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The PseEF Efflux System Is a Virulence Factor of *Pseudomonas syringae* pv. *syringae*

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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. The PseEF efflux system was located within a 3.3-kb operon that encodes a periplasmic membrane fusion protein (PseE), and an ABC-type cytoplasmic membrane protein (PseF). The PseEF efflux system exhibited amino acid homology to a putative ABC efflux system (MacAB) of *E. coli* W3104 with identities of 47.2% (i.e., PseE to MacA) and 57.6% (i.e., PseF to MacB). A nonpolar mutation within the *pseF* gene was generated by *nptII* insertional mutagenesis. The resultant mutant strain showed significant reduction in secretion of syringomycin (74%) and syringopeptin (71%), as compared to parental strain B301D. 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). 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%. In addition, mutant strain B301D-HK7 was less virulent by approximately 67% than parental strain B301D in immature cherry fruits. Mutant strain B301D-HK7 was not reduced in resistance to any antibiotics used in this study as compared to parental strain B301D. Expression (transcript levels) of the *pseF* gene was induced approximately six times by strain B301D grown on syringomycin minimum medium (SRM) supplemented with the plant signal molecules arbutin and D-fructose (SRM_{AF}), as compared to that of strain B301D grown on SRM (in the absence of plant signal molecules). In addition, during infection of bean plants by *P. syringae* pv. *syringae* strain B728a, expression of the *pseF* gene increased at 3 days after inoculation (dai). More than 180-fold induction was observed in transcript levels of the *pseF* gene by parental strain B728a as compared to strain B728a-SL7 (a *salA* mutant). Thus, the PseEF efflux system, an ABC-type efflux system, has an important role in secretion of syringomycin and syringopeptin, and is required for full virulence in *P. syringae* pv. *syringae*.

***Keywords*:** syringomycin, syringopeptin, phytotoxin, ABC efflux system, PseEF efflux system, *pseF*

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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 (Bradbury, 1986). SRE and $SP_{22}A$ are the major forms of syringomycin and syringopeptin, respectively (Bender *et al.*, 1999). 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 (Scholz-Schroeder *et al.*, 2001b). 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 (Scholz-Schroeder *et al.*, 2001b). SyrF protein mediates the SalA control of the *syr-syp* gene expression; SalA protein binds to the putative *syrF* promoter while SyrF protein binds to *syrB1* promoter (Wang *et al.*, 2006). 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 (Bender *et al.*, 1999; Gross *et al.*, 2003). The phytotoxins also have a role as key virulence determinants (factors), which significantly contribute to cytotoxicity against other microorganisms and plant tissues (Hutchison and Gross, 1997). In fact, both of the lipopeptide phytotoxins cause cytotoxicity to plant cells and they are harmful to the bacterial cells which produce them (Hutchison and Gross, 1997). 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 (Ren *et al.*, 2004), 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 (Nelson *et al.*, 2002). 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 (Buell *et al.*, 2003). Based on recent intensive searches of a variety of genomes (Ren *et al.*, 2004), 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 (Schneider and Hunke, 1998). 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 (Schneider and Hunke, 1998). 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*) (Llama-Palacios *et al.*, 2002), initiate induction of pectate catabolism (*P. chrysanthemi*) (Hugouvieux-Cotte-Pattat *et al.*, 2001), facilitate bacterial attachment to plant cells (*Agrobacterium tumefaciens*) (Matthysse *et al.*, 1996), and have a role in antagonism of the fungal pathogen, *Phytophthora parasitica* by *P. putida* (Lee and Cooksey, 2000). Similarly, in plant pathogenic fungi such as *Mycosphaerella gramincola* (Stergiopoulos *et al.*, 2003), *Magnaporthe grisea* (Urban *et al.*, 1999), and *Botryis cinerea* (Schoonbeek *et al.*, 2001), 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 (Quigley *et al.*, 1993) 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 (Gross *et al.*, 2003). 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 (Quigley *et al.*, 1993). The PseC in the PseABC efflux system exhibited significant amino acid sequence similarity to the RND transporter family (Gross *et al.*, 2003). 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 (Scholz-Schroeder *et al.*, 2001b). 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 (Gross *et al.*, 2003). 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 (Quigley *et al.*, 1993). 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 (Quigley *et al.*, 1993; Quigley and Gross, 1994). 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 (Mo and Gross, 1991b). 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*. We 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. We 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 1. DNA manipulations were performed with *E. coli* strain DH10B (Gibco-BRL) cultured at 37°C in Luria-Bertani (LB) broth or on LB agar (Sambrook, 1989). *P. syringae* pv. *syringae* strains were cultured routinely in nutrient-broth yeast extract (NBY) broth or agar media (Vidaver, 1967). PDA (potato dextrose agar) supplemented with 0.4% casamino acids and 1.5% glucose was used in bioassays for production of syringomycin and syringopeptin (Gross and DeVay, 1977). 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 (Sambrook, 1989). A 20-kb fragment from cosmid p116

and a 4.7-kb *Hin*dIII fragment from pJS091, containing the *pseE* and *pseF* genes were sequenced (Scholz-Schroeder *et al*., 2001b). 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) (Devereux *et al*., 1984). 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 (Scholz-Schroeder *et al*., 2003). 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) (Altschul *et al*., 1990). A motif search was performed using the Pfam server (http://motif.ad.jp/motif-bn/Srch_Motif_Lib) (Bateman *et al*., 2000). Hydropathy analysis was performed to predict transmembrane segment (TMS) using Protean (Lasergene) (Kyte and Doolittle, 1982) 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) (Corpet, 1988).

Construction of pHK503 for expression of MBP-PseF

In order to express the pseF gene, the malE gene encoding maltose binding protein (Lu *et al*., 2002b) 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. EcoRI or BamHI 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%) (Sambrook, 1989).

Mutagenesis of the *pseEF* gene

The *pseF* gene was disrupted by insertion of the *nptII* gene (Alexeyev, 1995). 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 *Eco*RV 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 (Cody *et al*., 1987). Transformants were selected on NBY agar supplemented with kanamycin. Double crossover mutations were confirmed by Southern analysis and by the polymerase chain reaction (PCR). The confirmed *pseF* mutant was labeled as B301D-HK7. A *syrB1* and *pseF* double mutant, labeled as BR132-HK7, was also generated by marker exchange of the *pseF*::*nptII* construct into the genome of *P. syringae* pv. *syringae* strain BR132 (a *syrB1* mutant) (Mo and Gross, 1991a).

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 (Scholz-Schroeder *et al*., 2001a), 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 (Scholz-Schroeder *et al*., 2001a). Each wounding site formed on cherry fruits was inoculated with 5×10^3 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

Table 1. Bacterial strains and plasmids used in this study

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
P. syringae pv. *Syringae*		
B301D	Wild type from pear	Cody *et al.* (1987)
BR132	*syrB1*::Tn3HoHo1 derivative of B301D-R; Pip^r Rif^r	Mo and Gross (1991a)
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	Lu *et al.* (2002a)
B728a	Wild type from bean	Quigley and Gross (1994)
B728a-SL7	*salA*::*nptII* derivative of BR132; Km^r	Lu and Gross, unpublished data
Plasmid		
pBSK(+)	Cloning vector; Ap^r	Strategene,
pBR325	Cloning vector; Cm^r Tc^r Ap^r	Prentki *et al.* (1981)
pBI101	Cloning vector containing *uidA* gene; Km^r	Clonetech
pGEMT-Easy	Cloning vector; Ap^r	Promega
pUCP26	Cloning vector; Tc^r	Olsen *et al.* (1982)
pBSL15	Kanamycin resistance gene cassette; Km^r	Alexeyev (1995)
p10B-9	Fosmid carrying a 30-kb *P. syringae* pv. *syringae* strain B301D genomic DNA fragment; Ap^r	Wang and 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 *npt*II 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 Eco*RV	This study
pMEKm12	pME10 carrying the 1.2-kb *nptII* gene inserted into the *Bam*HI site outside of the MCS; Km^r Ap^r	Lu *et al.* (2002b)
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.

and syringopeptin synthetase genes (Gibellini *et al.*, 2004). Bacterial RNA was extracted using the RNeasy Mini kit (QIAGEN) from *P. syringae* pv. *syringae* B301D cultured at 25°C for 72 h on syringomycin minimum medium (SRM) (Mo and Gross, 1991b) supplemented with the plant signal molecules arbutin and D-fructose (SRM_{AF}) (Mo and Gross, 1991b). 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 sec of primer annealing at 54°C, followed by 45 cycles of 15 sec of denaturation at 94°C, 30 sec of primer annealing at 54°C, and 30 sec of polymerization at 60°C. Primers were evaluated by following the manufacturer's instructions (QIAGEN). The fold induc-

Table 2. 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

tion 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 (Allen and McMurray, 2003).

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 (Livak and Schmittgen, 2001). 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 (Lorian, 1996). The susceptibilities of strain B301D-HK7 (a *pseF* mutant) were compared with those of parental strain B301D and *P. aeruginosa* strain PAO1 (Kim and Sundin, 2000). 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 (Edberg and Chu, 1975). The final cell concentration was adjusted to 4×10^4 CFU/ml per well. The *Pseudomonas* strains were cultured at 25°C for 18 h. The MIC was defined as the lowest concentration of antibiotics inhibiting visible growth (Sobel *et al.*, 2003).

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 (Mo and Gross, 1991b). *P. syringae* pv. *syringae* B301D was cultured on both SRM and SRM_{AF} plates at 25°C for 72 h (Mo and Gross, 1991b). 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 5×10^5 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 (Ott and Longnecker, 1999).

Results

Sequence analysis of the *pseE* and *pseF* genes

Sequencing of p116 and pJS091 revealed two open reading frames (ORFs) (Scholz-Schroeder *et al.*, 2001b). ORF1 and ORF2 were predicted to encode a periplasmic membrane fusion protein and a cytoplasmic ABC-type transporter, respectively (Fig. 1). 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 (Kobayashi *et al.*, 2001), 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 (Gilson *et al.*, 1990). The HlyD protein is a member of the membrane fusion protein family (Johnson and Church, 1999). 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 (Johnson and Church, 1999). PseE protein was predicted to contain one transmembrane segment (TMS) at the N terminus by hydropathy analysis (Putman *et al.*, 2000).

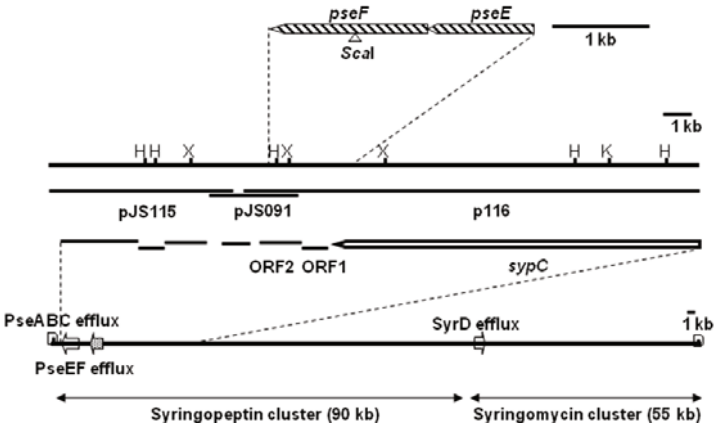

Fig. 1. Diagrammatic representation of the *syr-syp* genomic island on the chromosome of *P. syringae* pv. *syringae* B301D. The approximately 145-kb *Dra*I fragment consists of the syringopeptin (*syp*) gene cluster (90 kb) and the syringomycin (*syr*) gene cluster (55 kb) (Scholz-Schroeder *et al*., 2001b). 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, *Hin*dIII; K, *Kpn*I; and X, *Xho*I.

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 (Kobayashi *et al*., 2001).

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 (Kobayashi *et al*., 2001), and 32.1% identity (Z score, 45) to the SyrD protein in *P. syringae* pv. *syringae* B301D (Quigley *et al*., 1993). 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. 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) (Putman *et al*., 2000). 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. 2) (Schmitt and Tampe, 2002). The presence of these motifs

	Walker A	ABC signature	Walker B
PseF	042-GASGSGKST	146-LSGGQQQRV	167-ILA-DE
MacB	041-GASGSGKST	145-LSGGQQQRV	166-ILA-DE
SyrD	401-GGNGCGKST	515-LSYGQQKRL	533-IYLLDE
LmrA	382-GPSGGGKST	488-ISGGQRQRI	508-VLIFDE
MsbA	376-GRSGSGKST	482-LSGGQRQRI	502-ILILDE
Consensus	* ** ****	* ** *	**

Fig. 2. 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* (Kobayashi *et al*., 2001), the SyrD protein of *P. syringae* pv. *syringae* (Quigley *et al*., 1993), the LmrA protein of *Lactococcus lactis* (Venter *et al*., 2003), and the MsbA protein of *E. coli* (Chang and Roth, 2001). 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.

in PseF protein was consistent to those observed in the MacB protein (Kobayashi *et al*., 2001). PseF protein was predicted to contain four TMSs by hydropathy analysis (Putman *et al*., 2000).

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 (Scholz-Schroeder *et al*., 2001a). 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) (Figs. 3A and B). 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 (10.9 mm radius) (Figs. 3A and B). 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 (Figs. 3A and B). These data indicated complementation of mutant strain B301D-HK7 by pHK500, which carries the *pseEF* efflux genes. In spie that *pseF* mutation caused severe defect in secreting syringopeptin, we further dissected the role of *pseF* in secreting syringopeptin by making *pseF* mutant in *syrB1* mutation background because syringmycin is known to inhibit *B. megaterium* growth as well on the bioassay. The syrB1 mutation abolished syringomycin production but not syringopeptin production. The *pseF* and *syrB1* double mutation appeared to significant reduction in syringopeptin secretion, although the double mutant complemented with pHK500 *in trans* restored syringopeptin secretion (Fig. 4). This result indicated a functional PseF is required to secret syrngopeptin as well as syrngomycin.

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 (Bender *et al*., 1999). Parental strain B301D and strain B301D-HK7 (a *pseF* mutant) were

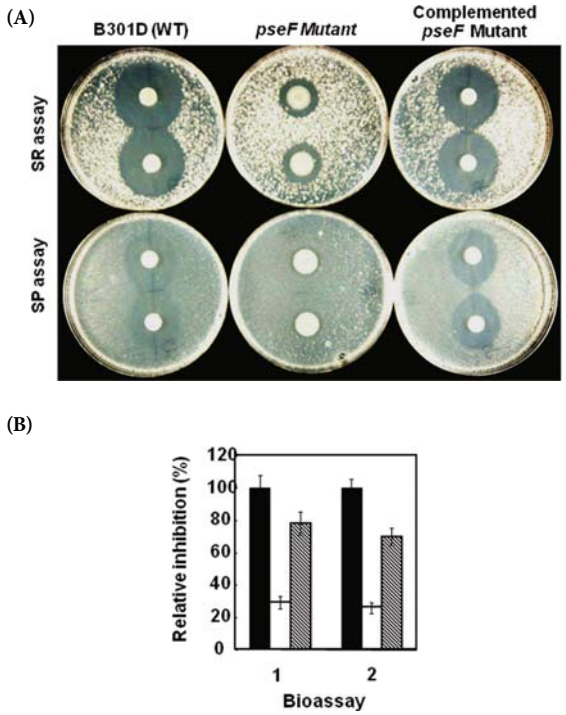

Fig. 3. Relative inhibition due to secretion of syringomycin and syringopeptin by strain B301D-HK7 (a *pseF* mutant). A bioassay for production of syringomycin and syringopeptin was performed by incubation of the strains on PDA medium containing 0.5 mM IPTG for 72 h and 48 h, respectively, followed by overspraying with *Geotrichum candidum* F-260, for the syringomycin bioassay, or *Bacillus megaterium* Km, for the syringopeptin bioassay. *pseF* mutant that was complemented with pHK500 plasmid showed the restoration of both toxin secretion (A). Zones of inhibition to *G. candidum* F-260 and *B. megaterium* Km due to the production of syringomycin and syringopeptin were measured (B). 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.

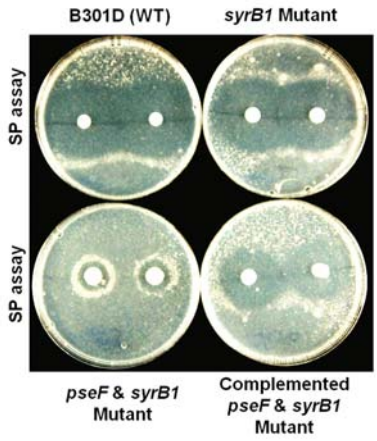

Fig. 4. Relative inhibition due to secretion of syringopeptin by strain BR132-HK7 (a *syrB1* and *pseF* double mutant). A bioassay for production of syringopeptin was performed by incubation of the strains on PDA medium containing 0.5 mM IPTG for 48 h, followed by overspraying with *Bacillus megaterium* Km, for the syringopeptin bioassay. The *syrB1* and *pseF* double mutant that was complemented with pHK500 plasmid showed the restoration of both toxin secretion. Zones of inhibition to *B. megaterium* Km due to the production of syringopeptin were compared between BR132 (*syrB1* mutant) and BR132-HK7 (*syrB1* and *pseF* double mutant).

cultured on SRM_{AF} medium for 72 h (Mo and Gross, 1991b), 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. 5).

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 (Quigley *et al.*, 1993). 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 (Figs. 6A and B).

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; 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

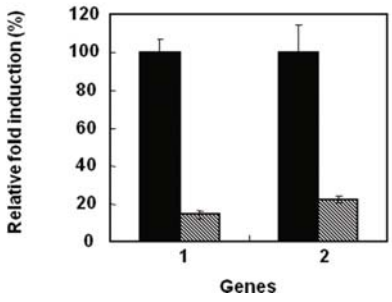

Fig. 5. Effects of the *pseF* mutation on expression of the *syrB1* (Mo and Gross, 1991a) and *sypA* (Scholz-Schroeder *et al.*, 2001a) synthetase genes. Using quantitative real-time RT-PCR, transcript levels of the synthetase genes in strain B301D-HK7 (*pseF* mutant, hatched bar) was compared to that in parental strain B301D (solid bar). Relative differences in expression of the synthetase genes were measured (Allen and McMurray, 2003). 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.

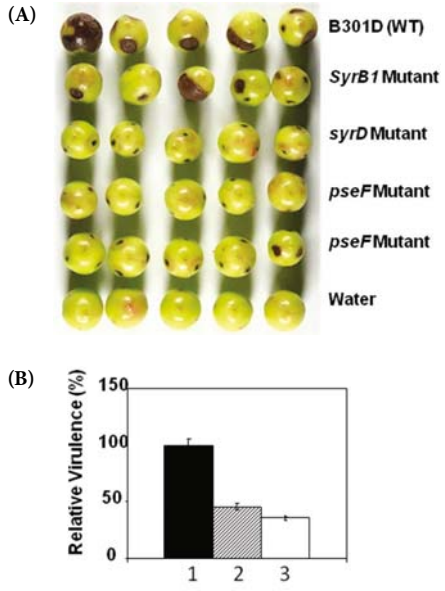

Fig. 6. The virulence of strains B301D-HK7 (a *pseF* mutant) and BR132-HK7 (a *syrB1* and *pseF* double mutant) was determined in immature Bing cherry fruits. The lesion diameters caused by mutant strains B301D-HK7 and BR132-HK7 were used to quantify relative virulence in the cherry fruits (A). The average lesion produced by pseF mutant strain (B301D-HK7) was comprared to that of parental strain B301D (B). Virulences of the *pseF* (column 2, B301D-HK7) and *syrD* (column 3) mutant strains were compared relative to that of parental strain B301D (column 1).

μg/ml, respectively. In comparison, *P. aeruginosa* strain PAO1 also exhibited MICs similar to those obtained from the previous MIC test; 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. These results suggested that the Pse EF efflux system was not involved in transporting antibiotics tested above.

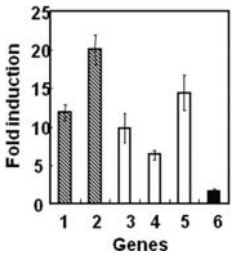

Fig. 7. Transcript levels of the synthetase genes (*syrB1* and *sypA*) and efflux genes (*syrD*, *pseF*, and *pseC*) in *P. syringae* pv. *syringae* strain B301D grown on SRM_{AF} (Mo and Gross, 1991b). Quantitative real-time RT-PCR was used to determine differences in transcript levels of the synthetase and efflux genes between strain B301D cells cultured separately on SRM and SRM_{AF} media at 25°C for 72 h (Mo and Gross, 1991b). 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 (Allen and McMurray, 2003). 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).

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 (Mo and Gross, 1991b) 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 25°C for 72 h (Mo and Gross, 1991b). 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. 7). 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 (Ravel and Cornelis, 2003). The *pvdS* gene encodes an alternative sigma factor that helps RNA polymerase initiate transcription of the *pvdA* gene encoding L-ornithine hydroxylase. The resultant hydroxylase is involved in pyoverdine biosynthesis (Hunt *et al.*, 2002). 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. 7).

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 (Gross *et al.*, 2003). 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 (Fig. 8A). The *salA* mutant fails to produce syringomycin (Kitten *et al.*, 1998) and is significantly reduced in expression of a *syrB1*::*uidA* reporter construct (Lu *et al.*, 2002a). 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) (Scholz-Schroeder *et al.*, 2001a). In fact, transcript levels of the synthetase and efflux genes in the background of strain B728a-SL7 (a *salA* mutant, a generous gift from Shin Liu, Mississippi state univ.) 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 (Fig. 8B). Using vacuum infiltration, the two strains were inoculated into bean leaves and then incubated

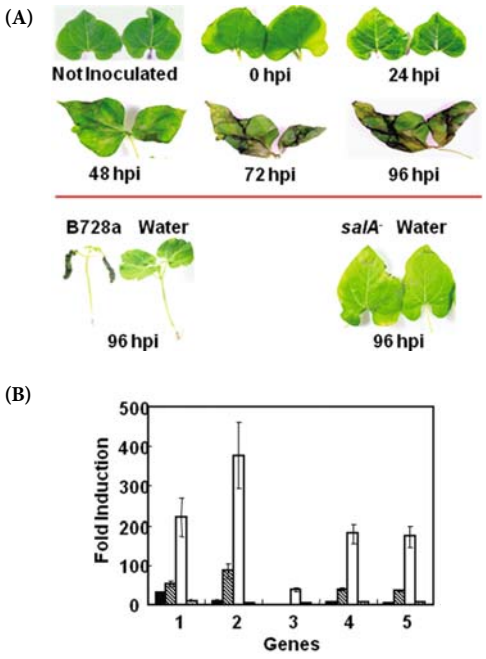

Fig. 8. Transcript levels of the synthetase genes (*syrB1* and *sypA*) and efflux genes (*syrD*, *pseF*, and *pseC*) of *P. syringae* pv. *syringae* strain B728a during infection of a susceptible plant, bean. Parental strain B728a and strain B728a-SL7 (a *salA* mutant) were inoculated into bean leaves, followed by incubation of the inoculated bean plants for indicated periods (up to 96 hours post inoculation) (A). As internal and negative controls, *salA* mutant strain that infected the bean plant did not show any legion on the plant. Total RNAs (plant and bacterial RNAs) were isolated from necrotic lesions of bacterial brown spot developed in leaves of the infected bean plants that were incubated for 72 h (parental strain B728a) or 24 h (mutant strain B728a-SL7), respectively (B). 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 (Allen and McMurray, 2003). 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.

for different incubation periods. Bacterial brown spot symptoms were observed in bean leaves 24 h incubation after inoculation with parental strain B728a. At longer incubation periods, the necrotic lesions characteristic of bacterial brown spot increased in number and size (Kitten *et al.*, 1998) (Fig. 8A). In contrast, no clear necrotic lesions were observed on bean leaves after inoculation with mutant strain B728a-SL7. 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. 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 (Ravel and Cornelis, 2003) 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).

Discussion

Production of two classes of lipopeptide phytotoxins, called syringomycin and syringopeptin, is characteristic of *P. syringae* pv. *syringae* strain B301D (Bender *et al.*, 1999). 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 (Gross *et al.*, 2003). Previous genetic studies demonstrated that the SyrD (ABC transporter) and PseABC (RND transporter) efflux systems are required for phytotoxin secretion (Quigley *et al.*, 1993). 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, membrane fusion protein) in *E. coli* W3104 (Kobayashi *et al.*, 2001). Like most membrane fusion protein family members, the hemolysin (Hly) D family secretion protein signature was identified in the amino acid sequence of the PseE protein. The PseF protein, a putative ABC transporter, also showed the highest similarity to a macrolide-specific ABC efflux carrier (MacB) in *E. coli* W3104 (Kobayashi *et al.*, 2001). We found the N-terminal region of the PseF protein contained several motifs that were characteristic of the ABC transporter family (Putman *et al.*, 2000). Acutally, a Walker A motif, the ABC signature, and a Walker B motif (Schmitt and Tampe, 2002) were identified in the PseF N-terminal region. The *pseE* and *pseF* genes were predicted to locate on an operon because two genes are arranged with 2 bp separation.

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 (Kobayashi *et al.*, 2001). 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. Based on functional genetic studies, a mutation in the *pseF* gene caused a significant reduction in secretion of lipopeptide phytotoxins, and consequently the mutation made strain B301D-HK7 (a *pseF* mutant) significantly less virulent than parental strain B301D. Therefore, the PseEF efflux system is required for both lipopeptide phytotoxin secretion and full virulence, like the SyrD efflux system.

MIC tests revealed 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 (Buell *et al.*, 2003), *P. putida* strain KT2440 (Nelson *et al.*, 2002), and *P. aeruginosa* strain PAO1 (Stover *et al.*, 2000), 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* strain B301D. MIC tests using *E. coli* strain AG100A [an *acrB* mutant, a generous gift from Akihito Yamaguchi (Osaka university, Japan)] were also conducted to determine whether the PseEF efflux system has a functional relationship with the MacAB-TolC efflux systems (data not shown). The strain AG100A carrying a nonpolar mutation in *acrB* gene (encoding the MacB protein) has defects in transporting macrolide compound (Kobayashi *et al.*, 2001), indicating the MacAB-TolC efflux system has a high specificity to macrolide compound. Unexpectedly, heterologous expression of the *pseEF* efflux genes in AG100A did not increase the resistance of mutant strain AG100A to erythromycin in this study. These data indicated that the PseEF efflux system might have a different substrate specificity from the MacAB-TolC efflux system, which has a high specificity to macrolide compounds (Kobayashi *et al.*, 2001).

The PseEF efflux system was functionally similar to either the SyrD or PseABC efflux systems in *Pseudomonas syringae* pv. *syringae*. Based on genetic studies, the PseABC efflux system prefers to screting syringopeptin (Kang and Gross, 2005) while the PseEF and SyrD efflux systems pump out both phytotoxins without any substrate preference. Any single mutation in all three effluxs actually caused a significant reduction in producing both syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase, indicating that all these efflux systems (SyrD, PseABC, PseEF) are required to secret the syringomycin and syringopeptin in maximum capacity. 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 (Wandersman and Delepelaire, 1990; Quigley *et al.*, 1993; Espinasse *et al.*, 2002). According to the negative feedback mechanism, the most important requirement for secretion of lipopeptide phytotoxins is likely that all three effluxs should be functional to efficiently secrete lipopeptide phytotoxins. A malfunction in one of the three effluxs 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*. Further study will test this hypothesis using double/triple mutant strains, which is eventually to dissect detailed functional role of each transporter in sereting the bacterial phytotoxons.

In constrast to the negative feedback mechanism, 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* (Hara and Beppu, 1982), *Vibrio fischeri* (Eberhard *et al.*, 1981), and *P. fluorescens* (Brodhagen *et al.*, 2004). 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 (Brodhagen *et al.*, 2004). The *pltHIJ* gene cluster is directly adjacent to the biosynthesis genes *pltABCDEFG* responsible for production of pyoluteroin (Nowak-Thompson *et al.*, 1997). 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, production of the pseEF, pseABC and syrD efflux systems might be dependent on the absolute amount of phytotoxins to be pumped out. These was suggesting that syringomycin and syringopeptin produced by *P. syringae* pv. *syringae* appear to serve as signaling molecules that induce expression of both synthetase genes 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.

As the plant signal molecules arbutin and D-fructose (Mo and Gross, 1991b) were shown to induce expression of the *syrB1* gene, we tested whether the plant signal molecules were able to induce expression of the efflux genes. The plant signal molecules significantly increased the *pseF* transcript production, suggesting that more transporters are produced to secerete more phytotoxins. Furthermore, since most efflux systems are known to virulence factor, we investigated if the *pseF* transcript was induced its production in a susceptible host, bean. The infection of bean plants by *P. syringae* pv. *syringae* strain B728a significantly increased the transcript level of the *pseF* gene. Taken together, 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*. Wassenaar and Gaastra proposed a new expanded concept of virulence genes (Wassenaar and Gaastra, 2001). 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. 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. In this context, 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 (Bender *et al.*, 1999). The efflux genes (*syrD*, *pseC*, *pseF*) placed in the secretory virulence gene class because mutations in the *syrD*, *pseC*, and *pseF* genes caused a significant reduction in virulence of *P. syringae* pv. *syringae* B301D (Gross *et al.*, 2003).

In summary, as shown in studies of the SyrD and PseABC efflux systems, the PseEF efflux system is required for se-

cretion 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.

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