CHARACTERIZATION OF TWO SIGMA FACTORS IN PLANT PATHOGENESIS BY *Pseudomonas syringae* pv. *syringae* B728a

A Thesis

by

POULAMI BASU THAKUR

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

MASTER OF SCIENCE

May 2011

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Dennis C. Gross
Committee Members,	Joshua S. Yuan
	Elizabeth A. Pierson
Head of Department,	Leland S. Pierson III

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ABSTRACT

Characterization of Two Sigma Factors in Plant Pathogenesis by

Pseudomonas syringae pv. *syringae* B728a. (May 2011)

Poulami Basu Thakur, B.S., University of Calcutta

Chair of Advisory Committee: Dr. Dennis C. Gross

Pseudomonas syringae pv. *syringae* B728a, an aggressive bacterial pathogen of bean, utilizes large surface populations and extracellular signaling to initiate a fundamental change from an epiphytic to a pathogenic lifestyle. Extracytoplasmic function (ECF) sigma (σ) factors serve as important regulatory factors in responding to various environmental signals. Bioinformatic analysis of the B728a genome has revealed 10 ECF sigma factors, five of which have high levels of sequence similarity to the FecI-type of ECF sigma factors and play a known role in the regulation of various iron transport systems. Because iron is essential for the induction of major virulence factors in B728a, I hypothesized that these FecI-type sigma factors may play a critical role in the bacterium's transition between lifestyles. Deletion mutants of two FecI-type sigma factors, *Psyr_1040* and *Psyr_1107*, in B728a have been created using homologous recombination based on the phage λ Red recombinase method.

This study shows that the B728a FecI-type sigma factors, *Psyr_1040* and *Psyr_1107* are affected by conditions of iron stress, and influence the expression of putative outer membrane receptors and transmembrane sensors associated with these

genes. Moreover, *Psyr_1107* contributes to the expression of a cluster of predicted pili assembly genes downstream of it. Mutations in *Psyr_1040* and *Psyr_1107* affect the population levels of B728a in bean plants, since i*n planta* growth of deletion mutants of B728a lacking *Psyr_1040* and *Psyr_1107* appears to be slower than wild-type B728a. In this thesis, the possible roles of *Psyr_1040* and *Psyr_1107* in the adaptation of B728a to a pathogenic lifestyle are addressed using a combination of phenotypic characterization and quantitative real-time PCR (qRT-PCR) analyses.

DEDICATION

To my parents, Manjusri and Bidyut Basu Thakur

To my husband, Dipanjan Mitra

To my sisters, Chandreyee and Sohini Basu Thakur

ACKNOWLEDGEMENTS

I would like to express my heartfelt gratitude and sincerest appreciation for my major advisor and mentor, Dr. Dennis Gross, for his guidance and enduring support and encouragement throughout my tenure at A&M. I would also like to thank members of my advisory committee, Dr. Elizabeth Pierson and Dr. Joshua Yuan, for their valuable suggestions and support throughout the course of this research.

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CHAPTER I

INTRODUCTION

Pseudomonas syringae is an agriculturally important bacterial pathogen of a variety of plant species worldwide. This species can be grouped into more than 50 pathovars on the basis of the host range of different strains (11, 13). *P. syringae* pv. *syringae* has been isolated from a wide range of plants exhibiting diverse symptoms, such as leaf or fruit lesions, cankers, blasts and galls (11). *P. syringae* pv. *syringae* B728a (B728a) is a highly versatile foliar pathogen of bean that causes brown spot on its host, a disease which manifests as water-soaked lesions on bean pods and leaves. This bacterium can survive as an epiphyte on the leaf surface for prolonged periods of time before invading the host apoplast. Thus, in order to adapt to the diverse environments encountered during epiphytic growth and host plant colonization, the bacterium has a critical need to be able to sense and respond to its extracellular environment. Extracytoplasmic function (ECF) sigma factors that function as transcriptional regulators of gene expression in response to specific environmental signals, offer a convenient mechanism to cater to this need (51).

ECF sigma factors are the largest and most diverse group of alternative sigma factors that regulate gene transcription in response to a wide range of environmental stimuli including heat-shock, osmotic and oxidative stress, and transport of metal ions (29). Signaling mediated by ECF sigma factors is often dependent on the ECF sigma

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This thesis follows the style of Journal of Bacteriology.

factor together with its cognate anti-sigma factor and an outer membrane receptor (26).

Bioinformatic investigation of the 6.09 Mb genome of B728a has revealed 15 different sigma factors (Fig. 1). These include the primary sigma factor *rpoD*, the σ^{54} *rpoN*, *fliA*, which controls the flagellar biosynthesis genes, *rpoS*, the starvation phase sigma factor, and *rpoH*, the heat shock sigma factor. In addition, Oguiza et al. have shown that the genomes of *P. syringae* pathovars encode 10 ECF sigma factors, all of which bear significant homology to those of other *P. syringae* pathovars (39). Five of these belong to the FecI-type of ECF sigma factors. These ECF sigma factors are arranged on the genome in clusters consisting of FecR-like transmembrane sensors and distinct outer membrane receptors, similar to the ferric citrate regulatory system of *E. coli* (39). The genomic organization of these genes along with their similarity to the *fec* system in *E. coli* suggests that they might be involved in the regulation of different iron transport systems.

The importance of iron in cellular processes as well as in the induction of major virulence factors has been established previously. Iron bioavailability exerts a strong influence on the expression of several virulence associated factors such as the type III secretion system (T3SS), biosynthesis of different siderophores and phytotoxins like syringomycin and syringopeptin in B728a and coronatine in *P. syringae* pv. *tomato* DC3000, as well as iron transport systems and a majority of the sigma factors (28, 56). Bacteria have evolved different strategies to sequester iron from the environment. Siderophore-mediated iron acquisition is among the most common forms of iron-uptake prevalent in bacteria (56). The ECF sigma factor, PvdS, has been well characterized in

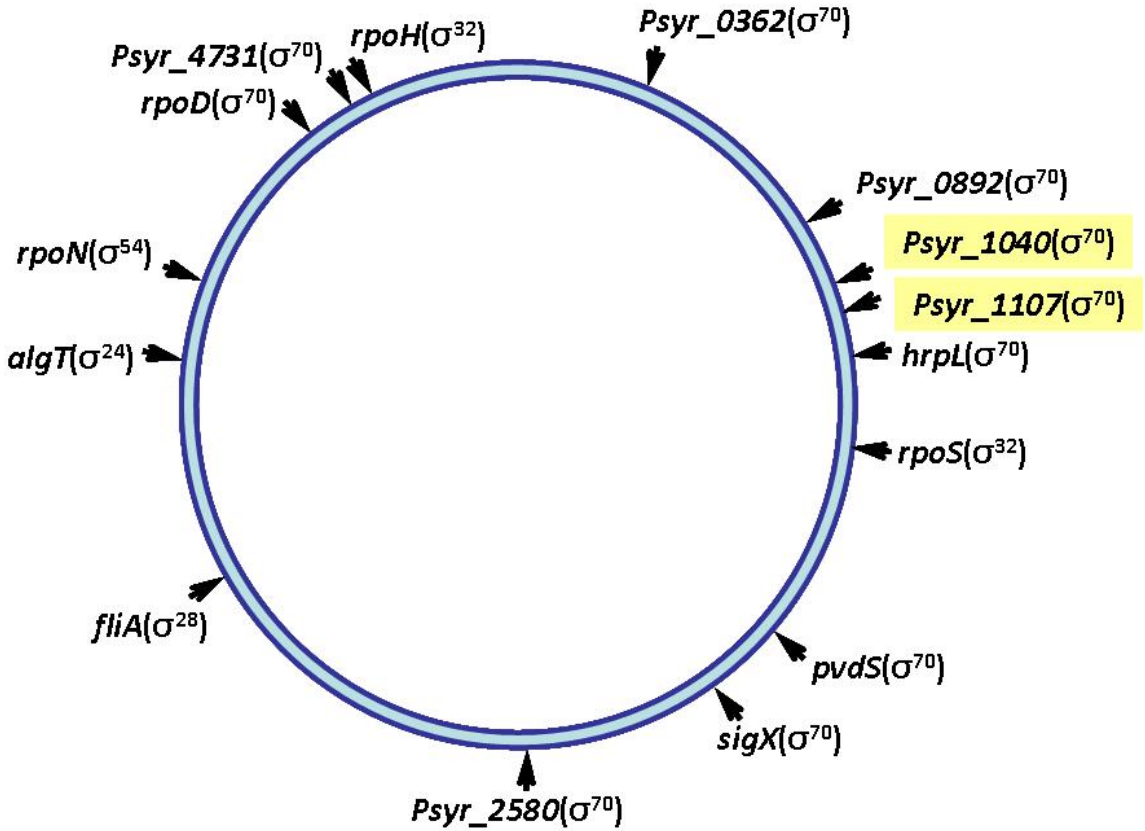


FIG. 1. Schematic representation of the B728a genome showing the different sigma factors. The 6.09 Mb genome of B728a encodes 15 different sigma factors. *rpoD* (*Psyr_4641*) is the essential housekeeping sigma factor for this bacterium belonging to the σ^{70} family, while *rpoN* (*Psyr_4147*) belongs to the σ^{54} type of sigma factors. The other sigma factors belong to the alternative sigma factor family and include *fliA* (*Psyr_3437*), which controls the flagellar biosynthesis genes, *rpoS* (*Psyr_1374*), the starvation phase σ factor, *rpoH* (*Psyr_4748*), the heat shock σ factor, and 10 ECF sigma factors, which are summarized in Table 1. Five of these belong to the FecI-type of ECF sigma factors, including *Psyr_1040*, *Psyr_1107*, *pvdS* (*Psyr_1943*), *Psyr_2580* and *Psyr_4731*. *Psyr_1040* and *Psyr_1107*, which are characterized in this thesis, are highlighted.

the opportunistic human pathogen *P. aeruginosa*, where it controls the transcription of genes for the biosynthesis and uptake of siderophores, and also regulates several virulence factors including exotoxin A and PrpL protease (52, 56). Homologs of *pvdS* have been identified in other fluorescent pseudomonads including B728a and the tomato pathogen, *P. syringae* pv. *tomato* DC3000, where it controls genes for siderophore biosynthesis. However, the influence of other iron-responsive sigma factors on bacterial virulence of B728a has not been elucidated. The overall goal of my research was to establish the roles played by *Psyr_1040* and *Psyr_1107*, both members of the FecI-type of ECF sigma factors, in shaping the B728a-host plant interaction. The predicted protein products of these genes share a significant level of identity with those of the other fluorescent pseudomonads, along with conserved domain organization. I hypothesized that these ECF sigma factors play a role in iron transport and are influenced by low iron conditions. In this thesis, I demonstrate that the expression of *Psyr_1040* and *Psyr_1107* are significantly up-regulated in conditions of iron stress and down-regulated in high iron. In addition, I show that Psyr_1040 and Psyr_1107 regulate expression of their associated transmembrane sensors and outer membrane receptors, and that Psyr_1107 contributes to the swarming activity of B728a.

CHAPTER II

FUNCTIONAL CHARACTERIZATION OF TWO ECF SIGMA FACTORS IN *Pseudomonas syringae* pv. *syringae* B728a

INTRODUCTION

B728a is a leaf-inhabiting Gram negative bacterium that can exist as an epiphyte on the leaf surface of bean plants, or as a highly aggressive plant pathogen (39). This bacterium is able to grow up to substantial numbers on the leaf surface, before entering the host through wound sites or natural openings, such as stomata. Colonization of the host is characterized by the rapid growth of the bacterium within the apoplast to large populations, which then serve to employ an array of virulence factors, including the Hrp type III secretion system (T3SS) and phytotoxins, to subvert host defense responses. The T3SS has been well studied and is known to inject effector proteins directly into the host cytoplasm (12, 54). B728a also produces two classes of cyclic lipopeptide phytotoxins, namely, syringomycin and syringopeptin. These macromolecules act as major virulence determinants for this strain (13). Both syringomycin and syringopeptin are potent necrosis-inducing toxins that form pores in plant plasma membranes, which results in passive transmembrane ion flux and eventually, cell death (1).

Recent advances in bacterial genome sequencing have enabled researchers to undertake genomic-based approaches to study *P. syringae* and related strains. The sequenced genome of B728a, spanning 6.09 Mb, is known to encode an array of regulatory genes. These include a high proportion of two-component systems and a

number of ECF sigma factors (29). This could mirror the remarkable ability of this bacterium to rapidly adapt to changing environments encountered during survival on the leaf surface and growth *in planta* (39). Identification of genes that might play a role in the adaptation of this strain to a plant pathogenic lifestyle is, therefore, essential in order to delineate the mechanisms by which B728a promotes disease on its host. ECF sigma factors, which control transcription of genes in response to extracellular stimuli, are important targets for the study of cell surface related signaling mechanisms of this organism.

Sigma factors are a class of proteins that reversibly bind the multi-subunit RNA polymerase core to initiate gene transcription in bacteria (41). These proteins provide promoter recognition specificity to the polymerase and contribute to DNA strand separation. Following transcription initiation, the sigma factor dissociates from the RNA polymerase core (18). All bacteria have one housekeeping sigma factor that controls the basal expression level of most genes during exponential growth, and a variable number of alternative sigma factors that possess different promoter-recognition properties (14, 50). The cell can choose from its array of sigma factors to alter its transcriptional program in response to various stimuli (14). In the absence of a stimulus, most alternative sigma factors are kept inactive by a cognate anti-sigma factor through direct protein-protein interaction (50).

Bacterial sigma factors can be grouped into two major categories, the σ^{70} and the σ^{54} type proteins (50). Members of the σ^{70} family are modular proteins consisting of up to four domains (14). Domain 1, on the N-termini of the σ^{70} proteins, has an

autoinhibitory region that masks DNA binding domains on free sigma factors and prevents them from binding to the promoter (14, 44). Domain 2 is highly conserved and comprises regions involved in core RNA polymerase binding, DNA melting and interaction with the -10 promoter element (44). Domain 3, which has a low degree of conservation, is known to interact with the extended -10 recognition determinants, when present (14, 44). The most C-terminal region of the σ^{70} type family is domain 4. This domain also has a high level of conservation among these proteins and contains a helix-turn-helix (H-T-H) motif for DNA binding to the -35 promoter region (44). In contrast to the σ^{70} family, the σ^{54} type proteins are known to recognize consensus sequences at -12 and -24 bp upstream of the transcription start site, and require an activator protein for gene transcription. These proteins have three functional domains. Domain 1 interacts with an upstream activator promoter to control promoter melting. Domain 2 comprises the core RNA polymerase and DNA binding motifs. Domain 3, on the C-terminus of σ^{54} proteins, has a highly conserved H-T-H motif that is critical for DNA binding (44). The σ^{54} proteins are required for diverse functions such as nitrogen assimilation, motility, phage-shock response and zinc tolerance (44). Although members of the σ^{54} family are widespread among bacteria, most bacterial genomes encode multiple proteins that are homologous to σ^{70}, and a single σ^{54} type representative (14, 44).

On the basis of sequence similarity and protein domain architecture, the σ^{70} family has been divided into several subgroups (14, 50). Group 1 includes primary sigma factors required for the transcription of essential housekeeping genes. Group 2 sigmas are closely related to the group 1 sigmas but are dispensable for growth (44). Members

of these two groups exhibit all the four distinct domains typical for the σ^{70} family, as well as a non-conserved region (NCR) adjacent to domain 2 (50). Proteins from the group 3 are more divergent in sequence than the group 1 sigma factors, and lack domain 1 and the NCR found in the group 1 and 2 sigma factors (50). The group 3 sigmas are known to activate specific regulons associated with heat shock, flagellar biosynthesis and sporulation (15, 44). Members of the fourth group constitute the ECF sigma factors, and exhibit the highly conserved domains 2 and 4 (Fig. 2). These sigma factors, which form the largest and most diverse subfamily of the σ^{70} type proteins, regulate gene transcription in response to a wide range of environmental stimuli including heat-shock, osmotic and oxidative stress, and transport of metal ions (29).

A limited number of ECF sigma factors have been characterized in the pseudomonads. These include HrpL, AlgT, SigX and PvdS. HrpL activates the expression of the well known *hrp/hrc* encoded T3SS and effector proteins that are directly transported into the host cytoplasm via the T3SS, and hence plays a key role in plant pathogenicity of *P. syringae* (12, 54). The stress response ECF sigma factor, AlgT, which is required for alginate production in *P. syringae*, increases heat tolerance of the bacterium and its ability to survive toxic compounds, such as, copper and H_2O_2 during plant tissue colonization (19, 20). SigX, which has been studied extensively in Gram-positive bacteria, is known to be associated with survival after stress. Strains of *Bacillus subtilis* lacking the *sigX* gene are impaired in their ability to survive at high temperature (17, 37). In *P. aeruginosa* and the root-colonizing *P. fluorescens*, this ECF sigma factor is involved in the regulation of a major outer membrane protein, OprF (5), which

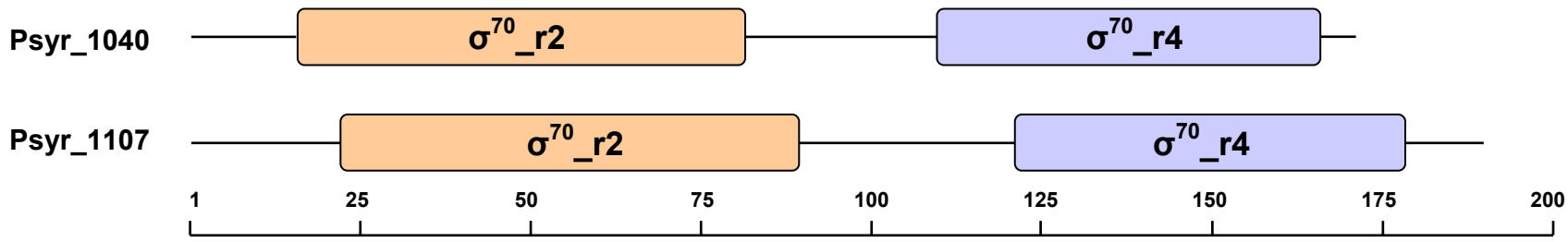


FIG. 2. Domain organization of the B728a FecI-type ECF σ factors, Psyr_1040 and Psyr_1107. The predicted protein products for Psyr_1040 and Psyr_1107 consist of 167 and 187 amino acids, respectively. Region 2 of the ECF σ factors is the most conserved region of the entire protein and contains both the -10 promoter recognition helix and the primary core RNA polymerase binding determinant. Region 4 interacts with the -35 promoter element. The scale bar indicates the protein length in numbers of amino acid residues.

functions as a nonspecific porin, involved in the maintenance of cell shape and growth in low-osmolarity environments (46). Another well-known ECF sigma factor is PvdS, which is associated with the regulation of genes involved in the biosynthesis of the siderophore, pyoverdine, in fluorescent pseudomonads and contributes significantly to the fitness of these bacteria during host-pathogen interactions (18, 52). Additionally, PvdS is also known to control the activity of genes encoding the virulence factors, exotoxin A and PrpL endoprotease in *P. aeruginosa* (18).

Sequenced genomes of fluorescent pseudomonads show frequent occurrence of the ECF sigma factors (Table 1) (29). However, bacteria with complex lifestyles involving different habitats appear to encode a greater number of ECF sigma factors than those with simple lifestyles living in stable niches (39). Thus, despite having similar genome sizes, the genomes of *P. aeruginosa* PAO1 and *P. putida* KT2440 are known to encode 19 ECF sigma factors (34), while that of *P. fluorescens* Pf-5 encodes 27 ECF sigma factors (32). In contrast, only 10 ECF sigma factors have been identified in *P. syringae* pathovars (39). Of these, five ECF sigma factors including HrpL, AlgT and SigX, are stress response sigma factors, while the other five which includes PvdS, are members of the iron responsive group of ECF sigmas (28). These iron responsive ECF sigma factors show a distinctive genomic arrangement consisting of transmembrane sensors and specific TonB-dependent siderophore receptors located in close proximity to the ECF sigma factors (39). The genomic organization of these ECF sigma factors closely resembles the well known ferric citrate regulatory system in *E. coli*, which is known to regulate transcription of the *fecABCDE* transport genes in response to ferric

TABLE 1. ECF sigma factors in B728a and their homologs in fluorescent pseudomonads[a]

P. syringae pv. *syringae* B728a	*P. syringae* pv. *phaseolicola* 1448A	*P. syringae* pv. *tomato* DC3000	*P. aeruginosa* PAO1	*P. putida* KT2440	*P. fluorescens* Pf-5
Psyr_0362	PSPPH_0345 (94%)	PSPTO_5176 (94%)	-	PP3006 (57%)	-
Psyr_0892	PSPPH_0927 (97%)	PSPTO_1043 (94%)	-	-	-
Psyr_1040	PSPPH_1093 (94%)	PSPTO_1209 (92%)	PA3899 (71%)	PP4611 (74%)	PFL0984 (76%)
Psyr_1107	PSPPH_1175 (92%)	PSPTO_1286 (88%)	-	PP1008 (70%)	PFL4625 (72%)
Psyr_1217 (HrpL)	PSPPH_1294 (92%)	PSPTO_1404 (88%)	-	-	-
Psyr_1943 (PvdS)	PSPPH_1909 (98%)	PSPTO_2133 (97%)	PA2426 (85%)	PP4244 (79%)	PFL4190 (87%)
Psyr_2096 (SigX)	PSPPH_2067 (98%)	PSPTO_2298 (98%)	PA1776 (94%)	PP2088 (90%)	PFL1875 (95%)
Psyr_2580	PSPPH_2747 (98%)	-	PA1912 (57%)	PP3577 (76%)	PFL2291 (55%)
Psyr_3958 (AlgT)	PSPPH_3955 (100%)	PSPTO_4224 (99%)	PA0762 (90%)	PP1427 (96%)	PFL1448 (97%)
Psyr_4731	PSPPH_4765 (94%)	PSPTO_0444 (92%)	PA0472 (78%)	PP0352 (82%)	PFL5704 (82%)

[a]Percent amino acid identities of homologs are indicated in parentheses (39).

citrate (4). This suggests that these FecI-type of ECF sigma factors are mainly involved in the regulation of different iron transport systems (39).

Acquisition of iron is crucial for microbial growth and survival, since it is an essential cofactor of many enzymes involved in metabolic activities (9). Most bacteria require micromolar levels of iron for optimal growth (43). However, the availability of free iron in nature is extremely low due to the rapid oxidation of the soluble form of iron (Fe^{2+}) to the insoluble ferric form (Fe^{3+}). Substantial amounts of iron contained in higher eukaryotes are bound to transport and storage proteins, and thus, not freely available to microbes (56). Consequently, bacteria have evolved different strategies to sequester iron from their extracellular environment. One of the most common iron uptake strategies employed by Gram negative bacteria involves the use of low molecular weight iron chelators called siderophores. Siderophore-mediated iron acquisition is known to play an important role in the virulence of *P. aeruginosa*, and in the fitness and biocontrol activity of fluorescent rhizosphere pseudomonads (43). Ferric iron bound siderophores are recognized by TonB-dependent siderophore receptors in the outer membrane, which act as gated porin channels. Transport of the ferrisiderophores into the cell is mediated by a complex of inner membrane proteins including TonB, ExbB and ExbD (8). However, iron uptake via siderophore production is an energetically expensive process that needs to be tightly regulated since the accumulation of iron can be deleterious to the cell, owing to the production of reactive oxygen species via the Fenton reaction (3, 5, 7). All fluorescent *Pseudomonas* spp. produce the yellow-green siderophore, pyoverdine, under conditions of iron limitation (9, 36). Pyoverdines have been shown to be important

for host tissue colonization and virulence in *P. aeruginosa* and *P. syringae* (9). PvdS, which controls expression of genes for pyoverdine biosynthesis and pathogenicity-associated factors in the pseudomonads, has been extensively studied in *P. aeruginosa* and *P. syringae*. Consequently, no work has been done to identify the roles that other members of the Foci-type of ECF sigma factors might play in the host-pathogen interaction. My research shows that the expression of the iron-responsive ECF sigma factors, *Psyr_1040* and *Psyr_1107*, along with their respective transmembrane sensors and outer membrane receptors are influenced by low iron conditions. This study also demonstrates the contribution of Psyr_1107 to bacterial swarming in B728a.

RESULTS

The B728a genes, *Psyr_1040* and *Psyr_1107* encode ECF sigma factors

An investigation of the predicted protein products of *Psyr_1040* and *Psyr_1107* using a combination of the bioinformatic resources, Pam (http://pfam.sanger.ac.uk/) and Interposal (http://www.ebi.ac.uk/Tools/pfa/iprscan/), revealed the presence of the two highly conserved regions, 2 and 4, commonly found in ECF sigma factors. A conserved domain search for Psyr_1040 and Psyr_1107 using NCBI Conserved Domains program (http://www.ncbi.nlm.nih.gov/Structure/cdd/) also confirmed the presence of these regions on the two proteins (Fig. 2). Analysis of the genomic neighborhoods of *Psyr_1040* and *Psyr_1107* showed the occurrence of FecR-type transmembrane proteins and TonB-dependent siderophore receptors in the immediate vicinity of both these genes, indicating that these ECF sigma factors belong to the FecI-type of ECF sigma

factors. BLAST analysis of these FecI-type ECF sigma factors using the blastp program at NCBI (http://www.ncbi.nlm.nih.gov//blast/Blast.cgi) revealed over 90% sequence homology to those of other *P. syringae* pathovars, and the presence of homologs in other fluorescent pseudomonads. Table 1 summarizes the percent amino acid sequence identities between ECF sigma factors in B728a and their corresponding homologs in other pseudomonads.

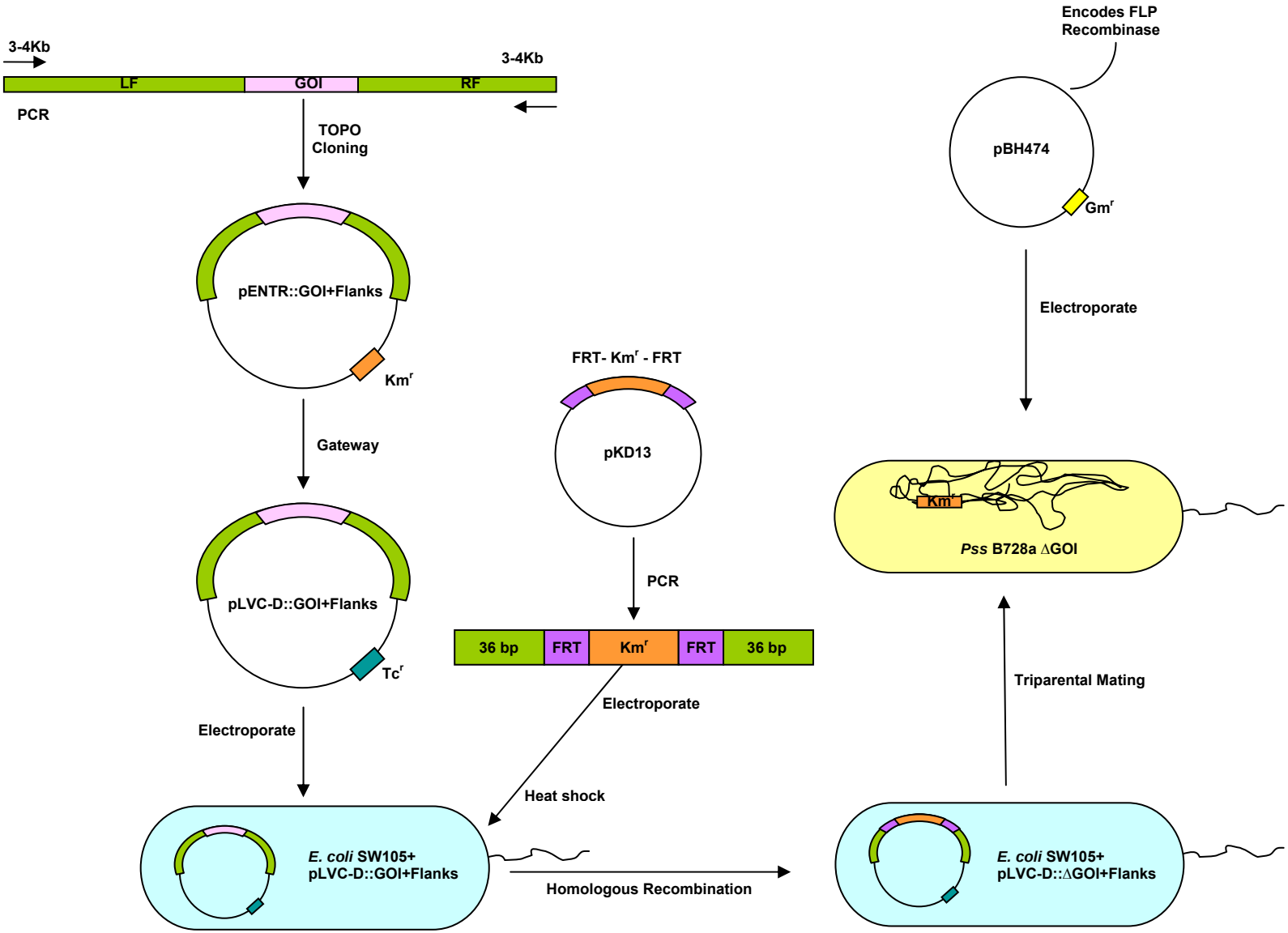
An effective method for markerless deletion mutatgenesis in B728a

Mutagenesis of *Psyr_1040* and *Psyr_1107* in B728a was conducted using a strategy based on recombination mediated by phage λ Red recombinase (10, 53, 59). The strategy is outlined in Fig. 3 and described in detail in Materials and Methods. *Psyr_1040* and *Psyr_1107* were deleted from the B728a genome using this method. Colony PCR and Southern analyses were conducted to confirm the deletions. The Km^r marker was removed from the resulting mutants using FLP recombination (10, 59), and the strains lacking *Psyr_1040* and *Psyr_1107* were named B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*, respectively.

The B728a *Psyr_1040* and *Psyr_1107* mutants are not affected in phytotoxin production

The lipopeptide phytotoxins, syringomycin and syringopeptin produced by B728a act as virulence factors for this strain (13) by forming pores in the host plasma membranes. This causes a leakage of electrolytes from the host cell, and ultimately leads to necrosis. (1). Besides being phytotoxic, these secondary metabolites also exhibit antimicrobial activity towards a wide variety of microorganisms. For example,

FIG. 3. Diagram of the strategy used for deletion mutagenesis of ECF sigma factor genes in B728a. The gene-of-interest (GOI, pink) along with 3-4 Kb flanking genomic regions (green) are PCR amplified, and cloned into the pENTR/D-TOPO vector carrying a kanamycin resistance marker (Km^r, orange), resulting in the pENTR::GOI+Flanks construct. Gateway cloning is used to move the GOI with its flanking regions into the pLVC-D destination vector carrying a tetracycline resistance marker (Tc^r, blue). This results in the pLVC-D::GOI+Flanks construct, which is transformed into *E. coli* SW105 (SW105) via electroporation. A linear cassette harboring a kanamycin resistance marker, flanked on either side by FLP recombinase recognition target (FRT-Km^r-FRT, purple, orange) sequences, is amplified from the pKD13 plasmid. The 5' end of the primers used to amplify the FRT-Km^r-FRT cassette had 36 bp tags homologous to the GOI flanks, to facilitate recombination. SW105 cells are briefly heat-shocked to induce expression of phage recombination proteins, and the linear cassette is subsequently transformed into these cells by electroporation. This results in SW105 cells carrying the pLVC-D::ΔGOI+Flanks construct. As detailed in the Materials and Methods section, triparental mating using a helper strain is used to catalyze replacement of the GOI in B728a with the FRT-Km^r-FRT cassette. The kanamycin marker is removed from B728a by electroporating vector pBH474, which encodes FLP recombinase enzyme.


3-4Kb
3-4Kb
LF
GOI
RF
PCR
TOPO
Cloning
pENTR::GOI+Flanks
Km^r
Gateway
pLVC-D::GOI+Flanks
Tc^r
Electroporate
FRT- Km^r - FRT
pKD13
PCR
36 bp
FRT
Km^r
FRT
36 bp
Electroporate
Heat shock
E. coli SW105+
pLVC-D::GOI+Flanks
Homologous Recombination
Encodes FLP
Recombinase
pBH474
Gm^r
Electroporate
Km^r
Pss B728a ΔGOI
Triparental Mating
E. coli SW105+
pLVC-D::ΔGOI+Flanks

syringomycin acts as an antifungal agent to filamentous fungi, such as *Geotrichum candidum*, and yeasts, such as *Rhodotorula pilimanae*. Similarly, the Gram-positive bacterium, *Bacillus megaterium*, and certain strains of *Botrytis cinerea* are highly sensitive to syringopeptin (1). To test the effects of the mutations in the ECF sigma factors, *Psyr_1040* and *Psyr_1107* on phytotoxin production, a bioassay was done to evaluate any differences in syringomycin levels produced by wild-type B728a and the two ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*. B728aΔ*gacS*, which lacks the GacS sensor kinase of the *gacA/gacS* two-component system, was used as a negative control. Wild-type B728a and mutant strains were incubated on Hrp minimal medium (HMM), and later sprayed with a suspension of *G. candidum* spores. Zones of inhibition resulting from the production of syringomycin were observed surrounding bacterial colonies of wild-type B728a and the ECF sigma factor mutant strains, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* (Fig. 4). Inhibition zones were absent in the B728a *gacS* mutant strain. This demonstrates that the ECF sigma factor mutants are not impaired in their ability to synthesize syringomycin.

The B728a *Psyr_1040* and *Psyr_1107* mutants are not reduced in exopolysaccharide production

B728a is known to produce at least two different EPS molecules, including alginate which is a co-polymer of *O*-acetylated β-1,4-linked D-mannuronic acid and L-guluronic acid, and levan, a polymer of fructofuranan (42, 60). Alginate is known to promote epiphytic fitness and virulence of *P. syringae* (38), while levan acts as an extracellular storage compound that is metabolized during periods of nutrient

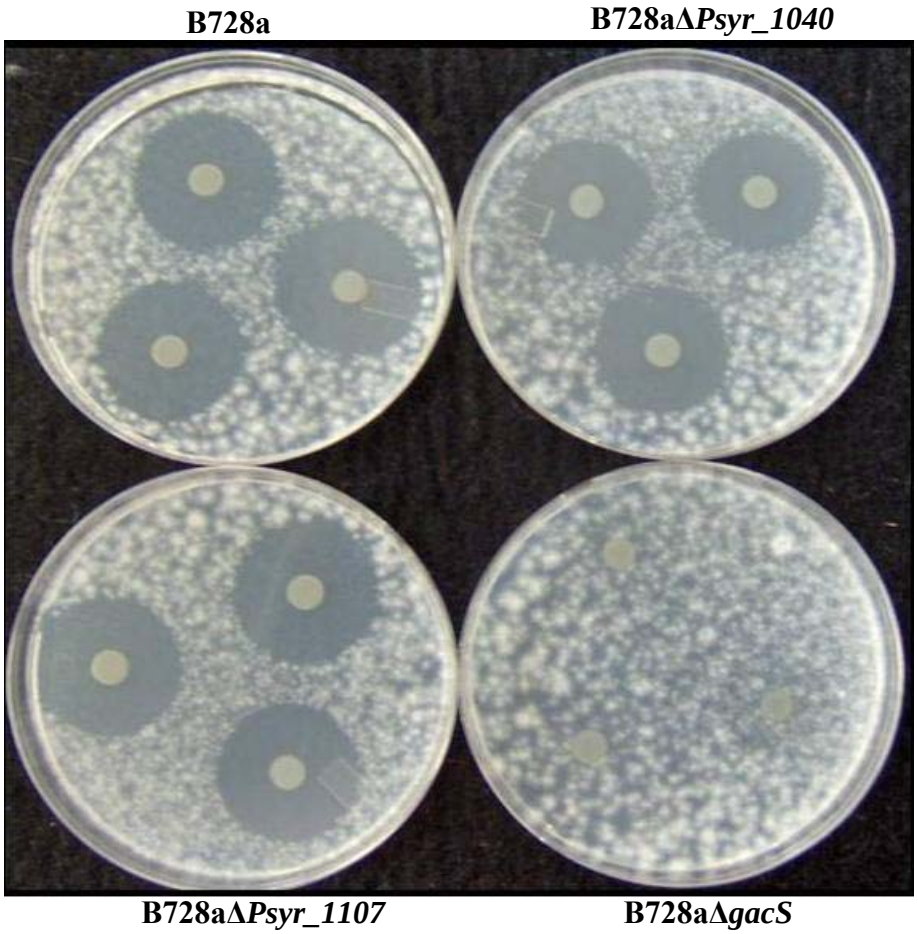


FIG. 4. Assay to evaluate syringomycin production in wild-type and mutant B728a. Wild-type B728a and mutant strains were spotted onto HMM agar and incubated at 25°C for three days, and sprayed with a suspension of *G. candidum.* Inhibition zones were measured a day later. The experiment was repeated three times.

deprivation (26, 47). Accordingly, wild-type B728a and the ECF sigma factor mutant strains, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* were assayed on mannitol glutamate medium supplemented with sorbitol and sucrose, to evaluate any differences in the levels of EPS produced by the different strains. Sorbitol and sucrose are known to induce the production of alginate and levan, respectively. Wild-type B728a and the ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*, appeared equally mucoid on media containing sorbitol and sucrose (data not shown), indicating the production of both alginate and levan by these strains. This suggests that the ECF sigma factors, Psyr_1040 and Psyr_1107, do not control EPS production in B728a.

The B728a *Psyr_1107* mutant exhibits a reduced swarming phenotype

Wild-type B728a and derivative strains, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*, were assessed for their ability to swarm on low agar medium. When inoculated onto filter discs in the center of semisolid NBY, wild-type B728a growth spread away from the disc in a dendritic pattern, thus indicating an ability to swarm (Fig. 5). B728aΔ*Psyr_1040* showed a similar movement pattern. However, B728Δ*Psyr_1107* is greatly reduced in swarming activity. The swarming distance of the different strains, as measured from the filter disk to the outer edge of bacterial growth, is summarized in Table 2. B728aΔ*gacS*, which was used as a negative control (22), showed no movement on the semisolid agar.

The B728a ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*, multiply *in planta* and produce disease symptoms similar to those caused by B728a

To determine the ability of B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* to cause

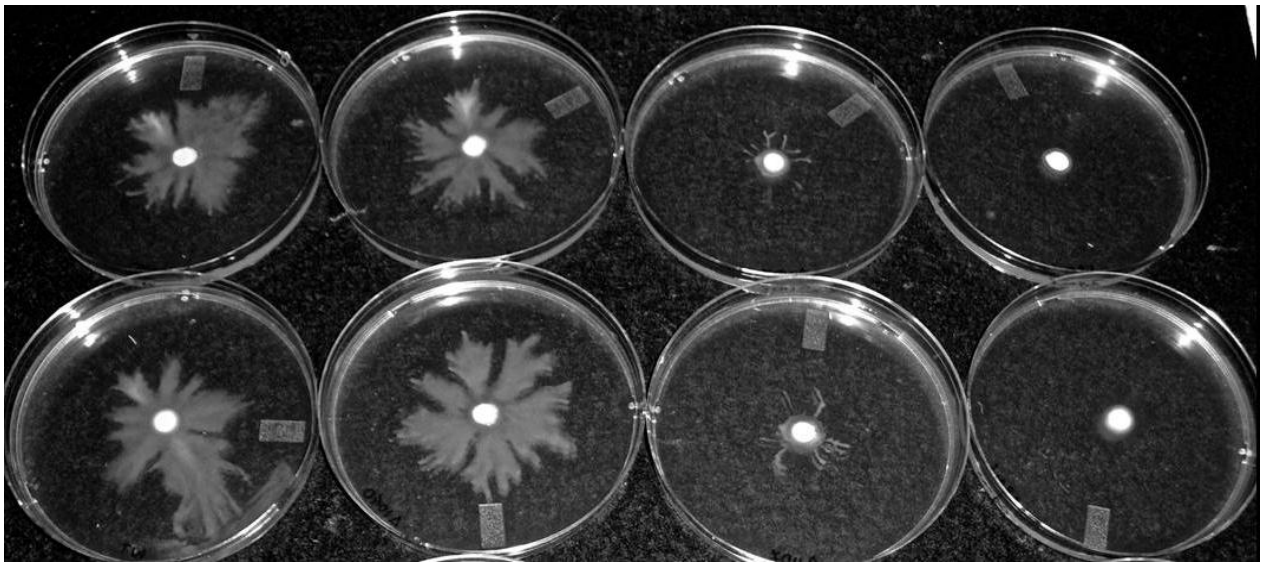


FIG. 5. Assay for swarming activity by *P. syringae* pv. *syringae* strains. Wild-type B728a and derivative strains were spotted on sterile filter discs placed in the center of semisolid NBY. The plates were incubated at 25°C in a humid chamber for 24 hours. Swarming distances of B728a and the ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*, were measured from the filter disk to the outer edge of bacterial growth. B728aΔ*gacS* was used as a negative control since it is unable to swarm.

TABLE 2. Swarming distance of wild-type B728a and the ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*[a]

Strain	Swarm Distance ± SE
B728a	33.57 ± 3.51
B728aΔ*Psyr_1040*	29.86 ± 2.19
B728aΔ*Psyr_1107*	11.86 ± 1.57
B728aΔ*gacS*	Does not swarm

[a]Values represent the average of three independent assays followed by the standard error (SE) of the mean.

disease on bean plants, pathogenicity assays were conducted using vacuum infiltration with bacterial suspensions of 10^6 CFU/ml. B728aΔ*gacS* was used as a control, since it is unable to cause disease (23). Each bacterial strain was tested on five individual plants and the experiment was repeated twice. B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* showed no difference in their ability to produce foliar disease symptoms, as compared to wild-type B728a (Fig. 6A).

Bacterial populations in infected plants were monitored over a 6-day period (Fig. 6B). At two days post-inoculation, bacterial titers for wild-type B728a were 5 x 10^7 CFU/cm^2, while those for B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* were 1.3 x 10^6 CFU/cm^2 and 4.5 x 10^5 CFU/cm^2, respectively. B728aΔ*gacS* grew up to 5 X 10^3 CFU/cm^2. At four days post-inoculation, wild-type B728a grew to 1.5 x 10^7 CFU/cm^2, whereas, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* grew to populations of 1.4 x 10^7 CFU/cm^2 and 6 x 10^6 CFU/cm^2, respectively. On day six, B728a, B728aΔ*Psyr_1040*, and B728aΔ*Psyr_1107* exhibited titers of 2.5 x 10^6 CFU/cm^2, 9 x 10^5 CFU/cm^2, and 3.8 x 10^5 CFU/cm^2, respectively. Titers for B728aΔ*gacS* at four and six days post-inoculation were 4.2 x 10^3 CFU/cm^2 and 4 x 10^3 CFU/cm^2, respectively. These results indicate that the ability of B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* to incite disease is delayed as compared to wild-type B728a.

***Psyr_1040* and *Psyr_1107* control the expression of genes involved in iron transport in B728a**

The genomic loci of *Psyr_1040* and *Psyr_1107* show the occurrence of specific siderophore receptors and transmembrane sensors adjacent to these ECF sigma factors.

A.

B.

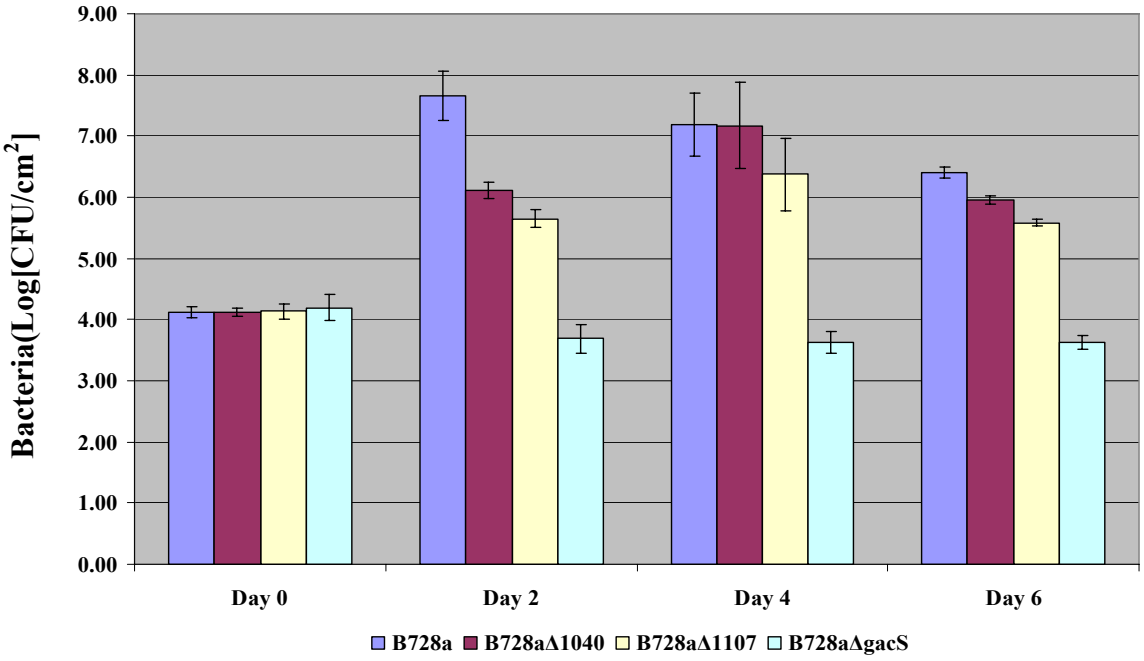


FIG. 6. Pathogenicity assay to evaluate contribution of *Psyr_1040* and *Psyr_1107* to disease development in bean. (A) Bean leaves were inoculated using vacuum infiltration with suspensions containing 10^6 CFU/ml of wild-type B728a, and the ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*. Plants were maintained at 25^{o}C in a growth chamber for 6 days. The experiment was performed twice; representative results are shown. (B) *In planta* populations of the different strains in panel A were monitored over a 6-day period. Bacterial populations in terms of the logarithm of CFU/ cm^2 of leaf surface are shown. Error bars represent the standard errors (SE) of the respective means.

In addition, the *Psyr_1107* locus shows the presence of a heme oxygenase gene (*Psyr_1104*) predicted to be involved in the uptake of heme, which acts as an essential source of iron for pathogenic bacteria like *P. aeruginosa*. After uptake, the heme molecule is degraded by heme oxygenases to yield ferrous iron for utilization by the cell (58). Data obtained from qRT-PCR analysis indicate a significant increase in expression of *Psyr_1040* along with the outer membrane receptor, *Psyr_1038* and the transmembrane sensor, *Psyr_1039* in low iron medium (Table 3). *Psyr_1107* does not show a significant level of expression in low iron conditions. However, *Psyr_1104*, *Psyr_1105* and *Psyr_1106*, which encode a heme oxygenase, an outer membrane receptor and transmembrane sensor, respectively, demonstrate that these genes are significantly induced in low iron medium (Table 3). These results suggest a role in iron uptake and transport for these genes.

To identify members of the *Psyr_1040* and *Psyr_1107* regulons in B728a, qRT-PCR analysis was used to determine transcript abundance of *Psyr_1038, Psyr_1039*, *Psyr_1104*, *Psyr_1105* and *Psyr_1106* in B728aΔ*Psyr_1040,* B728aΔ*Psyr_1107* and B728aΔ*gacS*, as compared to wild-type B728a in low iron conditions. The results indicate that expression of *Psyr_1038*, *Psyr_1039*, *Psyr_1104*, *Psyr_1105* and *Psyr_1106* are down-regulated in B728aΔ*Psyr_1040* (Table 4), indicating that *Psyr_1040* influences the expression of these genes. Interestingly, expression levels of *Psyr_1038*, *Psyr_1039*, *Psyr_1105* and *Psyr_1106* are significantly up in B728aΔ*Psyr_1107* (Table 4), suggesting *Psyr_1107* also plays a role in affecting expression of these genes. However, *gacS* does not appear to contribute significantly to

Table 3. Expression analysis of *Psyr_1040* and *Psyr_1107*, and putative iron-responsive genes in wild-type B728a in low and high iron media[a]

	Fold change in transcript levels ± SE	
Gene	**Expression in low iron**	**Expression in high iron**
Psyr_1040	6.31 ± 1.00	1.35 ± 0.20
Psyr_1038	2.67 ± 0.11	-2.68 ± 0.23
Psyr_1039	8.53 ± 0.66	-3.05 ± 0.07
Psyr_1107	1.87 ± 0.08	-1.02 ± 0.02
Psyr_1104	470.05 ± 18.87	26.14 ± 3.22
Psyr_1105	10.74 ± 1.57	-2.07 ± 0.11
Psyr_1106	2.25 ± 0.10	1.22 ± 0.09

[a]Values represent the average fold differences of three technical replicates of three biological samples. Gene expression was normalized to the *16s-rRNA* and *recA* internal control genes. Negative values indicate a decrease in expression levels as computed by taking the negative inverse of a fold change value less than 1.

Table 4. Expression analysis of putative iron-responsive genes in mutant strains of B728a as compared to wild-type B728a in low iron media[a]

	Fold change in transcript levels ± SE		
Gene	**B728aΔ*Psyr_1040***	**B728aΔ*Psyr_1107***	**B728aΔ*gacS***
Psyr_1038	-3.67 ± 0.19	-2.08 ± 0.03	1.39 ± 0.50
Psyr_1039	-10.58 ± 1.05	-5.76 ± 0.10	-1.51 ± 0.09
Psyr_1104	-127.57 ± 7.00	-1.33 ± 0.14	-2.98 ± 0.14
Psyr_1105	-17.73 ± 0.89	-159.79 ± 2.02	-1.09 ± 0.30
Psyr_1106	-2.87 ± 0.02	-2.61 ± 0.30	1.87 ± 0.26

[a]Values represent the average fold differences of three technical replicates of three biological samples. Gene expression was normalized to the *16s-rRNA* and *recA* internal control genes. Negative values indicate a decrease in expression levels as computed by taking the negative inverse of a fold change value less than 1.

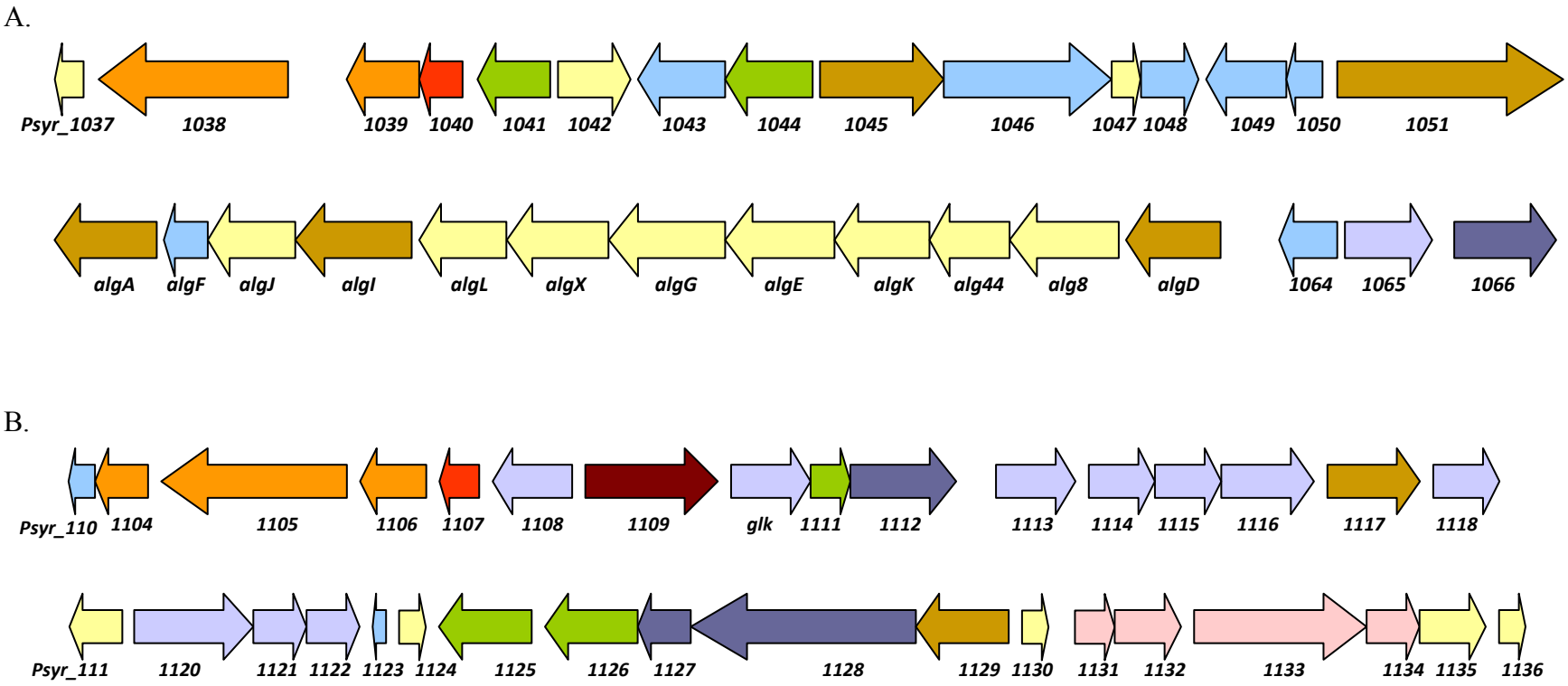


FIG. 7. Genomic neighborhood of *Psyr_1040* and *Psyr_1107*. The sigma factors, *Psyr_1040* and *Psyr_1107* are highlighted in red. (A) The TonB-dependent outer membrane receptor, *Psyr_1038* and the FecR transmembrane sensor, *Psyr_1039*, highlighted in orange, are adjacent to *Psyr_1040*. The alginate biosynthesis gene cluster (*algA-algD*) is located close to *Psyr_1040*. (B) *Psyr_1104*, *Psyr_1105*, and *Psyr_1106*, highlighted in orange, represent a heme oxygenase gene, a TonB-dependent outer membrane receptor, and a FecR transmembrane sensor, respectively, situated next to *Psyr_1107*. A pilus assembly/ fimbrial biogenesis gene cluster (*Psyr_1131-Psyr_1134*), highlighted in pink, is located in close proximity to *Psyr_1107*.

the expression of the genes, except for that of *Psyr_1104.* Taken together, these data suggest an overlap between the members of the*Psyr_1040* and *Psyr_1107* regulons.

The B728a *Psyr_1107* regulon includes genes associated with pili assembly in B728a

Investigation of the genomic regions flanking *Psyr_1107* revealed the occurrence of a cluster of genes (*Psyr_1131-1135*) involved in pili assembly (Fig. 7), organized in two operons. Bioinformatic analysis of these pilus-associated proteins revealed the presence of homologs in *P. syringae pathovars* (Table 5). Homologs of these proteins are absent in other *Pseudomonas* spp. Pili have been implicated in swarming behavior of *P. aeruginosa* (24). To determine the role of Psyr_1107 on the expression of these genes, qRT-PCR studies were performed to measure transcript abundance of *Psyr_1131-1134* in wild-type B728a and derivative strains. RNA was extracted from bacteria grown in liquid NBY and subjected to qRT-PCR using primers specific to *Psyr_1131-1134*. B728aΔ*Psyr_1107* exhibited a significant decrease in transcript levels of all the genes, while B728aΔ*Psyr_1040* did not show any apparent change in transcript levels (Table 6). The B728a *gacS* mutant showed greater reduction in expression levels. These results indicate that Psyr_1107 positively regulates expression of *Psyr_1131-1134.*

DISCUSSION

The genomes of *P. syringae* pathovars encode 10 ECF sigma factors, five of which belong to the FecI-type of ECF sigma factors, predicted to be involved in the regulation of iron transport systems (39). *Psyr_1040* and *Psyr_1107*, both of which encode putative ECF sigma factors, show the presence of TonB-dependent outer

Table 5. Putative pilus assembly/ fibril genes downstream of *Psyr_1107* in B728a[a]

***P. syringae* pv. *syringae* B728a**	***P. syringae* pv. *phaseolicola* 1448A**	***P. syringae* pv. *tomato* DC3000**	***P. fluorescens* Pf-5**
Psyr_1131 (Fimbrial protein)	PSPPH_1199 (95%)	PSPTO_1312 (94%)	PFL3922 (31%)
Psyr_1132 (Pili assembly chaperone)	PSPPH_1200 (83%)	PSPTO_1313 (73%)	PFL3923 (43%)
Psyr_1133 (Fimbrial biogenesis protein)	PSPPH_1201 (85%)	-	PFL3924 (49%)
Psyr_1134 (Pili assembly chaperone)	-	PSPTO_1317 (68%)	-

[a]Percent amino acid identities of homologs are shown in parentheses.

Table 6. qRT-PCR analysis of fimbrial gene expression in mutant B728a strains, as compared to wild-type B728a

	Fold change in transcript levels ± SE		
Gene	**B728aΔ*Psyr_1040***	**B728aΔ*Psyr_1107***	**B728aΔ*gacS***
Psyr_1131	1.41 ± 0.13	-3.07 ± 0.04	-7.06 ± 0.62
Psyr_1132	1.10 ± 0.26	-2.01 ± 0.10	-3.78 ± 0.28
Psyr_1133	1.05 ± 0.09	-3.47 ± 0.08	-4.72 ± 0.54
Psyr_1134	1.26 ± 0.07	-2.86 ± 0.02	-3.46 ± 0.17

[a]Values represent the average fold differences of three technical replicates of three biological samples. Gene expression was normalized to the *16s-rRNA* and *recA* internal control genes. Negative values indicate a decrease in expression levels as computed by taking the negative inverse of a fold change value less than 1.

membrane receptors and FecR-like transmembrane sensors adjacent to them, suggesting their possible role in iron sensing. Investigation of the domains of the predicted proteins of *Psyr_1040* and *Psyr_1107* showed the presence of the highly conserved regions 2 and 4, commonly associated with ECF sigma factors.

ECF sigma factors have been known to be key regulators of virulence in the pseudomonads. Examples include the previously characterized ECF sigma factors, HrpL, AlgT and PvdS, all of which participate in the virulence of *Pseudomonas* spp. B728a produces the cyclic lipopeptide toxins, syringomycin and syringopeptin, which are important pathogenicity factors for this bacterium. Phenotypic assays used to assess syringomycin production in the B728a mutants deficient in *Psyr_1040* and *Psyr_1107* revealed the presence of well-developed inhibition zones around the bacterial cultures (Fig. 4), indicating that these sigma factors do not contribute to the production phytotoxins.

B728a is known to produce at least two EPSs including the capsular polysaccharide, alginate and the polyfructan, levan (26, 47). Production of alginate has been associated with increased tolerance to environmental stresses, such as, heat and ROS, and therefore, implicated in the epiphytic fitness and virulence of *P. syringae* (19). Genes for alginate biosynthesis in *P. aeruginosa* and *P. syringae* are controlled by the ECF sigma factor, AlgT. Bioinformatic investigation of the genomic regions flanking *Psyr_1040* and *Psyr_1107* revealed the presence of the alginate biosynthetic cluster, *Psyr_1052 – Psyr_1063*. Hence, the ECF sigma factor mutant derivatives of B728a were assayed for the production of EPSs. Both B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107*

showed mucoid growth on MGY, when supplemented with sorbitol or sucrose (data not shown), indicating the production of alginate and levan.

Inoculation of bean plants via vacuum infiltration did not reveal a virulence defect in the ECF sigma factor mutants, B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107* (Fig. 6A). However, growth of B728aΔ*Psyr_1040* and B728aΔ*Psyr_1107 in planta* appears to be slower than wild-type B728a (Fig. 6B). It is possible that the Psyr_1040 and Psyr_1107 play a subtle role in the B728a-plant interaction, and require other experimental methods for detection.

Motility assays conducted with the ECF sigma factor mutants revealed that B728aΔ*Psyr_1107* is greatly reduced in its ability to swarm (Fig. 5) on semisolid agar surfaces. However, in comparison to the B728a *gacS* mutant, which is unable to swarm (22), movement in B728aΔ*Psyr_1107* is not completely abolished. My results indicate that Psyr_1107 positively contributes to B728a swarming activity. Consequently, there could be other components involved in the regulatory cascade that controls swarming in B728a. A study conducted by Köhler et al. has linked swarming in *P. aeruginosa* to flagella and pili, as well as nitrogen bioavailability and the production of the biosurfactant, rhamnolipid (24). Another study done by Kinscherf and Willis has demonstrated that the quorum sensing autoinducer signaling molecule, N-acyl-L homoserine lactone (AHL) (11), does not play a major role in swarming by B728a (22). However, Quiñones et al. have provided evidence that the quorum-sensing system comprising the AHL synthase, AhlI and the regulator, AhlR mediate the regulation of swarming motility in *P. syringae* pv. *syringae* (45). Thus, swarming motility in the

Pseudomonas spp. appears to be a complex process that involves multiple genes working in concert (22). Further investigation is required to elucidate the mechanism by which Psyr_1107 exerts its control on potential genes associated with swarming in B728a.

Expression analyses of *Psyr_1040* and *Psyr_1107* in low and iron media indicate that these FecI-type ECF sigma factors are indeed influenced by iron stress in the extracellular environment (Table 3). Moreover, expression profiles of the *Psyr_1040* and *Psyr_1107* associated outer membrane receptors (*Psyr_1038* and *Psyr_1105*) and FecR-type sensors (*Psyr_1039* and *Psyr_1106*) are also significantly up-regulated in iron-deficient conditions, and down-regulated in iron-replete conditions. *Psyr_1104*, which is located in close proximity to *Psyr_1107*, encodes a predicted heme oxygenase and shows the highest level of transcript abundance under low iron conditions. Based on the data obtained from qRT-PCR analysis of iron-responsive genes (*Psyr_1038*, *1039*, *1104*, *1105* and *1106*) in the ECF sigma factor mutants (Table 4), it appears that *Psyr_1040* positively regulates the expression of these genes. Surprisingly, *Psyr_1107* seems to control the expression levels of its own receptor, *Psyr_1105* and transmembrane sensor, *Psyr_1106*, as well as that of *Psyr_1038* and *Psyr_1039* associated with *Psyr_1040*. It is possible that *Psyr_1040* and *Psyr_1107* co-regulate the expressions of these iron-responsive genes. Transcript levels of the iron-responsive genes in Table 4 do not show a significant increase or decrease in the B728a *gacS* mutant, suggesting that *gacS* does not exert an influence on these genes.

Gene expression profiles of predicted pilus assembly proteins located in close proximity to *Psyr_1107* appear to be down-regulated in the B728a *Psyr_1107* mutant

(Table 6), indicating a potential role for this ECF sigma factor in the regulation of these genes. Interestingly, the B728a *gacS* mutant also exhibits a significant decrease in transcript levels of these pili-associated genes, suggesting a possible co-regulation of these genes by *gacS*. Pili have been reported to be involved in swarming motility, as well as in the adhesion and colonization of solid surfaces as early steps of biofilm formation by *P. aeruginosa* (24, 40). Motility has been strongly implicated in the virulence of *P. aeruginosa*, and is known to play a vital role in mobilization to and colonization of different environments, attachment of the bacteria to surfaces, and biofilm formation (40). It is possible that the expression of the pili biogenesis genes in B728a contributes to the complex phenomenon of swarming in this strain.

This thesis shows that Psyr_1040 and Psyr_1107 regulate virulence-associated activities in B728a. It is possible that these proteins act in concert with other regulators, such as the GacA/ GacS two-component system to orchestrate different effects on gene expression.

MATERIALS AND METHODS

Bacterial strains and media

The bacterial strains used in this study are listed in Table 7. *E. coli* Mach1 T1 cells were used following topoisomerase reactions, as per manufacturer's instructions (Invitrogen, Carlsbad, Calif.). *P. syringae* pv. *syringae* strains were routinely grown at 25^{o}C in nutrient broth-yeast extract (NBY) liquid or agar medium (55), or on King's B agar medium (KB) (21). Assays for syringomycin production were conducted on HMM

TABLE 7. Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial Strains		
E. coli		
DB3.1	F^- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(rB^- mB^-) *ara14 galK2 lacY1 proA rpsL20*(Sm^r) *xyl5* Δ*leu mtl1*	(2)
Mach1 T1	F^- Δ*recA1398 endA1 tonA* φ80(*lacZ*)ΔM15 Δ*lacX74 hsdR*(rK^- mK^+)	Invitrogen
SW105	DY380 (*cro-bioA*) <>*araC*-P_{BAD}*Cre* Δ*galK*	National Cancer Institute
P. syringae pv. *syringae*		
B728a	Wild-type, bean pathogen; Rif^r	(30)
B728aΔ*Psyr_1040*	*Psyr_1040* mutant derivative of B728a, Rif^r	This study
B728aΔ*Psyr_1107*	*Psyr_1107* mutant derivative of B728a, Rif^r	This study
B728aΔ*gacS*	*gacS* mutant derivative of B728a, Rif^r	(47)
Plasmids		
pBH474	*flp* constitutively expressed; Gm^r Suc^s	(16)
pENTR/D-TOPO	Gateway entry vector; Km^r	Invitrogen
pE1040	pENTR/D-TOPO carrying *Psyr_1040*, Km^r	This study
pE1107	pENTR/D-TOPO carrying *Psyr_1107*, Km^r	This study
pKD13	Template plasmid containing FRT-flanked *nptII*	(6)
pLVCD	Gateway destination vector for mating with *P. syringae*; pBR322 derivative with *mob* genes from RSF1010; Tc^r Ap^r Cm^r	(33)
pLV1040	pLVCD carrying *Psyr_1040*; Tc^r Ap^r	This study
pLV1107	pLVCD carrying *Psyr_1107*; Tc^r Ap^r	This study
pLV1040-FP	pLVCD carrying upstream and downstream regions of *Psyr_1040* fused to *nptII*; Tc^r Ap^r Km^r	This study
pLV1107-FP	pLVCD carrying upstream and downstream regions of *Psyr_1107* fused to *nptII*; Tc^r Ap^r Km^r	This study
pRK2073	Helper plasmid; Sp^r Trm^r	(27)

agar. Assays for mucoidy were performed on mannitol glutamte-yeast extract (MGY) agar supplemented with 0.6M sorbitol, or MGY supplemented with 5% sucrose (26). Assays for swarming activity were performed on NBY with 0.4% agar (19). Antibiotics were added at the following concentrations (μg ml^{-1}): rifampicin, 100; kanamycin, 75; tetracycline, 20; ampicillin, 100; gentamycin, 5; spectinomycin, 100.

General DNA manipulations

Restriction enzymes, T4 DNA ligase, and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Oligonucleotides were designed using the PrimerQuest and OligoAnalyzer applications of Integrated DNA Technologies (Coralville, Iowa) and purchased from them. The primer sequences used in this study are listed in Table 8. For cloning using the Gateway technology (25), target genes were amplified by PCR and cloned into the pENTR/D-TOPO vector (Invitrogen). LR clonase (Invitrogen) was used for recombination between pENTR constructs and Gateway destination vectors, according to the manufacturer's instructions. Plasmids were introduced into *E. coli* via chemical transformation or electroporation (48). Plasmids were introduced into *P. syringae* pv. *syringae* via tri-parental mating using the helper plasmid pRK2073 (27). Standard cycling conditions were used for PCR.

Construction of markerless *Psyr_1040* and *Psyr_1107* deletions in B728a

Clean deletion mutants of the two ECF σ factor genes, *Psyr_1040* and *Psyr_1107,* were created using a Red recombinase deletion method (10). This procedure utilizes homologous recombination in *E. coli* SW105 (SW105), which is mediated by recombination proteins provided from a defective λ prophage inserted into the SW105

TABLE 8. Primers used for PCR amplification

Name	Sequence (5' – 3')
P01	CACCCCAATTGCGTACGCCCATTCAGTT
P02	GCCCTGCGATTTGATTCCATGTGT
P03	CACCCGTCATCACTGACCTTGTAGTAGC
P04	AACTGACCTGATACAGCAACAGC
P05	GGATTTGTTTAAAGACTCGTTGTTCGGATTCATAGGGTGTAGGCTGGAGCTGCTTCG
P06	GAATCCACGCCACCAATGAGTCCCCGTGGATTGTTTATTCCGGGGATCCGTCGACC
P07	CGATATGAGTGTGGCAGCGTGCGGCAGGGTTCAGGGGTGTAGGCTGGAGCTGCTTCG
P08	GCGGACAAATATTTACAGATCCAGGCTTGACCCTCCATTCCGGGGATCCGTCGACC
P09	AAACATGCGCGCGAAGTGGTATTC
P10	AAAGCGAAAGGTTCATGGTGCAGG
P11	TCTGTCCTTTACTGCGCGTTATGC
P12	ACCGTCAATGGTGACCGTATTTCG

genome (57). Recombination proteins are transcribed from a promoter, which is repressed by the temperature-sensitive repressor *cI*857 at 32°C and de-repressed at 42°C. When bacteria containing this prophage are kept at 32°C no recombination proteins are produced. However, after a brief heat-shock at 42°C for 15-20 minutes, recombination proteins are produced.

In this strategy, a genomic fragment containing the gene-of-interest (GOI) along with 3-4 Kb flanking DNA, was PCR amplified using a long range proof-reading polymerase. The amplified PCR product was then directionally cloned into a TOPO cloning vector, pENTR/D-TOPO (Invitrogen) according to the manufacturer's instructions. LR clonase II (Invitrogen) was utilized to carry out recombination between the pENTR construct and the Gateway destination vector, pLVC-D. The pLVC-D plasmid was then introduced into SW105 cells via electroporation. A linear kanamycin cassette (FRT-Km-FRT) flanked on either side by FLP recognition target (FRT) sites, was amplified from the pKD13 plasmid using PCR primers with 36 bp extensions that were homologous to regions adjacent to the GOI. This linear cassette was moved into SW105 by electrotransformation following heat shock at 42^{o}C for 15 minutes. Homologous recombination mediated by the phage recombination proteins produced as a result of the brief heat shock replaced the GOI with the FRT-Km-FRT cassette. The pLVC-D plasmid with the linear cassette was then introduced into B728a via triparental mating using the helper plasmid pRK2073 (27). The kanamycin marker was later removed from mutant B728a by introducing the pBH474 vector carrying FLP recombinase.

Syringomycin assays

The production of syringomycin by B728a and derivative strains was evaluated on HMM agar. Bacteria were grown overnight in 2 ml NBY at 25°C with shaking. Cells were washed and resuspended in sterile water to OD_{600} = 0.3 (~2 x 10^8 CFU/ml), and spotted on HMM agar. The strains were incubated at 25°C for three days, following which they were finely sprayed with a suspension of *G. candidum* using sterile chromatography sprayers. Inhibition zones were measured a day later. The experiment was repeated three times.

Swarming motility assays

Swarming motility of strains was assessed on semisolid NBY containing 0.4% agar (19). Initially, bacteria were grown overnight in 2 ml NBY at 25°C with shaking. Cells were pelleted, washed and resuspended in NBY. 5 ml fresh NBY was then seeded with 6 µl of the washed culture and grown at 25°C with shaking, up to OD_{600} = 0.3. Sterile filter discs (Fisherbrand, Grade P8-Creped) sized to 6 mm with a standard 1-hole punch, were placed in the center of each plate and inoculated with a drop containing ~2 x 10^8 CFU/ml. Plates were incubated at 25°C for 24h in a moist chamber. The experiment was repeated three times.

Pathogenicity assays

The ability of the B728a *Psyr_1040* and *Psyr_1107* mutants to cause disease on bean plants was evaluated. B728a and derivative strains were grown overnight in 2 ml NBY at 25°C with shaking. The overnight cultures were used to inoculate fresh 100 ml NBY. Cultures were incubated at 25°C with shaking and grown to OD_{600} = 0.6. Two-

weeks-old Blue Lake 274 bean plants (*Phaseolus vulgaris*) were vacuum infiltrated with ~ 5 x 10^6 CFU/ml. The excess inoculum was gently washed off with sterile water. Disease symptoms were evaluated 2-3 days post inoculation. Each bacterial strain was tested on five individual bean plants, and the experiment was repeated twice.

For population analyses, five leaves were arbitrarily collected from each inoculated plant. A 2 ml screw cap microcentrifuge tube (Bio Plas Inc., San Rafael, Calif.) was used to punch out 14 leaf discs (8 mm diameter). The discs were homogenized in Silwet phosphate magnesium buffer. Serial dilutions were made in sterile water, and spread on KB agar. Colonies were enumerated after plates were incubated at 25°C for 48h.

RNA isolation for qRT-PCR studies

Bacterial strains were cultured overnight in 2 ml of NBY medium. Cells were harvested by centrifugation, washed in sterile water, and then 6 µl of washed culture were used to inoculate 2 ml of fresh NBY. The cultures were grown at 25°C with shaking to OD_{600} = 0.6 (approximately 5 x 10^8 CFU/ml). For iron-related studies, overnight cultures of strains were pelleted and washed in iron-free water, and then grown in modified liquid HMM without added iron, and supplemented with 10 µM and 100 µM $FeCl_3$ by shaking at 25°C to OD_{600} = 0.6. Cultures were grown in iron-free glassware to minimize iron contamination. Total RNA was extracted using the RNeasy Mini Kit in conjunction with RNAprotect reagent (Qiagen Inc., Valencia, Calif.) as per manufacturer's instructions. cDNA was generated using the SuperScript® VILO™ synthesis kit (Invitrogen, Carlsbad, Calif.). RNA samples were treated with TURBO

DNAse (Ambion, Austin, Texas) to remove any residual DNA in the samples.

qRT-PCR analyses

To determine the effects of low and high iron conditions on *Psyr_1040* and *Psyr_1107* in wild-type B728a and mutant strains, qRT-PCR was performed using primers specific for *Psyr_1040* and *Psyr_1107*. The effects of *Psyr_1040* and *Psyr_1107* mutations on the expression of their respective transmembrane sensors and outer membrane receptor genes was also analyzed using primers specific to *Psyr_1038*, *Psyr_1039*, *Psyr_1104*, *Psyr_1105* and *Psyr_1106*. To determine the effects of the *Psyr_1107* deletion on downstream fimbrial-type genes, primers specific to *Psyr_1131*, *Psyr_1132*, *Psyr_1133* and *Psyr_1134* were used to measure transcript abundance. Primer sequences are listed in Table 9. Gene expression was normalized to that of the housekeeping genes, *recA* and *16s-rRNA*. For each primer pair, the linearity of detection was confirmed to have a correlation coefficient of at least 0.98 ($r^2 > 0.98$) over the detection area by measuring a five-fold dilution curve with cDNA generated from reverse transcription of bacterial RNA. Reverse transcription was conducted by incubating samples at 25^{o}C for 10 minutes, followed by an incubation at 42^{o}C for 60 minutes. The reaction was terminated by incubating the samples at 85^{o}C for 5 minutes. Conditions for qRT-PCR consisted of incubation at 95^{o}C for 20 seconds, followed by 40 cycles comprising 3 seconds at 95^{o}C and 30 seconds at 60^{o}C. This was followed by melting curve analysis.

A comparative Ct (cycle threshold) method (49), also known as the ΔΔCt method, was used to determine an increase or decrease in transcript levels. Fold change

TABLE 9. Primers used for qRT-PCR analysis

Name	Sequence (5' – 3')
P13	TAGTGATTGCACAGCACACG
P14	CACACTGTCGAAGGCCTGTA
P15	GTCTGGAAAACGAAGGGTTC
P16	GTCGCACTCAGACTTCAACA
P17	GTGTAGTACTGGCGGTTGAAGATG
P18	CTTAATTTCGACAGCGCCTTCCAG
P19	AGCGACTCAACTCTGCGAGAAA
P20	ATTTGCCGGAGCCCTTGAAATAGC
P21	GTTTGATCAGGAATGCTGCACCGA
P22	TCAAACGATCATCAGTGACCTGCC
P23	GAAGGTCCAGTCATTCCAGCTGAT
P24	CAACGATCAAGCGCCAGAAAGTGA
P25	ATCGAAGAAGGCTTCACCCTGGTA
P26	GTTGTTCAGCTTCAGCAATCTGCC
P27	ACAACGGCACAATGGTCTTCAACG
P28	TCGAATCGGCTTTCGCTCGATACA
P29	TGACACTGAACTTGCCCTTCTGGA
P30	TAACCGGTTGTTTGTTGCCAAGGG
P31	AGACGTTCACCGATTCGCAGGTAT
P32	AACTTCCACGGTCGCATTGGTTTC
P33	CACCTGTATGTGCTTGAGATACCC
P34	TCATGAGTCAGTGTCCACAGCA

in gene expression was calculated according to the $2^{-\Delta\Delta Ct}$ equation, where $2^{-\Delta\Delta Ct}$ = [(Ct $_{\text{gene-of-interest}}$ − Ct $_{\text{internal control}}$) Treated sample − (Ct $_{\text{gene-of-interest}}$ − Ct $_{\text{internal control}}$) Untreated sample] (49). A fold change increase or decrease of 2 or more was considered to be significant (47). Fold change values of less than 1 indicated a reduction in transcript levels. The actual decrease in fold change was then computed by taking the negative inverse of the fold change value (49).

CHAPTER III

CONCLUSION

Cell surface signaling mediated by ECF sigma factors is commonly used by bacteria as a means to turn on specific regulons in response to various environmental stimuli. Studies have found that the sequenced genomes of bacteria encode a number of ECF sigma factors. Moreover, adaptation to different environments appears to be associated with the number of ECF sigma factors encoded by the bacterial genome (38). B728a, which is a highly fit colonist of bean, is known to encode 10 ECF sigma factors in its genome (38). This is considerably lower than that of other fluorescent pseudomonads including *P. aeruginosa* PAO1 and *P. putida* KT2440 which encode 19 ECF sigma factors (35), and *P. fluorescens* Pf-5 encoding 27 ECF sigma factors (32). Five of the 10 ECF sigma factors in B728a are members of the iron-starvation sigma factors with distinct siderophore receptors and transmembrane sensors located in close proximity to these genes. *Psyr_1040* and *Psyr_1107* are two such ECF sigma factors encoded by B728a. In this study, I addressed the hypothesis that *Psyr_1040* and *Psyr_1107* are involved in the control of iron transport in the cell, and hence play a role in the B728a-plant interaction.

The importance of iron acquisition by bacteria is reflected by the occurrence of multiple siderophore receptors associated with the iron-starvation sigma factors. The ability of *Pseudomonas* spp. to utilize heterologous siderophores of bacterial and fungal origin has been reported previously (31). My research confirms that *Psyr_1040* and

Psyr_1107 are expressed during conditions of iron deficiency. Furthermore, the data obtained from this study indicates potential roles of these ECF sigma factors in influencing the expressions of their respective siderophore receptors and transmembrane sensors (Table 4).

Phenotypic assays carried out to characterize the functional roles played by *Psyr_1040* and *Psyr_1107* in shaping the B728a-plant interaction, revealed a marked deficiency in swarming motility of the B728a *Psyr_1107* mutant (Fig.5). qRT-PCR analysis of genes downstream of *Psyr_1107*, predicted to encode putative pili assembly proteins (Table 5), indicates that this ECF sigma factor exerts a positive influence on the expression of these pili-associated genes (Table 6).

Results of the pathogenicity assays conducted with mutant strains of B728a lacking *Psyr_1040* and *Psyr_1107*, showed a slower growth rate of the ECF sigma factor mutants in bean plants, as compared to wild-type B728a (Fig. 6). *Psyr_1040* and *Psyr_1107* appear to play a subtle role in the B728a-plant interaction, and require more sensitive experimental methods for detection. It is possible that a decrease in the ability of the ECF sigma factor mutants to utilize bioavailable iron in the host apoplast, such as host iron bound proteins causes this delay in growth. The significant decrease in population numbers of the B728a *Psyr_1107* mutant could also be attributed to a decrease in the ability of the bacterium to adhere to its host, which delays multiplication and dissemination *in planta*.

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