secretion of lipopeptide phytotoxins and full virulence in *P. syringae* pv. *syringae* B301D.

CHAPTER III

THE ABC TRANSPORTER, CALLED THE PseEF EFFLUX SYSTEM, IS A VIRULENCE FACTOR OF *P. SYRINGAE* PV. *SYRINGAE*

INTRODUCTION

Pseudomonas syringae pv. *syringae* produces two classes of lipopeptide phytotoxins called syringomycin and syringopeptin, which cause necrosis on a broad range of monocot and dicot species (10). SRE and $SP_{22}A$ are the major forms of syringomycin and syringopeptin, respectively (8). A cyclic peptide is attached to a 3-hydroxy fatty acid tail to form SRE, whereas a cyclic peptide with 22 amino acids is attached to a 3-hydroxy fatty acid tail to form $SP_{22}A$. Syringopeptin and syringomycin are encoded by the *syr*-*syp* genomic island consisting of the *syr* and *syp* gene clusters (83). Synthetase genes, secretion genes, and regulatory genes are identified in the two gene clusters. Briefly, the cluster are composed of three syringopeptin synthetase genes (*sypA*, *sypB*, and *sypC*), four syringomycin synthetase genes (*syrB1*, *syrB2*, *syrC*, and *syrE*), four regulatory genes (*syrP*, *salA*, *syrF*, and *syrG*), and eight transporter-related genes (83). Following expression of the *syr* and *syp* genes, the resultant synthetases synthesize the lipopeptide phytotoxins via a nonribosomal mechanism, and then *P. syringae* pv. *syringae* secretes the phytotoxins through type I secretion systems (8, 31). The phytotoxins also have a role as key virulence determinants (factors), which significantly

contribute to cytotoxicity against other microorganisms and plant tissues (37). In fact, both of the lipopeptide phytotoxins cause cytotoxicity to plant cells and they are harmful to the bacterial cells which produce them (37). Therefore, as long as *P. syringae* pv. *syringae* produces the lipopeptide phytotoxins, it is important to efficiently pump the phytotoxins out of the bacterial cell.

In addition to the transport of cellular metabolites, transporter systems are required for full virulence of microbial pathogens. Consequently, it is important to characterize the functions of genes encoding transporter systems. Based on the genomes recently released (74), the ATP-binding cassette (ABC) transporter superfamily and resistance-nodulation-cell division (RND) transporter family are widespread throughout Gram-negative bacteria. For example, the *P. putida* KT2440 genome contains 350 predicted cytoplasmic membrane transport systems, including 117 ABC transporters and 18 RND transporters (60). The genome of the plant bacterial pathogen, *P. syringae* pv. *syringae* DC3000, exhibits 317 predicted cytoplasmic membrane transport systems, including 119 ABC transporters and 12 RND transporters (12). Based on recent intensive searches of a variety of genomes (74), the ABC transporters are found to exist in almost all the phyla of the three major kingdoms, and they form one of the largest protein families (80). The ABC transporters, powered by the energy of ATP hydrolysis, are involved in the transport (uptake or export) of a wide range of molecules including proteins, toxic metal ions, nutrients, and secondary metabolites (80). In Gram-negative bacteria including plant pathogens, the ABC transporters are required for full virulence and the transport of metabolites. For example, they provide fitness in planta (*Pectobacterium*

chrysanthemi) (48), initiate induction of pectate catabolism (*P. chrysanthemi*) (35), facilitate bacterial attachment to plant cells (*Agrobacterium tumefaciens*) (53), and have a role in antagonism of the fungal pathogen, *Phytophthora parasitica* by *P. putida* (46). Similarly, in plant pathogenic fungi such as *Mycosphaerella gramincola* (88), *Magnaporthe grisea* (91), and *Botryis cinerea* (84), ABC transporters are associated with pathogenesis in host plants. Therefore, ABC transporters are considered to be significant virulence factors in many plant pathogens.

Two transporter systems, the SyrD (71) and PseABC efflux systems, were recently characterized as two major efflux systems responsible for secretion of syringomycin and syringopeptin produced by *P. syringae* pv. *syringae* B301D (31). The SyrD protein is homologous to cytoplasmic membrane proteins of the ABC transporter family. Strain BR105 (a *syrD* mutant) shows a significant decrease in lipopeptide phytotoxin secretion, and is less virulent in immature sweet cherry fruits as compared to parental strain B301D (71). The PseC in the PseABC efflux system exhibited significant amino acid sequence similarity to the RND transporter family (31). Strain B301D-HK4 (a *pseC* mutant), similar to the *syrD* mutant, showed a large reduction in syringopeptin secretion, and was subsequently shown to be less virulent than parental strain B301D. Sequencing of cosmid p116 and plasmid JS091 revealed another ABC-type transporter homolog at the left border of the *syp* gene cluster of *P. syringae* pv. *syringae* strain B301D (83). This other ABC-type transporter homolog remains uncharacterized with respect to its function and contribution to virulence.

A 155-kb DNA region encompassing the whole *syr-syp* genomic island of *P.*

syringae pv. *syringae* B301D was recently sequenced (31). An RND-type transporter and two ABC-type transporters were identified in the genomic island. The two transporter systems (SyrD and PseABC) were demonstrated to be involved in secretion of lipopeptide phytotoxins and expression of full virulence of *P. syringae* pv. *sringae* B301D (71). Furthermore, transcript levels of the *syrB1* and *sypA* synthetase genes was reduced by mutations in the efflux genes, indicating a decrease in production of syringomycin and syringopeptin (70, 71). It was speculated that expression of the efflux genes (*syrD*, *pseC*, and *pseF*) might be associated with pathogenicity of *P. syringae* pv. *syringae* in a host plant, and production of lipopeptide phytotoxins was proposed to be upregulated during infection of a host plant (56). Furthermore, it was proposed that the phytobacterial pathogen increased expression of the efflux genes in order to facilitate secretion of lipopeptide phytotoxins. Therefore, it was necessary to test whether transcript levels of genes encoding efflux systems were increased when *P. syringae* pv. *syringae* infected a susceptible host, such as bean.

In this study, an ABC-type transport system, called the *Pseudomonas syringae* syringomycin and syringopeptin efflux system (PseEF efflux system), was characterized to test the hypothesis that the PseEF efflux system was involved in the export of syringomycin and syringopeptin. The objectives of this study were to determine the function of the putative PseEF efflux system and its contribution to virulence in *P. syringae* pv. *syringae* B301D and to test the roles of the efflux systems as virulence factors in *P. syringae* pv. *syringae*. I provided evidence that a mutation in the *pseF* gene causes a significant reduction in secretion of lipopeptide phytotoxins and a

substantial reduction in virulence of *P. syringae* pv. *syringae* B301D. I also demonstrated that the PseEF efflux system are required for full virulence in *P. syringae* pv. *syringae* B728a by showing that the bacterial genes responsible for the efflux systems are induced during infection of a susceptible host (bean) by strain B728a.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 6. DNA manipulations were performed with *E. coli* strain DH10B (Gibco-BRL) cultured at 37°C in Luria-Bertani (LB) broth or on LB agar (78). *P. syringae* pv. *syringae* strains were cultured routinely in nutrient-broth yeast extract (NBY) broth or agar media (94). PDA (potato dextrose agar) supplemented with 0.4% casamino acids and 1.5% glucose was used in bioassays for production of syringomycin and syringopeptin (30). Antibiotics (Sigma) were added to media when required at the following final concentrations: 100 μg/ml of ampicillin, 50 μg/ml of kanamycin, 200 μg/ml of chloramphenicol, and 6.25 to 25 μg/ml of tetracycline.

DNA manipulations and sequence analysis. Routine procedures were used for plasmid isolation from *E. coli*, restriction endonuclease digestion, and subcloning (78). A 20-kb fragment from cosmid p116 and a 4.7-kb *Hind*III fragment from pJS091, containing the *pseE* and *pseF* genes were sequenced (83). Sequence data were analyzed using the Wisconsin Sequence Analysis programs of Genetic Computer Group (GCG) package Version 10.0 and Lasergene expert sequence analysis software (Version 5.0; DNASTAR) (17). GCG programs FINDPATTERNS and TERMINATOR were

TABLE 6. Bacterial strains and plasmids used in chapter III

Strain or plasmid	Relative Characteristics[a]	Reference or source
E. coli strains		
DH10B	F⁻ *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*	Invitrogen
AG100	*ArgE3 thi-1 rpsL xyl mtl galK supE441* Δ*(gal-uvrB)* λ^-	(64)
AG100A	Kan^r, same as AG100 but Δ*acrAB::kan*	(64)
P. syringae pv. *Syringae*		
B301D	Wild type from pear	(14)
BR132	*syrB1*::Tn3HoHo1 derivative of B301D-R; Pip^r Rif^r	(55)
B301D-HK7	*pseF*::*nptII* derivative of B301D; Km^r	This study
BR132-HK7	*pseF*::*nptII* derivative of BR132; Pip^r Rif^r Km^r	This study
B301D-SL10	*syrF*::*nptII* derivative of B301D; Km^r	(50)
B728a	Wild type from bean	(70)
B728a-SL7	*salA*::*nptII* derivative of BR132; Km^r	S. Lu and D. C. Gross *unpublished data*
Plasmid		
pBSK(+)	Cloning vector; Ap^r	Strategene,
pBR325	Cloning vector; Cm^r Tc^r Ap^r	(68)
pBI101	Cloning vector containing *uidA* gene; Km^r	Clonetech
pGEMT-Easy	Cloning vector; Ap^r	Promega
pUCP26	Cloning vector; Tc^r	(65)
pBSL15	Kanamycin resistance gene cassette; Km^r	(3)
p10B-9	Fosmid carrying a 30-kb *P. syringae* pv. *syringae* strain B301D genomic DNA fragment; Ap^r	N. Wang and D. C. Gross *unpublished data*
pHK500	pUCP26 carrying the 9.2-kb *Pml*I fragment from p10B-9	This study
pHK501	pHK500 with the 1.2-kb *nptII* gene from pBSL15 inserted into the *Sma*I site of *pseF*	This study
pHK502	pBR325 carrying the 6.5-kb *Not*I-*Stu*I fragment from pHK501 at the *EcoR*V	This study
pMEKm12	pME10 carrying the 1.2-kb *nptII* gene inserted into the *BamH*I site outside of the MCS; Km^r Ap^r	(51)
pHK503	pMEKm12 carrying the *pseF* gene inserted into the MCS; Km^r Ap^r	This study

[a] Tc^r, Ap^r, and Km^r resistance to tetracycline, ampicillin, and kanamycin, respectively.

used to identify Shine-Dalgarno sequences and to predict rho-independent transcriptional terminators. Sequence randomization and calculation of Z scores were performed using the GAP program, which evaluates the significance of protein sequence similarity as described previously (82). Protein sequence similarity was considered to be significant and to indicate homology when the Z score value was greater than 6. Database searches for genes and proteins were performed using the BLAST servers of the National Center for Biotechnology Information (http://www.ncbi.nih.gov) and the Transporter Protein Analysis Database server (http://66.93.129.133/transporter/wb /index2.html) (5). A motif search was performed using the Pfam server (http://motif.ad.jp/motif-bn/Srch_Motif_Lib) (6). Hydropathy analysis was performed to predict transmembrane segment (TMS) using Protean (Lasergene) (45) and the hydropathy analysis server (http:// megaman.ucsd.edu/progs /hydro.php). Multi-alignment of nucleotide or protein sequences was performed using the MegAlign program (Lasergene) and the MultiAlign server (http://prodes.toulouse.inra.fr/multalin/ multalin.html) (15).

Construction of pHK503 for expression of MBP-PseF. In order to express the *pseF* gene, the *malE* gene encoding maltose binding protein (51) was translationally fused to the *pseF* gene, yielding plasmid pHK503. To amplify the *pseF* gene and confirm an in-frame fusion to the 3' terminus of the *malE* gene, specific primers (F-mal-*pseF* and R-mal-*pseF*) were designed. *Eco*RI or *Bam*HI restriction sites were included in the primers F-mal-*pseF* (5'-GTGGTG AATTCATGAGTCGAGCTCTTCTGG, forward) and R-mal-*pseF* (5'-AAAAAA GGATCCTCAATGCCGTGCCAGTGC,

reverse), respectively for direct cloning. The polymerase chain reaction (PCR) products amplifying the *pseF* gene were inserted into the multiple cloning site of pMEKm12 to generate plasmid pHK503, which expressed a MBP-PseF fusion protein. Sequencing was performed to confirm the in-frame insertion of the *pseF* gene at the 3' terminus of the *malE* gene. The protein was expressed in *E. coli* cultured in LB amended with Isopropyl-beta-D-thiogalactopyranoside (IPTG) to a final concentration of 0.3 mM and purified with maltose affinity chromatography as recommended by the manufacturer (New England Biolabs). Then, both the isolated MBP-PseF fusion protein, and the *E. coli* lysate that expressed the MBP-PseF fusion protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12%) (78).

Mutagenesis of *pseF*. The *pseF* gene was disrupted by insertion of the *nptII* gene (3). A 9.2-kb *Pml*I fragment from cosmid p10B-9 was subcloned into the *Sma*I site of pUCP26, yielding pHK500. A 1.2-kb *nptII* cassette from pBSL15 was inserted at the *Sca*I site of *pseF* in pHK500, generating pHK501. The insertion of the *nptII* gene was confirmed by sequencing pHK501. A 6.0-kb *Not*I-*Stu*I fragment from pHK501 was cloned into the *EcoR*V site of pBR325, yielding pHK502. To allow marker exchange mutagenesis to occur, *P. syringae* pv. *syringae* B301D was transformed with pHK502 by electroporation using a Gene Pulser II (Bio-Rad Laboratories) as described previously (14). Transformants were selected on NBY agar supplemented with kanamycin. Double crossover mutations were confirmed by Southern analysis and by the polymerase chain reaction (PCR) (Fig. 11). The confirmed *pseF* mutant was labeled as B301D-HK7. A *syrB1* and *pseF* double mutant, labeled as BR132-HK7, was also generated by

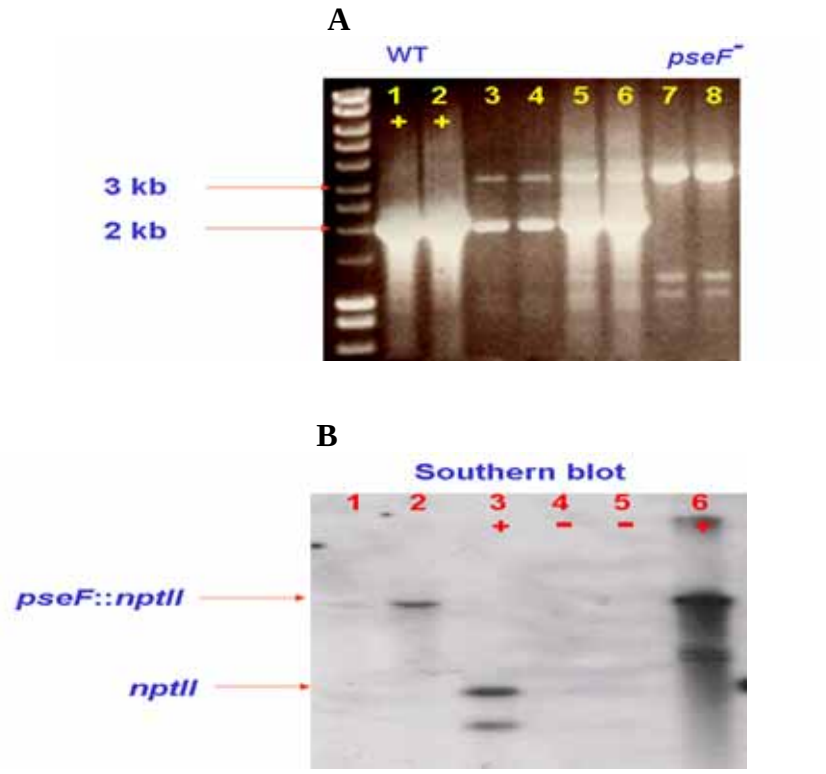

FIG. 11. Confirmation of strain B301D-HK7 (a *pseF* mutant) using PCR and Southern analysis. In panel A (PCR confirmation), the disrupted *pseF* gene amplified by PCR was detected due to its different size (3.2 kb) from that of the intact *pseF* gene (2.0 kb). Lanes 1 and 2 contain the *pseF* genes amplified from parental strain B301D and the amplified *pseF* genes were approximately 2.0 kb in size. Lanes 3, 4, 5, and 6 show both intact *pseF* genes (2.0 kb) and disrupted *pseF* genes (3.2 kb) by the *nptII* (1.2 kb) insertional mutation, which were amplified from single crossover *pseF* mutants. Lanes 7 and 8 demonstrated only disrupted *pseF* genes amplified from double crossover *pseF* mutants. In panel B (Southern analysis), the disrupted *pseF* gene was probed with the *nptII* gene in Southern analysis. Lanes 1, 2, 3, and 6 demonstrated the existence of the *nptII* genes in the genomes of double crossover *pseF* mutants (lanes 1 and 2), in pSL15 (3) containing only the *nptII* gene (lane 3), and in pBR325 containing the *pseC*::*nptII* construct (lane 6). Lanes 4 and 5 verified the absence of the *nptII* gene in the genome of parental strain B301D and the pBR325 vector, respectively.

marker exchange of the *pseF*::*nptII* construct into the genome of *P. syringae* pv. *syringae* strain BR132 (a *syrB1* mutant) (55).

Screening for syringomycin and syringopeptin secretion by the *pseF* mutant. Strains B301D-HK7 (a *pseF* mutant) and BR132-HK7 (a *syrB1* and *pseF* double mutant) were assayed for secretion of syringomycin and syringopeptin using standard bioassays as previously reported (81), except these strains were cultured on PDA (potato dextrose agar) medium plates. To assay syringomycin production, the plates were incubated for 72 h at 25˚C and the indicator fungus *Geotrichum candidum* F-260 was oversprayed and the plates were incubated at 25˚C for another 24 h. To assay syringopeptin production, the plates were incubated for 48 h at 25 ˚C and the indicator bacterium *Bacillus megaterium* Km was oversprayed and the plates were incubated at 25˚C for another 24 h. Resultant zones of growth inhibition to *G. candidum* and *B. megaterium* were measured. A low concentration of tetracycline (6.25 µg/ml) was added to PDA medium in order to maintain pHK500. IPTG (0.5 mM) was added to PDA medium to facilitate expression of the *pseF* gene, which was inserted in-frame and downstream of the *lacZ* promoter in a pUCP26 vector. The PDA plate bioassays were replicated six times.

Virulence assays in immature cherry fruits. Virulence assays of strains B301D-HK7 (a *pseF* mutant) and BR132-HK7 (a *syrB1* and *pseF* double mutant) were performed in immature cherry fruits as described previously (81). Each wounding site formed on cherry fruits was inoculated with 5 x 10^3 CFU of each strain of *P. syringae* pv. *syringae*. The inoculated fruits were incubated for 4 days at 20°C. Virulence was

determined by measuring the diameters of the necrotic lesions formed at each inoculation site. For each experiment, 10 cherry fruits were inoculated per treatment, and the experiment was repeated three times. Parental strain B301D and strain BR132 (a *syrB1* mutant) were used as controls.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was used to detect the effect of the *pseF* mutation on expression of the syringomycin and syringopeptin synthetase genes (25). Bacterial RNA was extracted using the RNeasy Mini kit (Qiagen) from *P. syringae* pv. *syringae* B301D cultured at 25ºC for 72 h on syringomycin minimum medium (SRM) (56) supplemented with the plant signal molecules arbutin and D-fructose (SRM_{AF}) (56). The purified RNA was prepared according to the manufacturer's instructions, which required DNase digestion using an RNase-Free DNase Set (Qiagen). Oligonucleotide primers were designed using PrimerSelect software (Version 5.0; DNASTAR). Quantitative real-time RT-PCR was accomplished using a QuantiTect SYBR Green RT-PCR kit (Qiagen) and the Smart Cycler (Cepheid).

Reaction components were prepared according to the manufacturer's instructions, except that each reaction was set up in 25 µl with 100 ng of template RNA and 1.25 pmole of each primer. The reverse transcriptase (RT) reaction was performed for 30 min at 94°C, 30 s of primer annealing at 54°C, followed by 45 cycles of 15 s of denaturation at 94°C, 30 s of primer annealing at 54°C, and 30 s of polymerization at 60°C. Primers were evaluated by following the manufacturer's instructions (Qiagen). The fold induction of mRNA was determined from the threshold values (C_T) that were

normalized for 16S rDNA expression (endogenous control) and then normalized to the C_T value obtained from parental strain B301D (4).

Before determining expression profiles of the *syrD*, *pseC*, *pseF*, and *pvdS* genes, the relative amplification efficiencies of the *syrD*, *pseC*, *pseF*, and *pvdS* primer pairs were assessed as described in the manufacturer's instructions (47). Differences in amplification efficiency of the *syrD*, *pseC*, *pseF*, and *pvdS* primer pairs were less than 0.1, which indicated that the amplification efficiencies were approximately equal. Primers used in quantitative real-time RT-PCR are listed in Table 2.

Minimum inhibitory concentration (MIC) tests. The antimicrobial susceptibilities to acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin and novobiocin (Sigma) were tested using a microtiter broth dilution method (49). The susceptibilities of strain B301D-HK7 (a *pseF* mutant) were compared with those of parental strain B301D and *P. aeruginosa* strain PAO1 (40). In addition to *Pseudomonas* spp., *E. coli* strains AG100A (an *acrB* mutant) and AG100 (*acrB*$^+$ parent) were transformed with pHK500, which carries the *pseE* and *pseF* genes. Resultant *E. coli* strains were used to test for whether the PseEF efflux system enhances the resistance to antibiotics of mutant strain AG100A. Parental strain AG100 was used as a control. Briefly, exponential phase bacterial cells were added to a sterile 96-well microtiter plate containing Mueller-Hinton (MH) broth and serial two-fold dilutions of antibiotics (19). The final cell concentration was adjusted to 4 x 10^4 CFU/ml per well. The *E. coli* strains were incubated at 37°C for 12 h. The *Pseudomonas* strains were cultured at 25°C for 18 h. The MIC was defined as the lowest concentration of antibiotics

TABLE 7. Primer sequences used for quantitative real-time RT-PCR (B301D-HK7)

Genes	Primer[a]
syrB1……..	F-RT-*syrB1*: TTAGCGCCGCGTCAGCCCCT CTCAAG
	R-RT-*syrB1*: GCTCAACGTCCGGGCTGCATCGCTCAC
sypA……..	F-RT-*sypA*: TGCGGGTCGAGGCGTTTTTG
	R-RT-*sypA*: GTTGCCGCGTCCTTGTCTGA
syrD……..	F-RT-*syrD*: GGAACTGCTGCCGGACCTCAA
	R-RT-*syrD*: GC CCTCAACCGCGCACTTCAC
pseC……..	F-RT-*pseC*: TCGGCGTGCCCAGGG ATTTG
	R-RT-*pseC*: GCCATGGAGCCGCGATAGTTTT
pseF……..	F-RT-*pseF*: TCACCGCGATCAACGACAG CAACA
	R-RT-*pseF*: GCAAAAGCGGCACGGGACCAAAGA
pvdS……..	F-RT-*pvdS*: GGAACACGTAATCACAAGTAAG
	R-RT-*pvdS*: GAGCGCAGTCTGAAAAAGGCA
16S……	F-RT-16S: ACACCGCCCGT CACACCA
	R-RT-16S: GTTCCCCTACGGCTACCTT

[a] F, forward ; R, reverse

inhibiting visible growth (87).

Determination of expression profiles of the efflux genes enhanced by plant signal molecules. Quantitative real-time RT-PCR was used to test whether the bacterial efflux genes (*syrD*, *pseC*, and *pseF*) were induced by the plant signal molecules arbutin and D-fructose (56). *P. syringae* pv. *syringae* B301D was cultured on both SRM and SRM_{AF} plates at 25ºC for 72 h (56). RNA was extracted using the RNeasy Mini kit (Qiagen). Transcript levels of the efflux genes were determined by using quantitative real-time RT-PCR. Expression of the efflux genes by parental strain B301D cultured on SRM was compared with that of parental strain B301D cultured on SRM_{AF}. Differences in transcript levels were expressed as fold induction as described in this study.

Plant inoculation and determination of expression of efflux genes *in planta*. Transcript levels of the bacterial efflux genes *syrD*, *pseC*, and *pseF* were determined when disease symptoms caused by *P. syringae* pv. *syringae* had developed in a susceptible plant. For experimental reproducibility, it was necessary to cultivate the susceptible plant in controlled culture conditions. Thus, rather than using immature cherry fruits, bean plants (Blue Lake 274) were used in this experiment because they are easily cultivatable in a growth chamber. Bean seeds were planted in soil and grown for three weeks in a growth chamber at 25°C, 95% humidity, and with a 16 h photoperiod. *P. syringae* pv. *syringae* B728a (a parental strain and a *salA* mutant strain) was cultured in NBY broth at 25˚C overnight. The bacterial cells were pelleted by centrifugation and washed twice with sterile water. The washed cells were resuspended in sterile

water to a concentration of 5 x 10^5 cells/ml. Cell suspensions of strain B728a (a parental strain and a *salA* mutant strain) were inoculated into bean leaves by vacuum infiltration (approximately 2 min). Then, the inoculated bean leaves were incubated at 25˚C in a growth chamber with 95% humidity for 72 h (a parental strain) or 24 h (a *salA* mutant strain). Total RNA (plant and bacterial RNAs) was isolated from lesions of bacterial brown spot, which developed in the infected bean leaves. Total RNA was extracted using a protocol for isolation of total RNA from plant cells and tissues infected by filamentous fungi as described by Qiagen. The integrity of total RNAs isolated from lesions of plant leaves was tested by running 7 μg of the RNAs on 1% agarose gels, staining with EtBr, and the RNAs appeared intact. Analysis of the RNA from *salA* mutant using Bioanalyzer (Bio-Rad), more sensitive assay, showed some degradation (28S to 18S radio 2.69), but the RNA was largely intact. Quantitative real-time RT-PCR was used to determine the expression profiles of the bacterial efflux genes under the same conditions described above, except that 300 ng of the total RNA were used as template.

Statistical analysis. Means in each test were compared with one another by conducting an ANOVA analysis and Tukey's W procedure (66).

RESULTS

Sequence analysis of the *pseE* and *pseF* genes. Sequencing of p116 and pJS091 revealed two open reading frames (ORFs) (83). ORF1 and ORF2 were predicted to encode a periplasmic membrane fusion protein and a cytoplasmic ABC-type transporter,

respectively (Fig. 12). The stop codon (TGA) of ORF1 was separated by 2 bp from the start codon (ATG) of ORF2.

The protein encoded by ORF1, PseE, was 385 amino acids in length. PseE protein showed 47.2% identity (Z score, 116) to the macrolide efflux protein MacA of *E. coli* W3104 (42), which belongs to the membrane fusion protein family. A probable Shine-Dalgarno sequence (TCGTGG) was identified 7 bp upstream of the start codon (ATG) of the *pseE* gene. There was no rho-independent transcriptional terminator identified downstream of the stop codon (TGA) of the *pseE* gene. A motif search predicted that PseE protein had a hemolysin (Hly) D family secretion protein signature (26). The HlyD protein is a member of the membrane fusion protein family (39). The signature corresponded to residues 59 through 217 of the PseE protein (E-value, 2.4e-10), and it is predicted to be associated with a periplasmic efflux protein (membrane fusion protein) that makes a bridge between an outer membrane protein and a cytoplasmic efflux protein (39). PseE protein was predicted to contain one transmembrane segment (TMS) at the N terminus by hydropathy analysis (69). Five positively charged residues were followed by 16 hydrophobic amino acid residues in the TMS of PseE protein. This sequential arrangement of positively charged and hydrophobic residues indicated the presence of a signal-like sequence in the PseE protein (42). The ABC-type transporter encoded by ORF2, called PseF, was predicted to be 653 amino acids in length. PseF protein showed 57.6% identity (Z score, 394) to the macrolide-specific ABC-type efflux carrier MacB in *E. coli* W3104 (42), and 32.1% identity (Z score, 45) to the SyrD protein

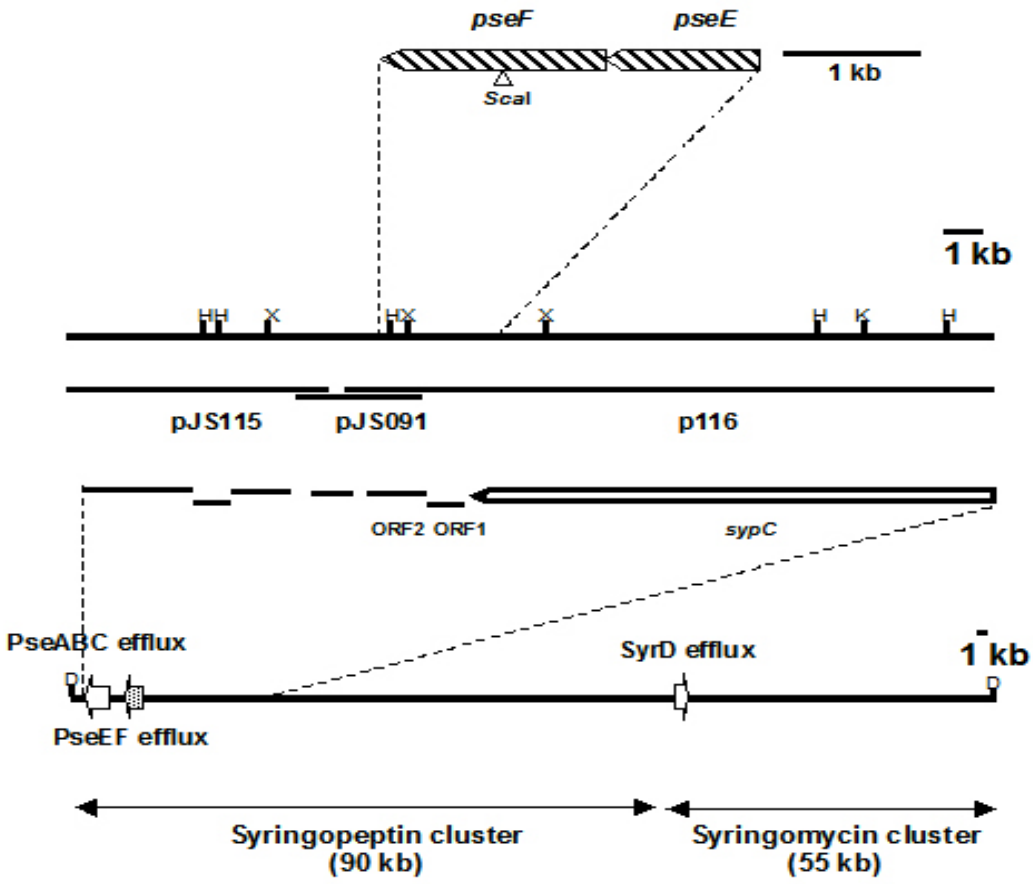

FIG. 12. Diagrammatic representation of the *syr-syp* genomic island on the chromosome of *P. syringae* pv. *syringae* B301D and location of the PseEF efflux system. The approximately 145-kb *Dra*I fragment consists of the syringopeptin (*syp*) gene cluster (90 kb) and the syringomycin (*syr*) gene cluster (55 kb) (83). The left border of *syp* gene cluster, 51-kb region, is mapped and shown on the *Dra*I fragment. The *pseE* (1.2 kb) and *pseF* (2.0 kb) genes are indicated on the map of the 51-kb region. Mutant strain B301D-HK7 was generated by disrupting the *pseF* (ORF2) gene by *nptII* insertional mutagenesis. The triangle identifies the restriction site in which the *nptII* cassette was inserted in the *pseF* gene. The stippled arrow represents the location of the PseEF efflux system, while two open arrows indicate the locations of the PseABC and SyrD efflux systems. The restriction enzyme sites are indicated as follows: D=*Dra*I, H= *Hind*III, K=*Kpn*I, and X=*Xho*I.

in *P. syringae* pv. *syringae* B301D (71). A probable Shine-Dalgarno sequence (CGGGGG) was found 8 bp upstream of the *pseF* gene. An approximately 110-kDa MBP-PseF fusion protein was overexpressed in *E. coli*, yielding a nearly 70-kDa PseF protein (Fig. 13). It was similar to the size (71.1-kDa) predicted by the Peptide Mass Server (http://us.expasy. org/cgi-bin/peptide-mass.pl). The secondary structure (TCCGGC GTTATCCCGG A) of a rho-independent transcriptional terminator was observed 747 bp downstream of the stop codon (TGA) of the *pseF* gene (secondary structure value, 43). A motif search predicted that the PseF protein contained motifs characteristic of the ABC transporter family in the N-terminal region (E-value, 1.4e-57) (69). The N-terminal region (34 to 222) of PseF protein was shown to contain a nucleotide-binding cassette domain that consists of a Walker A motif, an ABC transporter signature, and a Walker B motif (Fig. 14) (79). The presence of these motifs in PseF protein was consistent to those observed in the MacB protein (42). PseF protein was predicted to contain four TMSs by hydropathy analysis (Fig. 15) (69).

Screening for syringomycin and syringopeptin secretion by the *pseF* mutant. Strain B301D-HK7 (a *pseF* mutant) was screened for secretion of syringomycin and syringopeptin on PDA medium using *G. candidum* and *B. megaterium* as indicator microorganisms, respectively (81). Mutant strain B301D-HK7 produced zones of inhibition to *G. candidum* that showed an approximately 71% reduction (3.3 mm radius) as compared to those produced by parental strain B301D (11.3 mm radius) (Fig. 16 and 17). Zones of inhibition to *B. megaterium* produced by mutant strain B301D-HK7 (2.9 mm) showed a 74% reduction as compared to those produced by parental strain B301D

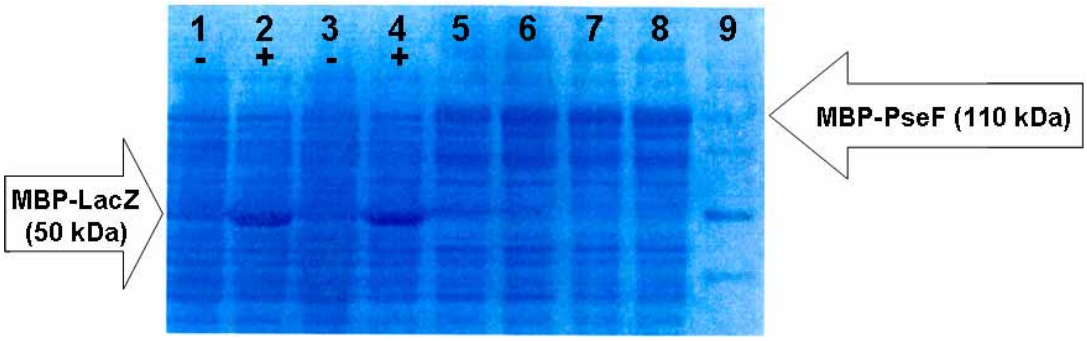

FIG. 13. SDS-PAGE analysis of *E. coli* DH10B producing a fusion protein of the maltose-binding protein and PseF protein. Cells were induced by 0.3 mM IPTG at 37˚C for 2 h (*E. coli)*. Proteins were separated by 12% acrylamide gels at 120 V and stained with Coomassie blue (51). Lanes: 1 and 3, DH10B, empty cells; 2 and 4, DH10B (pMEKm12) (51); 5 to 8, DH10B (pHK503); 9, protein molecular marker (10-250 kDa) (Bio-Rad).

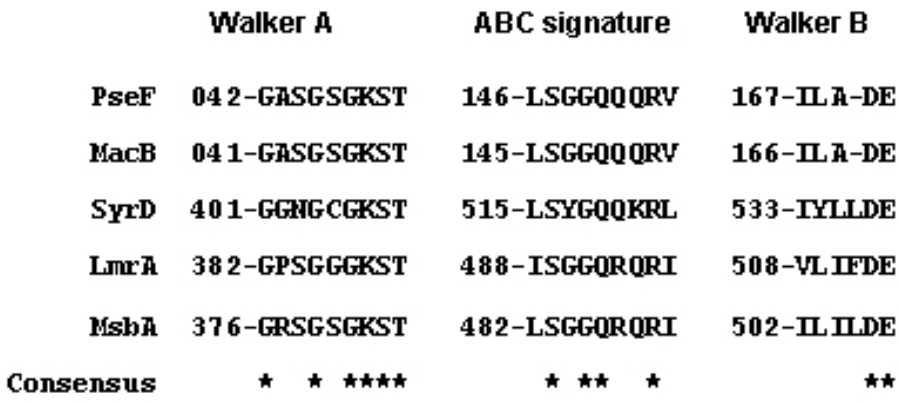

FIG. 14. Amino acid sequence alignment of the ATP-binding domains of the ABC transporters. The ATP-binding domain (Walker A motif, ABC signature, and Walker B motif) of the PseF protein was aligned with those of the MacB protein of *E. coli* (42), the SyrD protein of *P. syringae* pv. *syringae* (71), the LmrA protein of *Lactococcus lactis* (93), and the MsbA protein of *E. coli* (13). The Walker A motif, ABC signature, and Walker B motif are indicated above the amino acid sequences. The amino acid residue number for each protein is indicated at the beginning of each sequence. Consensus amino acid residues are marked with asterisks.

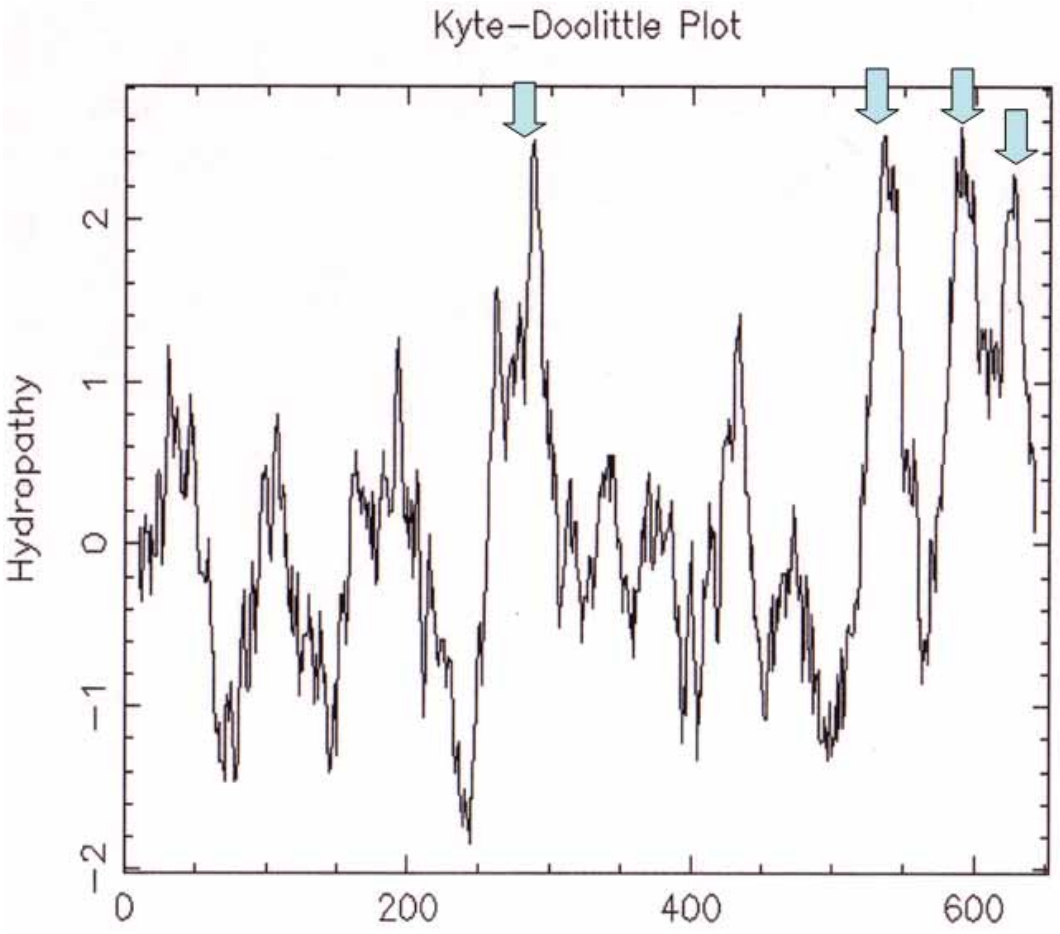

FIG. 15. Hydropathy analysis and prediction of the transmembrane segments (TMSs) of the PseF protein (45). The PseF was predicted to contain four TMSs. Arrows indicate locations of TMSs within the PseF protein.

(10.9 mm radius) (Fig. 16 and 17). However, mutant strain B301D-HK7 that carried pHK500 *in trans* exhibited large zones with the radius of the zone of inhibition to *G. candidum* and *B. megaterium* averaging approximately 9.8 mm and 7.7 mm, respectively (Fig. 17). These data indicated complementation of mutant strain B301D-HK7 by pHK500, which carries the *pseEF* efflux genes.

Effect of the *pseF* mutation on expression of the syringomycin and syringopeptin synthetase genes. Transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes were determined using quantitative real-time RT-PCR (8). Parental strain B301D and strain B301D-HK7 (a *pseF* mutant) were cultured on SRM_{AF} medium for 72 h (56), followed by determination of the expression profiles of the synthetase genes for the two strains using quantitative real-time RT-PCR. Expression of the *syrB1* and *sypA* genes in mutant strain B301D- HK7 was approximately 14.6% and 6.9%, respectively, of those in parental strain B301D (Fig. 18).

The *pseF* mutant showed significant reduction in virulence. The virulence of strains B301D-HK7 (a *pseF* mutant) and BR132-HK7 (a *syrB1* and *pseF* double mutant) was determined in immature Bing cherry fruits using methods described previously (Fig. 19) (71). The lesion diameters were used to quantify relative virulence in the cherry fruits. Mutant strains B301D-HK7 and BR132-HK7 produced lesions nearly 2.2 mm in diameter, which corresponded to approximately 33% ($P = 0.05$) of those formed by parental strain B301D (7.0 mm). In comparison, the average lesion produced by strain BR132 (a *syrB1* mutant) (5.0 mm) corresponded to approximately 60% of that observed for parental strain B301D.

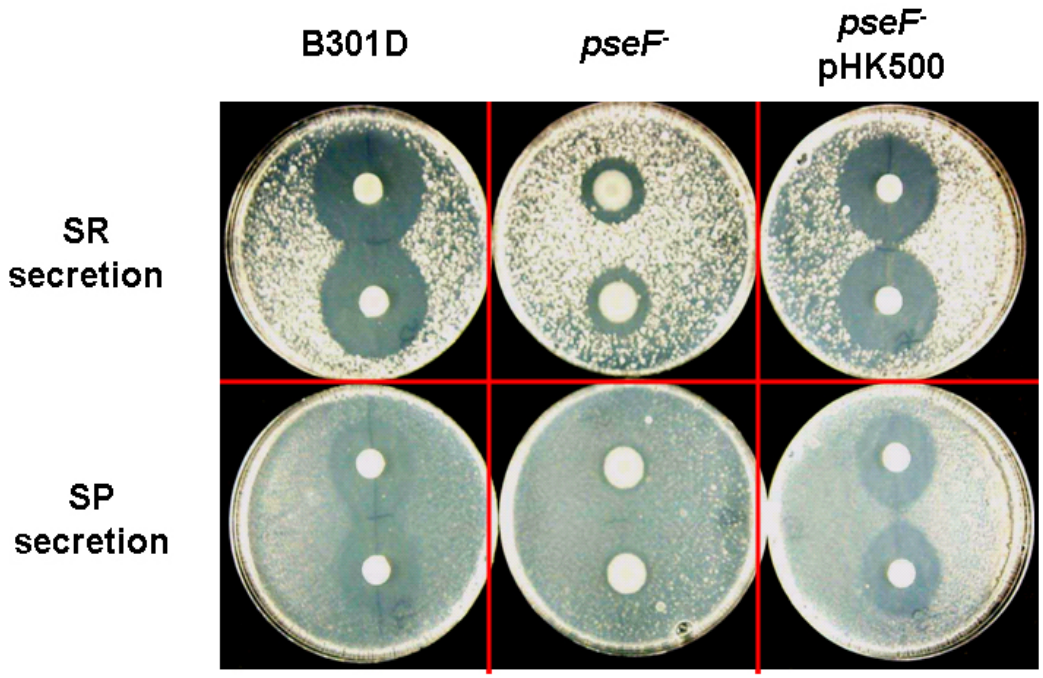

FIG. 16. Inhibition due to syringomycin and syringopeptin secretion by strain B301D-HK7 (a *pseF* mutant). A bioassay for syringomycin was performed by incubation of the strains on PDA medium for 3 days and then overspraying with *G. candidum*. For the bioassay of syringopeptin, the strains were incubated for 2 days on PDA medium, followed by overspraying with *B. megaterium*. Syringomycin and syringopeptin are abbreviated as SR and SP, respectively. pHK500 is pUCP26 carrying the *pseEF* efflux genes. *pseF*⁻ indicates strain B301D-HK7 (a *pseF* mutant).

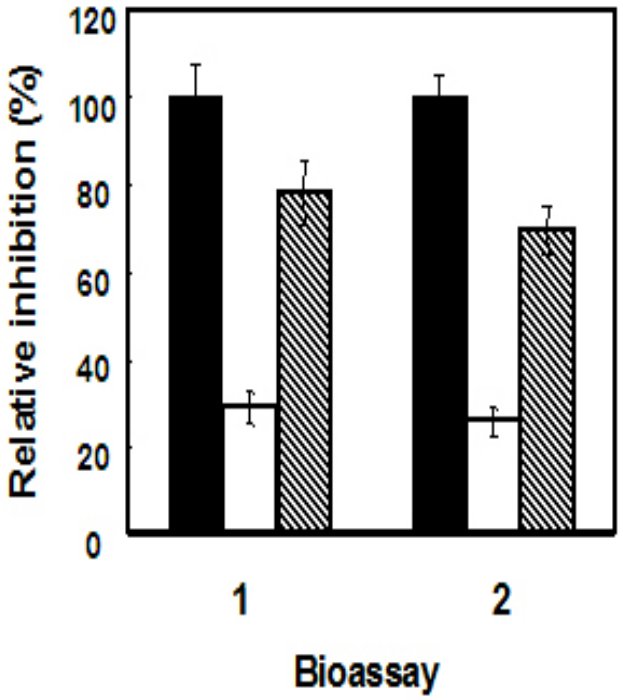

FIG. 17. Relative inhibition due to secretion of syringomycin and syringopeptin by strain B301D-HK7 (a *pseF* mutant). A bioassay for production of syringomycin and syringopeptin was performed by incubation of the strains on PDA medium containing 0.5 mM IPTG for 72 h and 48 h, respectively, followed by overspraying with *Geotrichum candidum* F-260, for the syringomycin bioassay, or *Bacillus megaterium* Km, for the syringopeptin bioassay. Zones of inhibition to *G. candidum* F-260 and *B. megaterium* Km due to the production of syringomycin and syringopeptin were measured. Inhibition zones to *Geotrichum* and *Bacillus* due to lipopeptide phytotoxins produced by mutant strain B301D-HK7 (open bar) and B301D-HK7 carrying pHK500 (hatched bar) were compared with those by parental strain B301D (solid bar). Column 1, bioassay for syringomycin production; and 2, bioassay for syringopeptin production.

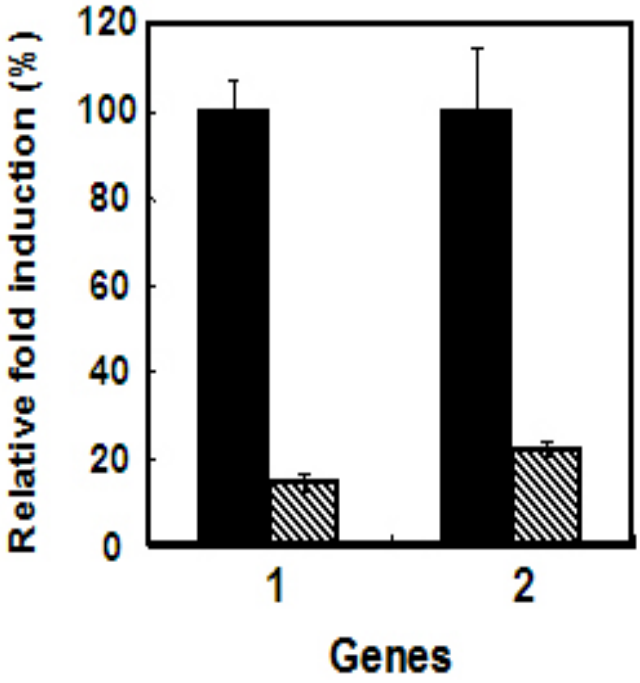

FIG. 18. Effects of the *pseF* mutation on expression of the *syrB1* (55) and *sypA* (81) synthetase genes. Using quantitative real-time RT-PCR, transcript levels of the synthetase genes in strain B301D-HK7 (*pseF* mutant, hatched bar) was compared to that in parental strain B301D (solid bar). Relative differences in expression of the synthetase genes were measured (4). The relative level of mRNA was determined from the threshold values (C_T) that were normalized for 16S rDNA expression (endogenous control) and then the WT (parental strain B301D) value was defined as 100%. Error bars represent the standard error of the means. Expression of the *syrB1* (column 1) and *sypA* (column 2) genes by mutant strain B301D-HK7 were compared relative to that of parental strain B301D.

Antibiotic susceptibility of the *pseF* mutant. Antimicrobial susceptibility tests were performed to determine whether the PseEF efflux system contributed to antibiotic resistance in *P. syringae* pv. *syringae* strains. The susceptibilities to all the tested antibiotics were observed to be similar between strains B301D-HK7 (a *pseF* mutant), B301D-HK4 (a *pseC* mutant) and parental strain B301D (Table 8); MICs for acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin, and tetracycline were 12.5 μg/ml, 100 μg/ml, 200 μg/ml, 50 μg/ml, 12.5 μg/ml, 6.3 μg/ml, and 0.2 μg/ml, respectively. In comparison, *P. aeruginosa* strain PAO1 also exhibited MICs similar to those obtained from the previous MIC test (Table 8); MICs for acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin and tetracycline were 200 μg/ml, 50 μg/ml, 50 μg/ml, 200 μg/ml, 100 μg/ml, 12.5 μg/ml, and 3.1 μg/ml, respectively.

In a previous study (Chapter II) it was shown that heterologous expression of the *pseA, pseB,* and *pseC* genes in *E. coli* strain AG100A (an *acrB* mutant) increased resistance to gentamicin two times as compared to that of the mutant strain AG100A without the expression of the *pseA, pseB,* and *pseC* genes. Thus, it was tempting to test whether the PseEF efflux system has a functional relationship with the PseABC efflux system. Using the same method described previously, mutant strain AG100A was transformed with pHK500 and then assessed for susceptibility to aztreonam, chloramphenicol, acriflavine, erythromycin, gentamicin, novobiocin, and tetracycline (Table 9). Similar to the PseABC efflux system, the PseEF efflux system failed to increase resistance of mutant strain AG100A to the tested antibiotics except for

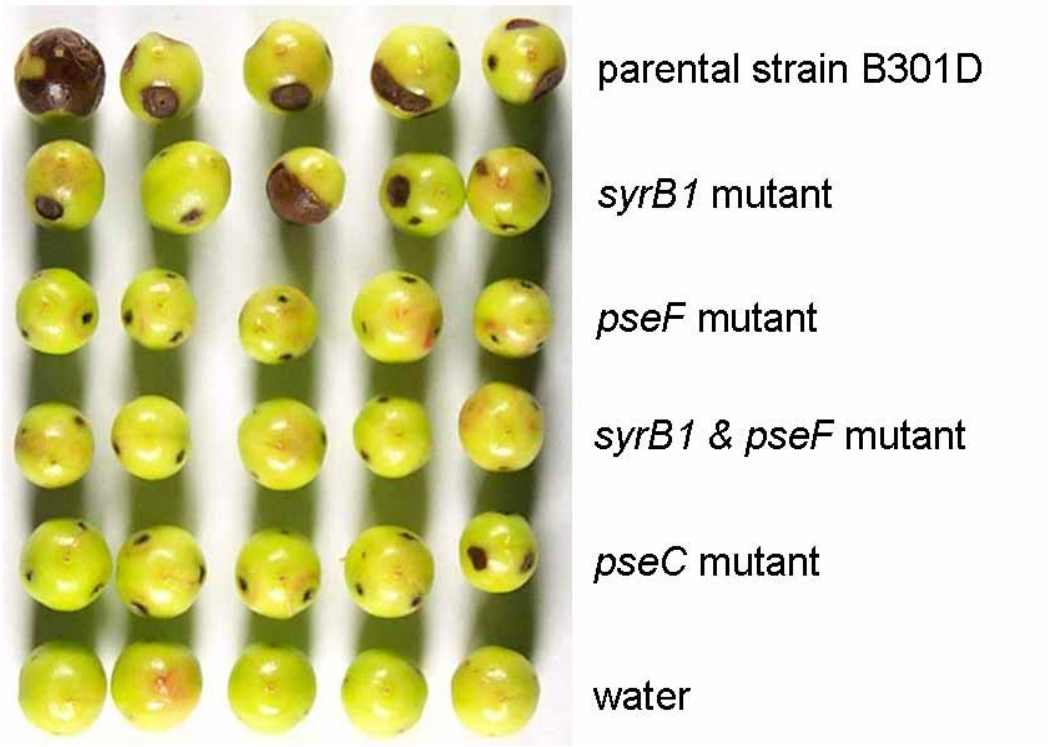

FIG. 19. Virulence test of the *pseF* mutants. Virulence of strains B301D-HK7 (a *pseF* mutant), BR132 (a *syrB1* mutant), BR132-HK7 (a *syrB1* and *pseF* double mutant), and B301D-HK4 (a *pseC* mutant) was compared to parental strain B301D in immature cherry fruits. Each fruit was incubated at three sites with 5 x 10^3 cells per site. Control fruits were injected with sterile water. Symptoms were observed 4 days after inoculation.

TABLE 8. Antibiotic susceptibility of *P. syringae* pv. *syringae* strains B301D, B301D-HK7 and *P. aeruginosa* strain PAO1

Strains	MIC (μg/ml)[a]						
	AF	AZ	CA	CM	EM	GM	TC
B301D[b]	12.5	100	200	50	12.5	6.25	0.20
B301D-HK7[c]	12.5	100	200	50	12.5	6.25	0.20
PAO1[d]	200	50	50	200	100	12.5	3.13

[a]Abbreviations: AF, acriflavine; AZ, aztreonam; CA, carbenicillin; CM, chloramphenicol; EM, erythromycin; GM, gentamicin; TC, tetracycline.

[b] *P. syringae* pv. *syringae* strain B301D (a parental strain).

[c] *P. syringae* pv. *syringae* mutant strain B301D-HK7 (a *pseF* mutant).

[d] *P. aeruginosa* strain PAO1 (wild type).

gentamicin. Resistance to gentamicin was increased two times in mutant strain AG100A with heterologous expression of the *pseE* and *pseF* genes as compared to mutant strain AG100A without expression of the *pseE* and *pseF* genes. Mutant strain AG110A carrying pHK500 produced MICs for gentamicin of 0.4 μg/ml, whereas mutant strain AG100A carrying only a pUCP26 vector exhibited MICs for gentamicin of 0.2 μg/ml. In comparison, *E. coli* parental strain AG100 carrying a pUCP26 vector was used as a control, and its MIC for gentamicin was 0.8 μg/ml.

Expression of the efflux genes (*syrD, pseC,* and *pseF*) was induced by plant signal molecules. It was tested whether the plant signal molecules arbutin and D-fructose (56) were able to induce expression of the efflux genes. Transcript levels of the efflux genes were determined using quantitative real-time RT-PCR. Bacterial RNAs were extracted separately from *P. syringae* pv. *syringae* B301D that was cultured on SRM and SRM_{AF} media at 25ºC for 72 h (Fig. 20) (56). Expression of the synthetase (*syrB1*, and *sypA*) and efflux genes (*syrD*, *pseF*, and *pseC*) in strain B301D grown on SRM_{AF} (in the presence of plant signal molecules) was compared with that in strain B301D grown on SRM (in the absence of plant signal molecules) (Fig. 21). As predicted, expression of the synthetase genes was enhanced due to the presence of plant signal molecules (12- and 20-fold induction for *syrB1* and *sypA*, respectively). Expression of the efflux genes was also induced significantly on SRM_{AF} (10-, 6-, and 14-fold induction for *syrD*, *pseF*, and *pseC*, respectively). Expression of the *pvdS* gene was used as a control (73). The *pvdS* gene encodes an alternative sigma factor that helps RNA polymerase initiate transcription of the *pvdA* gene encoding L-ornithine

TABLE 9. Antibiotic susceptibility of *E. coli* mutant strain and its transformants expressing the *pseEF* efflux genes

Strains (plasmid)	MIC (μg/ml)[a]						
	AF	AZ	CM	EM	GM	NV	TC
AG100[b] (pUCP26)	100	0.10	3.1	25	0.8	100	100
AG100A[c] (pUCP26)[d]	50	0.05	1.6	12.5	0.2	100	25
AG100A (pHK500)[e]	50	0.05	0.8	1.6	0.4	100	25

[a]Abbreviations: AF, acriflavine; AZ, aztreonam; CM, chloramphenicol; EM, erythromycin; GM, gentamicin; NV, novobiocin; and TC, tetracycline.
[b] *E. coli* strain AG100 (a parental strain) (64).
[c] *E. coli* strain AG100A (an *acrB* mutant) (64).
[d] pUCP26 carrying the tetracycline resistance genes (65).
[e] pUCP26 carrying the *pseEF* efflux genes.

FIG. 20. Inhibition due to syringopeptin (SP) secretion by *P. syringae* pv. *syringae* strain B301D on syringomycin minimum medium supplemented with plant signal molecules (SRM_{AF}). A bioassay for syringopeptin was performed by incubation of the strain on SRM_{AF} for 3 days and then oversprayed with *B. megaterium*.

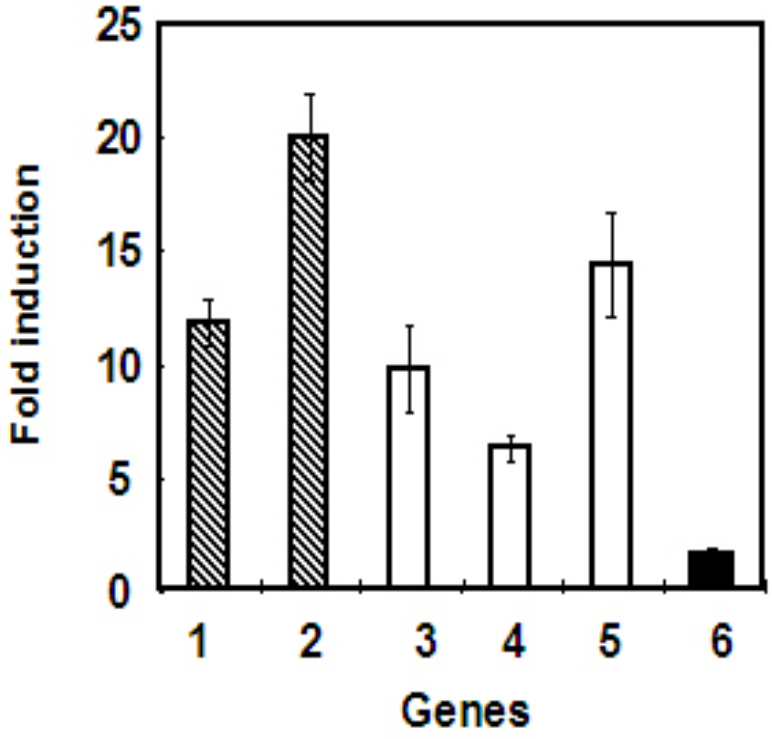

FIG. 21. Transcript levels of the synthetase genes (*syrB1* and *sypA*) and efflux genes (*syrD*, *pseF*, and *pseC*) in *P. syringae* pv. *syringae* strain B301D grown on SRM_{AF} (56). Quantitative real-time RT-PCR was used to determine differences in transcript levels of the synthetase and efflux genes between strain B301D cells cultured separately on SRM and SRM_{AF} media at 25ºC for 72 h (56). The fold induction of mRNA was determined from the threshold values (C_T), which were normalized for 16S rDNA expression (endogenous control), and then expressed relative to the normalized C_T value obtained from strain B301D grown on SRM (4). Column 1, *syrB1* gene (hatched bar); 2, *sypA* gene (hatched bar); 3, *syrD* gene (open bar); 4, *pseF* gene (open bar); 5, *pseC* gene (open bar); and 6, *pvdS* gene (control, solid bar).

hydroxylase. The resultant hydroxylase is involved in pyoverdine biosynthesis (36). Expression of the *pvdS* gene by strain B301D grown on SRM_{AF} was compared with that of strain B301D grown on SRM, and it was relatively low (less than 2-fold induction) as compared to expression of the synthetase and efflux genes (Fig. 21).

The *pseF* and *pseC* genes were highly induced during infection of *P. syringae* pv. *syringae* B728a in bean. Comparison of the *syr-syp* genomic islands of strain B301D and B728a revealed that the genomic islands of the two strains are highly conserved (31). Supporting the conservation of the *syr-syp* genomic island, genes encoding the efflux systems (SyrD, PseABC, and PseEF efflux systems) were shown to share 99% nucleotide identity between the two strains. The transcript levels of the bacterial efflux genes (*syrD*, *pseC*, and *pseF*) during infection of a susceptible host were determined for strain B728a using quantitative real-time RT-PCR.

The *salA* mutant fails to produce syringomycin (41) and is significantly reduced in expression of a *syrB1*::*uidA* reporter construct (50). Thus, it was speculated that expression of the *syr* and *syp* genes might be as low as the *syrB1* and *sypA* double mutant (strain BR-DBL1) (81). In fact, transcript levels of the synthetase and efflux genes in the background of strain B728a-SL7 (a *salA* mutant) were low (C_T values, 37 to 43). This characteristic of mutant strain B728a-SL7 was used to normalize transcript levels (C_T values) of the synthetase and efflux genes in parental strain B728a.

Parental strain B728a and mutant strain B728a-SL7 harvested from infected bean plants were evaluated for transcript levels of the synthetase and efflux genes. Then, comparisons of transcript levels were made between the two strains. Using vacuum

infiltration, the two strains were inoculated into bean leaves and then incubated for different incubation periods. Bacterial brown spot symptoms were observed in bean leaves 24 h incubation after inoculation with parental strain B728a (Fig. 22). At longer incubation periods, the necrotic lesions characteristic of bacterial brown spot increased in number and size (41). In contrast, no clear necrotic lesions were observed on bean leaves after inoculation with mutant strain B728a-SL7 (Fig. 23). Total RNAs (plant and bacterial RNAs) were then isolated from the lesions after inoculation. Expression profiles of the bacterial efflux genes were determined. As predicted, it was observed that expression of the synthetase and efflux genes was enhanced as necrotic lesions of bacterial brown spot developed in the infected bean leaves (Fig. 24). As severity of necrotic lesions increased, expression of the *syrB1* and *sypA* genes by parental strain B728a was high at 3 day after inoculation (dai) (222- and 377-fold induction for *syrB1* and *sypA*, respectively). Similarly, expression of the *pseF* and the *pseC* genes in parental strain B728a was high at 3 dai (180- and 172-fold induction for *pseF* and *pseC*, respectively). However, expression of the *syrD* gene in parental strain B728a was at low levels at 3 dai (39-fold induction) as compared to expression of the *pseC* and *pseF* genes. As a control, expression profiles of the *pvdS* gene (73) by parental strain B728a and mutant strain B728a-SL7 were determined. Differences in levels of *pvdS* expression between the two strains were insignificant (5-fold induction for the *pvdS* gene at 3 dai, data not shown).

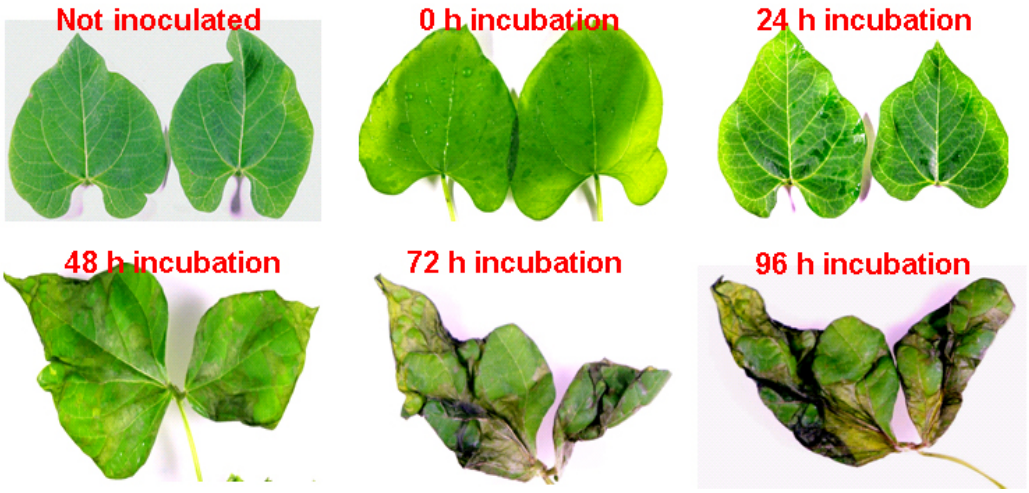

FIG. 22. Gradual development of necrotic lesions of bacterial brown spot of bean caused by P. *syringae* pv. *syringae* strain B728a. Bean leaves were inoculated with strain B728a using vacuum infiltration. Resultant bean leaves were incubated at a growth chamber (95% humidity at 25˚C). Necrotic lesions became larger and widespread in bean leaves as incubation periods lasted longer. "Not inoculated" indicates bean leaves uninoculated with strain B728a. The time periods 0 to 96 h indicate each incubation period after inoculation with strain B728a.

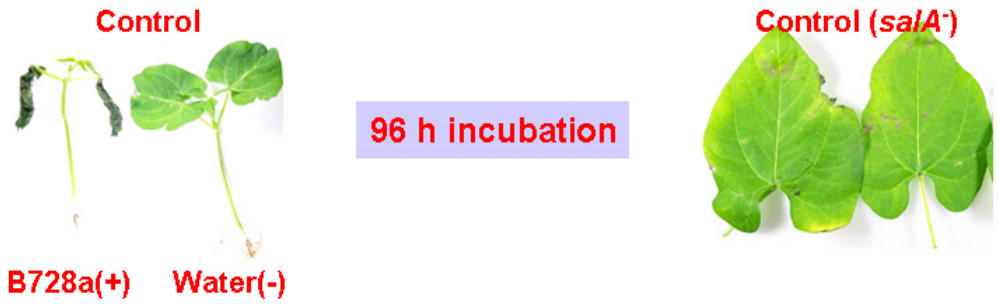

FIG. 23. Differences in development of necrotic lesions in bean leaves infected with parental strain B782a and strain B728a-SL7 (a *salA* mutant). Bean leaves were inoculated with strains B728a, B728a-SL7, and sterile water using vacuum infiltration. Resultant bean leaves were incubated in a growth chamber (95% humidity at 25˚C) for 96 h. Severe necrotic lesions were observed in bean leaves infected with parental strain B728a (left control), while mild symptoms of bacterial brown spot of beans (browning) developed in bean leaves infected with mutant strain B728a-SL7 (right control). In comparison, bean leaves infiltrated with sterile water failed to develop symptoms in 96 h after inoculation.

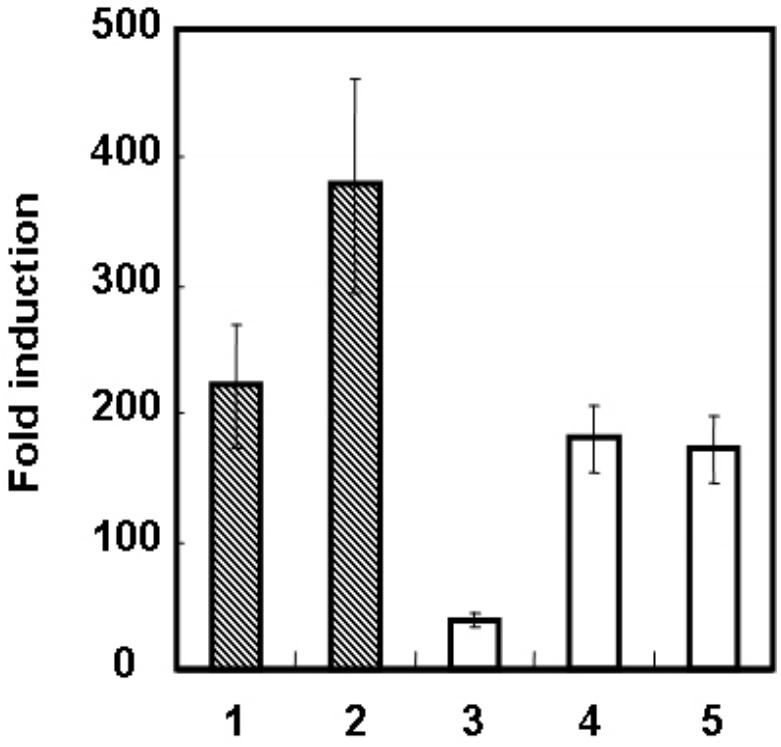

FIG. 24. Transcript levels of the synthetase genes (*syrB1* and *sypA*) and efflux genes (*syrD*, *pseF*, and *pseC*) of *P. syringae* pv. *syringae* strain B728a during infection of a susceptible plant, bean. Parental strain B728a and strain B728a-SL7 (a *salA* mutant) were inoculated into bean leaves, followed by incubation of the inoculated bean plants for 72 h (parental strain B728a) or 24 h (mutant strain B728a-SL7). Total RNAs (plant and bacterial RNAs) were isolated from necrotic lesions of bacterial brown spot developed in leaves of the infected bean plants. Expression of the synthetase genes and efflux genes by parental strain B728a was compared with that of mutant strain B728a-SL7 using quantitative real-time RT-PCR. The fold induction of mRNA was determined from the threshold values (C_T) that were normalized for 16S rDNA expression (endogenous control) and then normalized to the C_T value obtained from mutant strain B728a-SL7 (4). Column 1, fold induction of the *syrB1* gene; 2, the *sypA* gene; 3, the *syrD* gene; 4, the *pseF* gene; and 5, the *pseC* gene.

DISCUSSION

Production of two classes of lipopeptide phytotoxins, called syringomycin and syringopeptin, is characteristic of *P. syringae* pv. *syringae* strain B301D (8). The *syr-syp* genomic island encodes two nonribosomal peptide synthetases that synthesize syringomycin and syringopeptin under control of the GacS/GacA global regulatory system. Three efflux systems encoded by the *syr-syp* genomic island facilitate the secretion of lipopeptide phytotoxins (31). Previous genetic studies demonstrated that the SyrD (ABC transporter) and PseABC (RND transporter) efflux systems are required for phytotoxin secretion (71). Another putative ABC transporter among the three efflux systems, called the PseEF efflux system, was characterized in this study. The PseE protein showed the closest similarity to a macrolide efflux protein, MacA, of *E. coli* W3104 (42); the MacA protein belongs to the membrane fusion protein family. A hemolysin (Hly) D family secretion protein signature, characteristic of the membrane fusion protein family, was identified in the amino acid sequence of the PseE protein. The PseF protein, a putative ABC transporter, showed the highest similarity to a macrolide-specific ABC efflux carrier, MacB, of *E. coli* W3104 (42) and 42% similarity to the SyrD protein (71). The N-terminal region of the PseF protein was predicted to contain motifs characteristic of the ABC transporter family (69). A Walker A motif, the ABC signature, and a Walker B motif (79) were identified in the N-terminal region of the PseF protein. Based on the arrangement of the *pseE* and *pseF* genes, which are separated by only 2 bp, these two genes were predicted to be transcribed as an operon.

The export of erythromycin (macrolide) involves the MacB protein (ABC

transporter), the MacA protein (membrane fusion protein), and the TolC protein (outer membrane protein), which form a three-component functional complex called the MacAB-TolC efflux system (42). Similar to MacB protein, the PseF protein might form a three-component complex by binding with the PseE protein and one of two outer membrane proteins (PseA or the OprM homolog) identified in the *syr-syp* genomic island. A genetic study demonstrated that a mutation in the *pseF* gene caused a significant reduction in secretion of lipopeptide phytotoxins and strain B301D-HK7 (a *pseF* mutant) was substantially less virulent than parental strain B301D. Therefore, the PseEF efflux system is classified as an ABC-type transporter system and is required for lipopeptide phytotoxin secretion and full virulence, as shown for the SyrD efflux system.

Similar to strain B301D-HK4 (a *pseC* mutant), strain B301D-HK7 (a *pseF* mutant) was as sensitive to a series of antibiotics as parental strain B301D in MIC tests. These data indicated that the PseEF efflux system of *P. syringae* pv. *syringae* B301D is not involved in the export of the tested antibiotics. Due to the presence of multiple genes encoding multidrug exporters in the genomes of *Pseudomonas* spp., such as *P. syringae* pv. *tomato* strain DC3000 (12), *P. putida* strain KT2440 (60), and *P. aeruginosa* strain PAO1 (89), other genes encoding multidrug exporters are predicted to be present and their products might be responsible for the export of antibiotics and metabolites in *P. syringae* pv. *syringae* B301D.

MIC tests with *E. coli* strain AG100A (an *acrB* mutant) were performed to determine whether the PseEF efflux system has a functional relationship with the PseABC, AcrAB-TolC (64), and MacAB-TolC efflux systems (42). In terms of substrate specificity and

spectrum, the PseEF efflux system was different from the AcrAB-TolC and MacAB-TolC efflux systems, but it was functionally related to the PseABC efflux system. Heterologous expression of the *pseEF* efflux genes did not increase resistance of mutant strain AG100A to most antibiotics tested in this study except gentamicin. These data suggested that the PseEF efflux system might have a narrow spectrum of substrates, which is different from that of the AcrAB-TolC efflux system, which has a broad spectrum of substrates. Although the PseEF efflux system has high amino acid similarity with the MacAB efflux system, increased resistance to erythromycin was not observed in mutant strain AG100A by heterologous expression of the *pseEF* efflux genes. Thus, the PseEF efflux system appears not to export macrolide compounds. However, it was functionally related to the PseABC efflux system in that heterologous expression of both the *pseABC* or the *pseEF* efflux genes enhanced resistance of mutant strain AG100A to gentamicin, although the expression of the *pseA, pseB,* and *pseC* genes is also involved in reducing susceptibility of mutant strain AG100A to tetracycline. In order to confirm whether the PseEF efflux system increases resistance to gentamicin, another set of MIC tests was performed. Because pUCP26 and pHK500 carry the *tet* gene encoding a tetracyline efflux protein (65) and mutant strain AG100A is susceptible to 1/16 the level of tetracycline as the parental strain AG100 (64), for plasmid maintenance, a constant concentration of tetracycline (12.5 μg/ml) was added into 96 wall plates that contained different concentration of gentamicin. Results of this MIC test were similar to those of the previous MIC tests performed without adding the constant concentration of tetracycline, except that parental strain AG100 and mutant

strain AG100A carrying only the pUCP26 vector (0.39 μg/ml and 0.10 μg/ml of MICs for gentamicin, respectively) became more susceptible to a mixture of gentamicin and tetracycline compared to the same transformants grown without tetracycline (0.78 μg/ml and 0.20 μg/ml of MICs for gentamicin, respectively). These data suggested that the PseEF efflux system might be involved in the export of gentamicin. In addition to the export of gentamicin, there were other similarities between the PseEF and the PseABC efflux systems. In spite of its preference for syringopeptin, the PseABC efflux system was shown to secrete both lipopeptide phytotoxins (syringomycin and syringopeptin). Likewise, the PseEF efflux system was required for secretion of both lipopeptide phytotoxins. Thus, the PseEF and PseABC efflux systems were similar in that they appear to have high substrate specificity for both syringomycin and syringopeptin.

Similar to mutations in the SyrD and PseABC efflux systems (71), a *pseF* mutation caused a significant reduction in expression of the syringimycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes. Transcript levels of the *sypA* and *syrB1* genes in mutant strain B301D-HK7 were greatly decreased compared to parental strain B301D (91% reduction for *syrB1* and 86% reduction for *sypA*). Thus, inactivation of the *pseF* gene had strong effects on expression of the synthetase genes whose products catalyze synthesis of the lipopeptide phytotoxins. These data supported the hypothesis that a negative feedback mechanism may be involved in a regulatory coupling between toxin production and expression of efflux gene, as proposed in other studies (21, 71, 97).

Based on the concept of virulence genes described previously (98), these virulence life-style genes are subclassified into seven gene classes: true virulence genes (factors),

colonization genes, defense system evasion genes, processing virulence genes, secretory virulence genes, virulence housekeeping genes, and regulatory genes. The plant signal molecules arbutin and D-fructose added to syringomycin minimal medium (SRM) cause to enhanced transcript levels of the *syrB1* gene (processing virulence gene) and subsequently increase syringomycin production (true virulence factor) (56). Apparently, the supplementation of SRM with plant signal molecules induces the virulence life-style genes in *P. syringae* pv. *syringae*. Subsequently, the *syrB1* gene is shown to be a plant signal-upregulated gene on SRM. However, it was interesting to test whether plant signal molecules are also involved in induction of the bacterial efflux genes responsible for secretion of lipopeptide phytotoxins. Transcript levels of synthetase genes were enhanced by adding plant signal molecule (11 times for *syrB1* and 20 times for *sypA*), and transcript levels of three efflux genes (*syrD*, *pseC*, and *pseF* genes) were also increased by 6 to 14 times in strain B301D grown on SRM_{AF} as compared to that of strain B301D on SRM. These data suggest that expression of the efflux genes is induced in order to facilitate secretion of lipopeptide phytotoxins whose production is enhanced due to plant signal molecules. Eventually, plant signal molecules seem to induce in concert expression of the processing virulence genes (synthetase genes), true virulence genes (syringomycin and syringopeptin), and secretory virulence genes (efflux genes). In short, expression of the virulence-associated genes is proposed to be induced whenever expression of true virulence genes is stimulated.

Although expression of the synthetase and efflux genes are enhanced by supplementation of SRM with plant signal molecules, it was a question whether the

bacterial efflux genes are upregulated during infection of *P. syringae* pv. *syringae* in the host plant. For the purpose of determining transcript levels of the bacterial efflux genes in a susceptible plant, *P. syringae* pv. *syringae* B728a causing bacterial brown spot in bean was used to investigate expression profiles of the various bacterial efflux genes. Because of the ease of preparation of infected bean plants and because the *syr-syp* genomic island is conserved between strains B301D and B728a (31), strain B728a was used in the plant experiments. Transcript levels of the efflux genes of strain B728a-SL7 (a *salA* mutant) were utilized for normalizing transcript levels of the efflux genes of parental strain B728a because a mutation in the *salA* gene causes a significant reduction in expression of the synthetase and efflux genes. Regardless of incubation periods, transcript levels of the synthetase and efflux genes of strain B728a-SL7 in bean plants after inoculation were at levels very low for detection by quantitative real-time RT-PCR. Symptoms of bacterial brown spot (necrotic lesions) were observed in bean leaves at 1 dai with parental strain B728a. The necrotic lesions increased in size at longer incubation periods. Transcript levels of the synthetase and efflux genes were high at 3 dai. Thus, the synthease genes required for biosynthesis of syringomycin and syringopeptin were clearly induced during development of the necrotic lesions in the infected bean leaves. The bacterial efflux genes were also highly expressed in the infected bean leaves at 3 dai, indicating facilitation of the transport of lipopeptide phytotoxins. On the basis of these observations and gradual development of necrotic lesions in the infected bean leaves, production of syringomycin and syringopeptin is predicted to be augmented in bean plants infected by strain B728a. Therefore, infection

of bean plants by strain B728a results in increased expression of a true virulence factor (lipopeptide phytotoxins), as well as increased expression of processing virulence genes (*syrB1* and *sypA*) and secretory virulence genes (*syrD*, *pseC*, and *pseF*). Together with induction of the efflux genes by plant signal molecules, these data indicate that expression of the efflux genes is increased to meet the requirement for secretion of lipopeptide phytotoxins throughout infection. Furthermore, these results supported evidence that the efflux genes belong to the up-regulated virulence-associated genes (98).

Simultaneous induction of the synthetase and efflux genes led to a suggestion that syringomycin and syringopeptin might positively autoregulate expression of the synthetase and efflux genes. Positive autoregulatory systems are reported to be present in *Streptomyces griseus* (33), *Vibrio fischeri* (18), and *P. fluorescens* (11). A-factor, acyl homoserine lactones, and pyoluteorin produced by *Streptomyces griseus*, *Vibrio fischeri*, *P. fluorescens*, respectively, serve as signaling molecules (autoregulator) that induce their own expression and subsequently increase production of the signaling molecules. In addition to the autoinduction, exogenous pyoluteorin also enhances transcript levels of the *pltI* and *pltJ* genes, which encode members of the ABC transporter family (11). The *pltHIJ* gene cluster is directly adjacent to the biosynthesis genes *pltABCDEFG* responsible for production of pyoluteroin (63). Such enhancement in transcription of the ABC transporter genes by pyoluteroin indicates that the regulatory mechanism for production of pyoluteroin is tightly coordinated with transport of pyoluteorin. Similarly, syringomycin and syringopeptin produced by *P. syringae* pv. *syringae* appear to serve as signaling molecules that induce expression of the synthetase

and efflux genes required for production and export of the phytotoxins. However, to determine whether these is autoregulation by lipopeptide phytotoxins, it is necessary to do further studies on the regulatory network controlling production and export of syringomycin and syringopeptin.

In summary, this study demonstrated that the PseEF efflux system is required for secretion of lipopeptide phytotoxins and full virulence in *P. syringae* pv. *syringae*. Furthermore, it provided important clues about the overall mechanism for secretion of syringomycin and syringopeptin.

CHAPTER IV

CONCLUSIONS

A tripartite RND (resistance-nodulation-cell division) transporter system, called the PseABC efflux system, was identified at the left border of the *syr-syp* genomic island of *Pseudomonas syringae* pv. *syringae* strain B301D (31, 58). A 5.7-kb operon contained the *pseA*, *pseB*, and *pseC* genes, encoding an outer membrane protein (PseA), a periplasmic membrane fusion protein (PseB), and an RND-type cytoplasmic membrane protein (PseC). The PseA protein showed 48.2% identity to an outer membrane protein of *Ralstonia solanacearum* GMI1000 (77), and 23.9% identity to the TolC protein of *E. coli* K-12 (9). The PseB protein showed 51.2% identity to a putative membrane fusion protein of *R. solanacearum* GMI1000 (77), and 23.4% identity to the AcrA protein of *E. coli* K-12 (9). The PseC protein showed 61.6% identity with a probable transporter transmembrane protein of *R. solanacearum* GMI1000 (77), and 27.2% identity to the AcrB protein of *E. coli* K-12 (9). A mutation within the *pseC* gene was generated by *nptII* insertional mutagenesis. The resultant mutant strain B301D-HK4 reduced the secretion of syringopeptin (67%) and of syringomycin (41%) as compared to parental strain B301D. In order to determine the regulatory relationship between the GacS/A system and *pseA* expression, a translational fusion of the *pseA* gene with the *uidA* gene, encoding β-glucuronidase, was used as a reporter. A β-glucuronidase (GUS) assay with the *pseA::uidA* reporter construct indicated that the GacS/A two-component system

controls expression of the *pseA* gene. Transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes in strain B301D-HK4 (a *pseC* mutant) was determined by using quantitative real-time RT-PCR. Transcript levels of the *sypA* gene in mutant strain B301D-HK4 corresponded to approximately 13% of that in parental strain B301D, while *syrB1* gene expression in mutant strain B301D-HK4 was nearly 61%. The virulence of strain B301D-HK4 (a *pseC* mutant) was determined in immature Bing cherry fruits using methods described previously (71). Mutant strain B301D-HK4 was reduced in virulence by about 58% as compared to parental strain B301D. In order to determine whether the PseABC efflux system contributes to antibiotic resistance in *P. syringae* pv. *syringae* strains, antimicrobial susceptibility tests were performed. Mutant strain B301D-HK4 did not reduce resistance to any antibiotics used in this study as compared to parental strain B301D. However, a drug-supersensitive *acrB* mutant of *E. coli* (64) showed 2-fold increased resistance to gentamicin and tetracycline, respectively, by heterologous expression of the *pseABC* efflux genes.

The RND superfamily is composed of eight recognized phylogenetic families, which are correlated with substrate specificity (74). In Gram-negative bacteria, RND families 1 to 3 form a three-component efflux system conjugated with a membrane fusion protein and an outer membrane protein. Family 1 is involved in the export of heavy metals (22), family 2 in the export of multiple drugs (58), and family 3 in the export of lipooligosaccharides involved in plant nodulation by rhizobia (66). Based on this phylogenetic study, the PseABC efflux system appeared to be a unique RND-type

transporter system in that it secreted specific lipopeptide phytotoxins, which are natural secondary metabolites produced by a Gram-negative bacterium, *P. syringa*e pv. *syringae* (31). This study was the first report that demonstrates involvement of an RND-type efflux system in secretion of phytobacterial toxins. In summary, the PseABC efflux system is an important RND-type transporter system involved in secretion of syringomycin and syringopeptin and is required for expression of full virulence of *P. syringae* pv. *syringae* B301D.

An ATP-binding cassette (ABC) transporter, called the PseEF efflux system, was identified at the left border of the *syr-syp* genomic island of *Pseudomonas syringae* pv. *syringae* strain B301D (31, 79). A 3.3-kb operon contained the *pseE* and *pseF* genes, encoding a periplasmic membrane fusion protein (PseE) and an ABC-type cytoplasmic membrane protein (PseF). The PseE protein showed 47.2% identity to the macrolide efflux protein MacA of *E. coli* W3104 (42), which belongs to the membrane fusion protein family. The PseF protein showed 57.6% identity to the macrolide-specific ABC-type efflux carrier MacB in *E. coli* W3104 (42), and 32.1% identity to the SyrD protein in *P. syringae* pv. *syringae* B301D (71). A mutation within the *pseF* gene was generated by *nptII* insertional mutagenesis. The resultant mutant strain, B301D-HK7, had reduced secretion of syringomycin (74%) and syringopeptin (71%) as compared to parental strain B301D. To confirm the reduction in secretion of lipopeptide phytotoxins due to mutation in the *pseF* gene, quantitative real-time RT-PCR was used to determine transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes in strain B301D-HK7 (a *pseF* mutant). Transcript levels of the *syrB1*

and *sypA* genes in mutant strain B301D-HK7 were approximately 6.9% and 14.6%, respectively, as compared to that of parental strain B301D. The virulence of strain B301D-HK7 (a *pseF* mutant) was determined in immature Bing cherry fruits using methods described previously (71). Mutant strain B301D-HK7 was approximately 67% less virulent than parental strain B301D. Similarly to the studies on the PseABC efflux system, antimicrobial susceptibility tests were performed to determine whether the PseEF efflux system contributed to antibiotic resistance in *P. syringae* pv. *syringae* strains. Mutant strain B301D-HK7 did not show altered resistance to any antibiotics used in this study as compared to parental strain B301D. However, a drug-supersensitive *acrB* mutant of *E. coli* (64) showed increased resistance to gentamicin by heterologous expression of the *pseEF* efflux genes.

The plant signal molecules arbutin and D-fructose (56) were shown to induce expression of the *syrB1* gene. Thus, it was reasonable to test whether the plant signal molecules were able to induce expression of the efflux genes. Using quantitative real-time RT-PCR, it was observed that the transcript level of the *pseF* gene was induced approximately six times in strain B301D grown on SRM_{AF} (in the presence of plant signal molecules) as compared to the level in strain B301D grown on SRM (in the absence of plant signal molecules). Furthermore, quantitative real-time RT-PCR was performed to investigate transcript levels of the bacterial efflux genes (*syrD*, *pseC*, and *pseF*) when the strain B728a parasitizes a susceptible host, bean. During infection of bean plants by *P. syringae* pv. *syringae* strain B728a, transcript level of the *pseF* gene by parental strain B728a was observed to be high at 3 dai as compared to strain B728a-SL7

(a *salA* mutant). In comparison, transcript levels of the *syrB1* and *sypA* genes by parental strain B728a also were high at 3 dai. Similarly, expression of the *syrD* and the *pseC* genes by parental strain B728a increased at 3 dai. In summary, the PseEF efflux system has an important role in secretion of syringomycin and syringopeptin, and is required for full virulence in *P. syringae* pv. *syringae*.

Expression of the *syrD* gene was not induced as high as expression of *pseC* or *pseF* genes during infection of parental strain B728a in bean plant. However, the *syrD*, *pseC*, *pseF* genes were induced to similar levels in strain B301D grown on SRM_{AF}. This suggests that the PseABC and PseEF efflux systems might be the major efflux systems for secretion of lipopeptide phytotoxins during the bacterial infection of bean plants. A previous study showed that the *sypA* (syringopeptin synthetase) mutant showed a greater reduction in virulence in immature sweet cherry fruits than the *syrB1* (syringomycin synthetase) mutant, and it suggested that syringopeptin contributes more to virulence of *P. syringae* pv. *syringae* B301D than syringomycin (81). Accordingly, on the basis of their location in the *syp* gene cluster and functional study of mutants of the *pseC* and *pseF* genes, the PseABC and PseEF efflux systems were proposed to be more important for secretion of syringopeptin than the SyrD efflux system. However, an important role of the SyrD efflux system in virulence cannot be excluded because strain BR105 (a *syrD* mutant) is substantially less virulent to immature cherry fruits than parental strain B301D (71). Therefore, it was suggested that expression of the *syrD* gene may not be induced as much as that of the *pseC* and *pseF* genes, although its expression is essential for full virulence of *P. syringae* pv. *syringae* B301D. The most important requirement

for secretion of lipopeptide phytotoxins is likely that all three efflux systems should be functional to efficiently secrete lipopeptide phytotoxins. A malfunction in one of the three efflux system is enough to trigger a negative feedback inhibition mechanism that subsequently reduces expression of the synthetase genes required for production of syringomycin and syringopeptin, followed by decreased production of lipopeptide phytotoxins. Eventually, the reduced production of lipopeptide phytotoxins (true virulence factors) causes a significant reduction in virulence of *P. syringae* pv. *syringae*.

These studies are an important foundation for understanding the overall mechanism for secretion of syringomycin and syringopeptin. Based on these and previous studies (31, 50, 71, 81), an overall mechanism for secretion of syringomycin and syringopeptin is proposed (Fig. 25). The GacS/A two-component system captures an environmental signal and subsequently triggers production of syringomycin and syringopeptin. The signal may be delivered to the SalA and SyrF transcriptional regulators, which activate expression of the syringomycin and syringopeptin synthetase genes. The synthetases catalyze syringomycin (SR) and syringopeptin (SP) synthesis. The lipopeptide phytotoxins induce expression of the efflux genes (*syrD*, *pseABC*, and *pseEF* efflux genes). The efflux systems are associated with the cytoplasmic membrane and are involved in the export of lipopeptide phytotoxins. On the basis of functional studies with mutants of the efflux genes, the PseABC efflux system is suggested to transport both phytotoxins, but to have a preference for syringopeptin. In contrast, the PseEF and SyrD efflux systems are proposed to pump out both phytotoxins without preference for either substrate.

Finally, in order to emphasize the roles of virulence genes in pathogenesis, a recent report (98) described the concept of virulence genes. The proposed concept is extended from classic virulence genes to virulence life-style genes that include all genes essential to complete a pathogenic life cycle (98). These virulence life- style genes are subclassified into seven gene classes: true virulence genes (factors), colonization genes, defense system evasion genes, processing virulence genes, secretory virulence genes, virulence housekeeping genes, and regulatory genes. Applying this concept of virulence life-style genes to *P. syringae* pv. *syringae*, syringomycin and syringopeptin would be classified as true virulence factors because they directly interact with host plant cells and cause necrotic symptoms in the host plant (8). Synthetase genes for production of syringomycin and syringopeptin would be considered processing virulence genes (8). Regulatory genes found in the *syr-syp* genomic island include the *syrP*, *salA*, *syrG* and *syrF* genes (50). Finally, based on the fact that mutations in the *syrD*, *pseC*, and *pseF* genes caused a significant reduction in virulence of *P. syringae* pv. *syringae* B301D (31), these efflux genes were placed in the secretory virulence gene class.

In conclusion, the SyrD, PseABC, and PseEF efflux systems are required for secretion of lipopeptide phytotoxins and are important virulence factors in *P. syringae* pv. *syringae*.

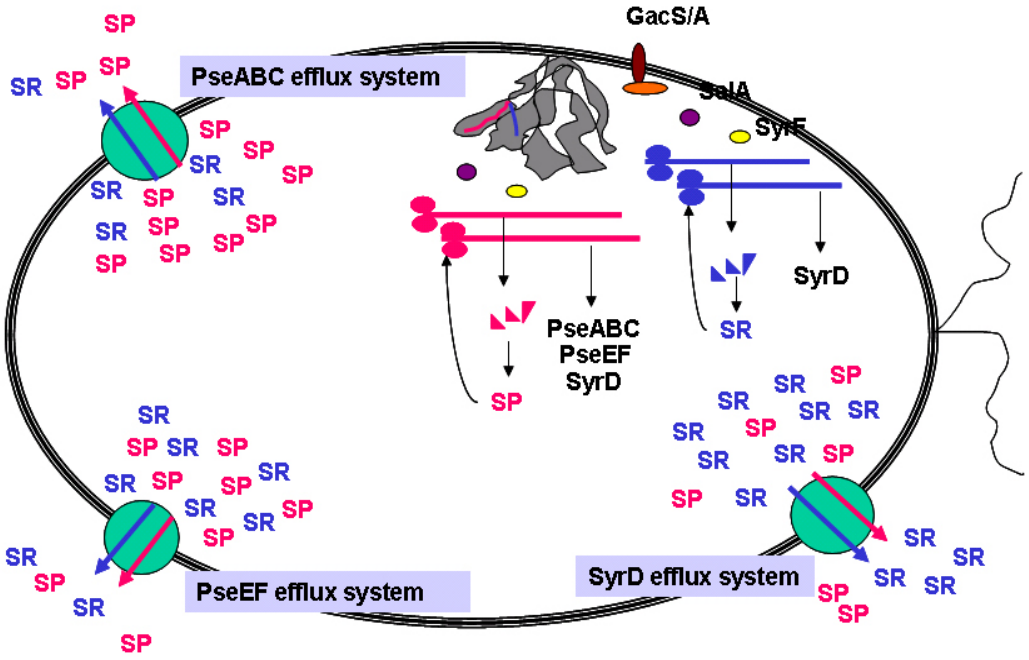

FIG. 25. Proposed overall mechanism for secretion of syringomycin and syringopeptin. The GacS/A two-component system captures an environmental signal and subsequently triggers signal transduction for production of syringomycin and syringopeptin. The signal may be delivered to the SalA and SyrF transcriptional regulators, which induces expression of syringomycin and syringopeptin synthetase genes. Resultant synthetases catalyze syringomycin (SR) and syringopeptin (SP) syntheses. The lipopeptide phytotoxins induce expression of the efflux genes (*syrD*, *pseABC*, and *pseEF* efflux genes). The efflux systems are associated with the cytoplasmic membrane and are involved in the export of lipopeptide phytotoxins. On the basis of functional studies with mutants of the efflux genes, the PseABC efflux system is suggested to transport both phytotoxins with preference for syringopeptin. In contrast, the PseEF and SyrD efflux systems are proposed to pump out both phytotoxins without preference for either substrate.

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VITA

Hyojeung Kang

Permanent Address
751-1, Keumamri, Jinjeunmyun,
Masan, Kyungnam, 631-835, Republic of Korea.
82-055-271-7010

Email
sigtranb@yahoo.com

Education

Doctor of Philosophy
Texas A&M University
College Station, TX 77843-2132
Major: Plant Pathology
December 2004

Master of Science
Korea University
Seoul, 136-701 Republic of Korea
Major: Biochemistry
February 2000

Bachelor of Science
Korea University
Seoul, 136-701 Republic of Korea
Major: Agrobiology
February 1998