CHARACTERIZATION OF THE *rpoN* GLOBAL REGULATORY GENE OF *Pseudomonas syringae* pv. *syringae* B728a AND ITS IMPACT ON THE PLANT-PATHOGEN INTERACTION

A Thesis

by

AMBER LORGE

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 2009

Major Subject: Plant Pathology

CHARACTERIZATION OF THE *rpoN* GLOBAL REGULATORY GENE OF *Pseudomonas syringae* pv. *syringae* B728a AND ITS IMPACT ON THE PLANT-PATHOGEN INTERACTION

A Thesis

by

AMBER LORGE

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

Approved by:

Chair of Committee,	Dennis Gross
Committee Members,	Carlos Gonzalez
	Alan Pepper
Head of Department,	Dennis Gross

May 2009

Major Subject: Plant Pathology

ABSTRACT

Characterization of the *rpoN* Global Regulatory Gene

of *Pseudomonas syringae* pv. *syringae* B728a and Its Impact on

the Plant-Pathogen Interaction. (May 2009)

Amber Lorge, B.S., Marian College

Chair of Advisory Committee: Dr. Dennis Gross

Gene regulation in bacteria is highly complex and requires the activity of sigma factors that function as transcriptional regulators. In *Pseudomonas syringae* pv. *syringae* B728a, 14 sigma factors have been identified. One of the more interesting is *rpoN*, encoding Sigma 54, which was initially described for its role in nitrogen utilization and later shown to be involved in regulating adhesion, motility, toxin production, and pathogenicity. The only commonality identified amongst these genes is that gene regulation by Sigma 54 is not essential for normal growth and development because mutational inactivation of *rpoN* is not lethal. Unlike Sigma 70, which recognizes promoter sites located at positions -10/-35 upstream of the transcription initiation site, Sigma 54 recognizes sites located at positions -12/-24. *P*.s. pv. *syringae* B728a encodes an RpoN that shares 80-98% identity with other *Pseudomonas* species. Promoter scans were conducted on the B728a genome to look for probable binding sites of RpoN. Analysis revealed that RpoN may be involved in regulating genes encoding ABC transporters, drug efflux pumps, flagella proteins, nitrate transporters, and several

regulatory proteins. An insertional mutation in the *rpoN* gene was constructed in the B728a genome and a phenotypic analysis was initiated. Decreased swarming and adhesion ability of the *rpoN* mutant was observed as compared to B728a. The ability to utilize sole nitrogen sources was also affected. The *rpoN* mutant showed little or no growth on sole nitrogen sources such as alanine, histidine, lysine, and serine. Pathogenicity was shown to require a functional RpoN, as both HR and disease development was effected by an *rpoN* mutation. *Pseudomonas syringae* pv. *syringae* is most known for the production of two phytotoxins. Unlike RpoN in other species, in *P.s.* pv. syringae B728a it appears to indirectly down regulate toxin production of syringomycin and syringopeptin. The goal of this study was to characterize some of the important roles RpoN is known to possess and to understand its role in the plant pathogenic and epiphytic lifestyle of *P. s.* pv. *syringae* B728a.

DEDICATION

To my parents, who taught me to never walk away from what I started. And my future husband, Jason Enderle, for all the help and support he gave me.

ACKNOWLEDGEMENTS

I would like to thank my major advisor and mentor, Dr. Dennis Gross. Throughout my time at Texas A&M, I always appreciated his support in all my decisions. I would also like to thank the rest of my committee members, Dr. Carlos Gonzalez and Dr. Alan Pepper.

I would also like to thank many members of the Plant Pathology & Microbiology department who have supported me in many ways. These persons include Dr. Angela Records, Dr. Brian Shaw, Dr. Charles Kenerley, Jessie Williams, Charles Greenwald, and Suzanne Segner.

And finally, I would like to thank all the friends I have met while at Texas A&M, friends from my past, and my family for their support throughout this process.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION TO GENE REGULATION IN BACTERIA

Gene regulation in bacteria is highly complex and requires the activity of sigma factors that function as transcriptional regulators. Prokaryotes utilize several different sigma factors to aid in proper gene expression in coordination with RNA polymerase (RNAP). Briefly, a sigma factor is a prokaryotic transcription initiation factor which is required to interact with RNA polymerase for specific binding to promoter sites on DNA upstream of a specific gene. These sigma factors aid RNA polymerase in gene transcription by allowing RNAP to initiate unwinding and melting of the DNA strand, and begin transcription after activation. It is the substitution of different types of sigma factors that redirects RNAP to activate transcription of different genes, which would not otherwise be transcribed (59). Once gene transcription begins, the sigma factor is released and available to activate another RNA polymerase subunit (57). Since RNAP is able to interact with many sigma factors and disassociate from RNAP after activation, the sigma cycle is able to adjust transcription levels rapidly in response to appropriate internal and external cues. The types of genes transcribed are therefore not based on RNAP, but by the activation of specific sigma factors (40).

Bacteria encode multiple sigma factors based on the complexity of its genome and life cycle. The obligate pathogen *Mycobacterium leprae* encodes the least number,

---

This thesis follows the style of Journal of Bacteriology.

only four sigma factors (49). In contrast, 14 sigma factors have been identified in the non-obligate pathogen *Pseudomonas syringae* pv. *syringae* B728a genome. In addition to the common sigma factor family, the Sigma 70 family, many alternative sigma factors exist to aid in gene regulation. Some of these include: Sigma B, Sigma S, Sigma 28, Sigma E, and Sigma 54 (27). Sigma 54, which is encoded by the gene *rpoN* (initially named *ntrC*), is a relatively rare alternative sigma factor that is not found in all bacterial genomes. Several prokaryotes utilize Sigma 54 to control transcription of a diverse set of genes, although a physiological theme has not yet been identified (27). Initially described for its role in nitrogen utilization, it was later shown to be involved in regulating adhesion, motility, toxin production, and pathogenicity (48). The only commonality identified amongst these gene classes is that gene regulation by Sigma 54 is not essential for normal growth and development because mutational inactivation of *rpoN* is not lethal (54).

Sigma 54 is both structurally and functionally unique from Sigma 70 (27). Its main differences include over-all protein structure, a unique promoter recognition site, and a requirement for activation. Sigma 54 consists of three domains that are important in the regulatory process. Domain I, the activator interacting domain (AID), interacts with various activator proteins. In order to initiate transcription, it is required that Sigma 54 is activated by an outside activator protein, which will allow for an open promoter complex. Domain II, the core binding domain, directly contacts RNAP to form an enhancer dependent holeoenzyme. Finally, domain III, the DNA binding domain, binds to the appropriate promoter sequence on the DNA strand. The promoter site recognized

by Domain II of Sigma 54 is very unique, highly conserved, and consists of two short sequences at positions -24 and -12, upstream of the transcription initiation site. Unlike the traditional TATA box recognized by Sigma 70, Sigma 54 recognizes TGGCAC-N5-TTGC, where the underlined regions identify the -24/-12 position upstream of the gene of interest and are the most conserved nucleotides (4, 6).

Sigma 54 is constitutively expressed and actively inhibiting gene transcription until activated. Since this sigma factor governs expression of such a diverse set of genes, there are several activators present in the genome. Specifically, *Pseudomonas aeruginosa* possesses 21 activators, 12 of which have a known function (55). Regulation of Sigma 54 and RNAP requires specific binding to the appropriate promoter sequence and activation by specific activators known as enhancer binding proteins (EBP). These EBPs bind to Domain 1 and have ATPase activity, which promotes conformation of changes of Sigma 54 by phosphorylation and allows for transcription to begin (18, 65). EBP regulation is controlled by its own signal transduction mechanism and involves cellular and environmental signals (31, 41, 53). Leaky expression rarely occurs in the absence of EBP due to its involvement in DNA melting (10).

As a species, about 50 *Pseudomonas syringae* pathovars exist with a wide range in pathogenicity and host range (23). The complete sequence of *P.s.* pv. *syringae* B728a has been recently published. Sequencing of this pathovar shows that it contains a 6.1 Mb circular genome with no plasmid DNA. It was predicted that 5,217 genes exist in its genome: 3,840 with known function, 1,271 with unknown function, and 80 RNA genes. When compared with the genome of *P.s.* pv. *tomato* strain DC3000, the *P.s.* pv. *syringae*

B728a genome contains an additional 976 genes and shares 4,273 genes (11), making this a very unique and specialized pathovar.

In *P.s.* pv. *syringae* B728a, which causes brown spot in beans, 14 sigma genes have been identified. Among these sigma factors, *P.s.* pv. *syringae* B728a encodes an RpoN (Sigma 54) which shares 80-98% identity with other *Pseudomonas* species. Comparison of RpoN from several *Pseudomonas* sp. and *Vibrio fisheri* indicated that the *P.s.* pv. *syringae* B728a RpoN is highly conserved and closely related to both *P.s.* pv. *glycinea* and *P.s.* pv. *phaseolicola* (Fig 1.1). Although much work has been conducted on *rpoN* in other bacterial species, a detailed analysis of the genes controlled by Sigma 54 in *P.s.* pv. *syringae* B728a has not been reported. I hypothesized that *rpoN* in this species will fill roles similar to its homolog in other species. It was the goal of this work to further characterize the *rpoN* gene in *P.s.* pv *syringae* B782a and define its phenotype. This study also evaluated additional roles that RpoN plays in *P.s.* pv. *syringae* B782a gene regulation and assessed its involvement in the plant-pathogen interaction.

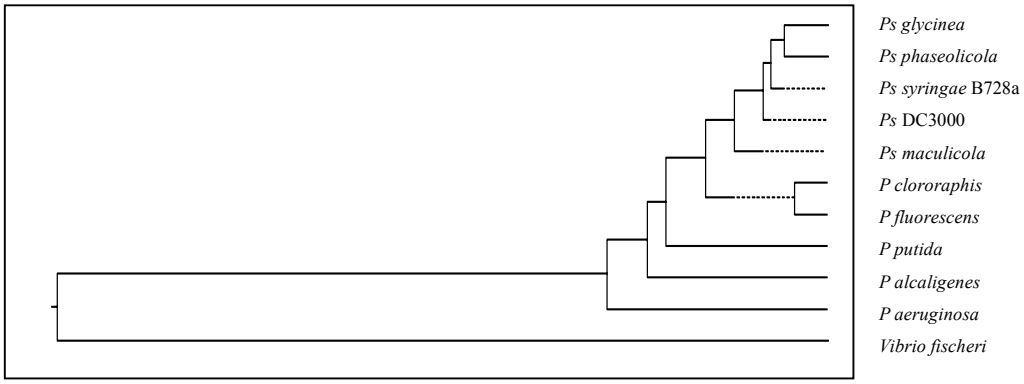


Fig 1.1. RpoN comparison between several species of *Pseudomonas* and *Vibrio fischeri*. Phylogenetic tree constructed based on a ClustalW alignment of the protein sequences of RpoN. Species used in this analysis include: *P.s.* pv. *glycinea*, *P.s.* pv. *phaseolicola* 1448A, *P.s.* pv. *syringae* B728a, *P.s.* pv. *tomato* DC3000, *P.s.* pv. *maculicola*, *P. chlororaphis*, *P. fluorescens* Pf-5, *P. putida* GB-1, *P. alcaligenes*, *P. aeruginosa* PAO1, and *Vibrio fischeri* ES114.

CHAPTER II

CHARACTERIZATION OF THE *rpoN* GLOBAL REGULATORY GENE OF *Pseudomonas syringae* pv. *syringae* B728a

INTRODUCTION

Pseudomonas syringae is a prevalent world wide pathogen in a wide range of crops. Symptoms vary from leaf spots, foliar blight, stem cankers, and water soaked lesions. As such a versatile pathogen, they are required to respond quickly to vastly changing environment conditions via proper gene regulation by use of various sigma factors. These sigma factors rely on activators, which sense changing environmental conditions, to aid in regulation of necessary genes. One such sigma factor is RpoN (Sigma 54), which has been identified in *P.s.* pv. *syringae* B278a (Fig 2.1). This sigma factor is known to be involved in regulating a vast type of unrelated genes (2, 54), recognizes a unique promoter region (2), and is of particular interest to the understanding of the global regulatory network.

Much of the early work conducted on the Sigma 54 promoter recognition site was accomplished in *Klebsiella pneumoniae*, and it was proposed that Sigma 54 does not recognize the traditional -35/-10 promoter site (2). Instead, it was shown that the recognition site is located at positions -24/-12 upstream of the transcriptional start site (1, 2). As noted earlier, the conserved sequence Sigma 54 recognizes is: TGGCAC-N5-TTGC. Disruption to this conserved sequence often inhibits transcription (61). The C-terminal region of Sigma 54 has been shown to be mostly involved in the binding to the

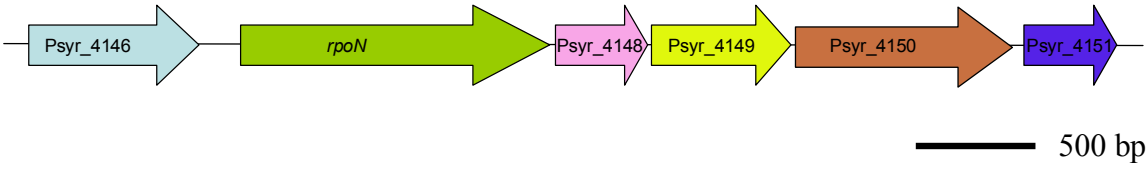


Fig 2.1. Genome organization surrounding *rpoN* in *P.s.* pv. *syringae* B728a. Psyr_4147 (*rpoN*) is located at the beginning of an operon. Psyr_4146 is located 141-bp upstream, therefore *rpoN* likely has its own promoter region. The closeness of genes located downstream of *rpoN* indicates that they are potentially regulated by the same promoter. This is likely since Psyr_4146 is a modulation protein for *rpoN*, and Psyr_4149 is a nitrogen regulatory protein.

promoter site (6). DNA cleavage assays were conducted to show that the conserved RpoN box located in the C-terminal recognizes the -24 promoter element (5). The -12 promoter element is involved in both binding to Sigma 54 and interaction with an activator (66). Knowledge of this unique promoter region was shown to be useful for prediction roles of RpoN in gene regulation.

Sigma 54 was initially described for its involvement in nitrogen assimilation and the ability to utilize certain sole nitrogen sources (24). The bacterial cell utilizes nitrogen assimilation to obtain intracellular nitrogen donors. Sigma 54 is involved in regulation of several nitrogen-regulated (Ntr) genes. These genes are specific to the nitrogen source they transport: for example *argT-hisJMPQ* transports arginine, lysine, ornithine, and histidine (48). In several *Pseudomonas* species, decreased ability to utilize sole nitrogen sources has been observed (21) (Table 2.1). In *Pseudomonas putida*, an *rpoN* mutant was unable to grow on plates containing nitrite, urea, alanine, glycine, isoleucine, or serine as the sole nitrogen source (29). Also, *Pseudomonas aeruginosa* is unable to utilize proline, histidine, glutamate, and nitrite as its sole nitrogen source when containing a mutation in *rpoN* (56). Similar results were observed in *P.s.* pv. *maculicola* (19), indicating that Sigma 54 is important to proper bacterial cell metabolism.

As previously stated, an *rpoN* mutant often exhibits decreased motility and adhesion capability. In *P. putida*, an *rpoN* mutation resulted in lose of motility (29). Based on such results, it was concluded that either Sigma 54 is involved in expressing genes directly involved in flagella biosynthesis or it disrupts bacterial cell surface

Table 2.1 Summary of an *rpoN* mutant's ability to utilized sole nitrogen sources identified in previous studies

Nitrogen Source	Nitrogen utilization of *Pseudomonas* strains containing mutation in *rpoN*[a]:		
	P.s. pv. m*aculicola* (DC3000) (20)	*P. putida* KT2440 (29)	*P. aeruginosa* PA14 (21, 56)
Alanine	-	-	ND
Ammonia	+	+	+
Arginine	+	+	+
Asparagine	+	+	ND
Aspartate	-	+	ND
Glutamate	+	+	-
Glutamine	+	+	+
Glycine	ND	-	ND
Histidine	-	+	-
Isoleucine	-	-	ND
Leucine	-	-	ND
Lysine	+	+	ND
Methionine	-	ND	ND
Proline	-	+	-
Serine	-	-	ND
Threonine	-	ND	ND

[a] Growth (+) or lack of growth (-) on minimal media plates containing the specified nitrogen source.

components that must be present during flagella biosynthesis. When grown on low agar plates (0.4%), this mutant showed decreased swarming ability due to loss of motility (29). Lack of quorum sensing ability may also be attributed to this phenotype. This suggestion is probable since RpoN is involved in *rpoS* regulation, and RpoS it known to be involved in regulation of quorum sensing genes (67). Although this is generally the case in pseudomonads, a *Xylella fastidiosa rpoN* mutant showed no significant difference in motility after several days of growth (43).

The ability for bacteria to attach to a plant cell surface and form a biofilm is important for pathogenicity (45). Past studies have shown that Sigma 54 is involved in pili production via regulation of the *hrp* genes (12, 15). Studies with a *P.s.* pv. *maculicola* (DC3000) *rpoN* mutant show decreased adhesion and infection capability due to defects in Hrp regulation (20). In contrast, a *Xylella rpoN* mutant showed an increase in biofilm formation (43).

In *Pseudomonas aeruginosa*, which is able to colonize cystic fibrosis patient's lungs, Sigma 54 has been shown to be involved in virulence (3). Alginate is a major virulence factor for this pathogen, and it has been shown to be regulated by Sigma 54 (3). It is known that Sigma 54 also regulates genes involved in both pili and flagella formation, and both are required for complete virulence (21, 56). An *rpoN* mutant in *P. aeruginosa* showed decreased adhesion to host cells due to the inability to form pili (7, 56). Hendrickson *et al* (21) showed that a decrease in flagellar motility occurs in this mutant. It is the combination of these two phenotypes, adhesion and motility, which is thought to decrease the ability of *Pseudomonas* to attach to the host cell, therefore

inhibiting infection. Studies conducted in mice show that disease does not occur even when using a high dose of bacteria containing a mutation in *rpoN*, as compared to a low dose response in the wild type strain (21). It is hypothesized that a decrease in disease development will be observed in bean plants infected in the *rpoN* mutant as compared to wild type strain.

It was suggested that RpoN is involved indirectly as a regulator of the hypersensitive response and pathogenicity (*hrp)* gene cluster, which encodes pathogenicity-related genes (12, 15). These genes are required for pathogenicity and elicitation of a hypersensitive response by the plant (35). A study by Hendrickson (19), showed that an *rpoN* mutant is unable to cause HR in *Nicotiana tabacum.* Although, when the *hrpL* gene is expressed under an *E. coli lacZ* promoter in an *rpoN* mutant, HR was restored. This indicated an involvement of RpoN in *hrp* regulation, which the authors suggest may be a result of the inability of the *hrp* genes to synthesis coronatine. Therefore, it will be important for this study to determine if RpoN is involved in *P.s.* pv. *syringae* B728a toxin regulation. It was also noted that expression of *hrpL* through the *lacZ* promoter did not complement other *rpoN* phenotypes, including lack of motility, and nitrogen utilization (19). Based on these studies, it is anticipated that the *P.s.* pv. *syringae* B728a *rpoN* mutant formed in this study will be unable to cause HR in *N. tabaccum.*

It is known that RpoN plays a role in toxin production in several species, although its regulation may not be direct. Coronatine is a phytotoxin known to be synthesized under the *hrp* pathway and contributes to a pathogenic phenotype in plants

(20). *P.s.* pv. *syringae* is known for production of the toxins syringomycin and syringopeptin, and has been well characterized (13, 17, 25, 37, 38, 50-52, 62-64, 68). These are lipopeptide toxins; know for causing necrosis on plant tissue. Although many studies have been conducted on toxin synthesis, it is not known if RpoN is involved in its regulation.

In recent years, many studies have been conducted on the newly sequenced genome of *P.s.* pv. *syringae* B728a. Sigma 54 is one of 14 sigma factors which have been identified is this bacterial species. Although several studies have been conducted on Sigma 54 in other species, studies have not been conducted in *P.s.* pv. *syringae* B728a. Therefore, it is important to begin to identify the role Sigma 54 plays in *P.s.* pv. *syringae* B728a. This knowledge will allow us to understand what functions RpoN plays in both the non-pathogenic and pathogenic lifestyles of this organism.

It is expected that similar phenotypic characteristics listed above will be observed in *P.s.* pv. *syringae* B728a, but some differences are likely. The ability to utilize some nitrogen sources is likely to be a direct result of and *rpoN* mutation. While a decreased swarming ability and adhesion may be part of a more complex regulatory pathway, involving the co-regulation with the *hrp* genes and *rpoS*. This knowledge can then be used to unravel and further understand the gene regulatory network of *P.s.* pv. *syringae* B728a in which Sigma 54 may be involved.

RESULTS

Sequence analysis of Psyr_4147 indicates that is encodes the alternative sigma factor RpoN

Genome analysis of *P.s.* pv. *syringae* B728a indicated that it encodes the alternative sigma factor RpoN and can be identified as gene number Psyr_4147 (YP_237215). Its location within the genome is at base pair 4934535-4936070 and is transcribed into 511 amino acids. Using the blastP program at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), it was indentified that the amino acid sequence of this gene shares 80-98% homology to RpoN in other pseudomonads.

In silico characterization of the *P.s.* pv. *syringae* B728a genome for potential RpoN-dependent binding sites

In order to indentify genes directly regulated by RpoN, *in silico* analysis was conducted using two programs which were developed based on the known RpoN binding site. Two programs have been developed to search bacterial genomes for this specific promoter region sequence: PromScan (55) and Virtual footprint (42). Both of these programs were utilized to search the entire genome of *P.s.* pv. *syringae* B728a for potential promoter sites recognized by RpoN. Data from PromScan was discarded unless it received a score of at least 90 out of 100. Virtual footprint had a preset error score of 0.8 out of 1.0, therefore all data was retained. Positive sequence matches from the retained data was then used to aid in identifying genes in which RpoN many regulate (Table 2.2). In nearly all case, these positive hits were located -24/-12 location upstream

Table 2.2. Predicted RpoN-dependent promoter bind sites located in *P.s.* pv. *sryingae* B728a

Location	Direction	Sequence[a]	Gene Number	Gene Description	Program Used[b]
102387	Forward	GCACCGCCGGGGTCAGC GCCCTGGCAGCACGCTT GCTGATAAAGGG	Psyr_RNA1	Arg tRNA	VP
1134618	Forward	TCCATAGCGCTTTTCCTG TACTGGCATGGATGCTG CTAATGCCTGC	Psyr_1001	Basic membrane lipoprotein	VP
144171	Forward	CTGGCACAGCGGTTGCA	Psyr_0135	TonB, C-Terminal	PS
538024	Forward	TTGGCATGGTCGTTGCT	Psyr_0496	Hypothetical proteins	PS
657262	Forward	ACGGCACGGCGCTTGCT	Psyr_0578	Histidine kinase, HAMP region: Bacterial chemotaxis sensory transducer	PS
685619	Forward	ATGGCACGCGCCTTGCT	Psyr_0597	Extracellular ligand-binding receptor	PS
905365	Forward	CTGGCACGACCTGTGCT	Psyr_0799	Fimbrial protein pilin	PS
941151	Reverse	TTGGCACAGCTTCTGCT	Psyr_0830	Poly (A) polymerase	PS
1214598	Forward	CTGGTACGGGCTTTGCT	Psyr_1072	Extracellular solute-binding protein, family 3	PS
1237891	Reverse	CTGGCACGCCGCCTGCT	Psyr_1094	Cold-shock protein, DNA-binding	PS
1244655	Reverse	CTGGCAGAGCTATTGCT	Psyr_1098	PAS:GGCEF	PS
1289330	Reverse	CTGGCGCGCAACTTGCT	Psyr_1137	Hypothetical protein	PS
1394104	Reverse	ATGGCACGCACCTTGCC	Psyr_1233	Inositol monophosphatase	PS
1523443	Forward	CTGGCAAGATCGTTGCT	Psyr_1344	Elongation factor Ts	PS
1646807	Forward	AGCGAGGGGATTACGCA ACATTGGCAGATACGTT GCTGTTTACTTA	Psyr_1462	GCN5 related N-acetyltransferase	VP
1974053	Forward	CTGGCACGAATGTCGCT	Psyr_1737	ABC transporter	PS

Table 2.2 Continued

Location	Direction	Sequence[a]	Gene Number	Gene Description	Program Used[b]
2078367	Reverse	TTGGCGCAGCTCTTGCT	Psyr_1804	Hypothetical protein	PS
2116204	Reverse	CTGGCACGGCCTCTGCA	Psyr_1835	Amino acid permease associated region	PS
2144186	Forward	GTGGCACGGGCTTTGCG	Psyr_1869	Protein of unknown function (DUF404)	PS
2150813	Reverse	CTGGCACGGCGCATGCT	Psyr_1874	Mg2+ transporter protein, CorA-like	PS
2453954	Reverse	TTGGCCCGTCTATTGCT	Psyr_2102	Nitrate transporter	PS
2458060	Reverse	GTGGTTTCAAGCAAGGC ATATGCCATCGCCGGGG GAGGGCGAGCAA	Psyr_2105	Hypothetical protein	VP
2563050	Reverse	TTGGCATTGCCCTTGCT	Psyr_2205	Extracellular ligand-binding receptor	PS
2638698	Forward	CTGGCAAGCGCCTTGCA	Psyr_2273	Glutamate ammonia ligase	PS
2647366	Forward	ATGGCACGGTTGCTGCT	Psyr_2280	Alkanesulfonate monooxygenase	PS
2817294	Forward	ATTATGACT*TTATGCGGC AGTTGGCACTGGACTTGC T*GGTAAACCT	Psyr_2433	Hypothetical protein	VP & PS
2865018	Reverse	ATTACAGGGGTTTTGAA AAC*CTGGCACGGGTCCT GCT*ATATCCTTT	Pysr_2470	Isovaleryl-CoA dehydrogenase	VP & PS
2962594	Reverse	TTCAGCCACTCGATAAA GCC*GTGGCACGAAGCTTG CT*CGAAACTTG	Psyr_2546	DMT superfamily multiple drug efflux pump	VP & PS
2962924	Forward	GTAGACGATGATCAATA ACG*GTGGCACGCCACTTG CT*GTGTACTCC	Psyr_2549	Hypothetical protein	VP & PS
3066882	Reverse	GTGGCCCGGCAGTTGCT	Psyr_2607	LuxR	PS
3246528	Forward	TTGGCATCGCTCTTGCA	Psyr_2675	Regulatory protein, GntR	VP
3278070	Forward	ACGATATTCCTGGTAGC TCTTTGGCAGAACGCTT GCTCTTTGAACG		Next several ORFs are Reverse	VP
3325898	Forward	ATGGCAGGCCACTTGCA	Psyr_2740	Transcriptional activator FtrA	PS

Table 2.2 Continued

Location	Direction	Sequence[a]	Gene Number	Gene Description	Program Used[b]
3430333	Reverse	CGAGGCAGGAGCAGAA AGCATGCCACTGGAAGA ATTCAT	Psyr_2858	Isochorismatase hydrolase	VP
3875299	Reverse	CTGGCGCGCCAGTTGCT	Psyr_3235	Lipoprotein, putative	PS
4111532	Reverse	CAGAGGCAGAGCAAGT GTTATGCCAGAGTTGTA CGAAACGGGGATG	Psyr_3446	Flagellar protein	VP
4111557	Reverse	CTGGCATAACACTTGCT	Psyr_3448	Flagellar protein	PS
4152582	Reverse	TACTGACAAAGCA*ATCG GCGTGCCATGTTGT*AAAA GCCAATGTTTG	Psyr_3481	Flagellar basal body rod protein	VP
4275837	Reverse	ATGGCACTGACGTTGCT	Psyr_3592	Nitroreductase	PS
4394778	Reverse	TTTGATATCAAAATGAC TCC*ATGGCACGCGTATTG CT*ATCTACATG	Pysr_3707	C4-dicarboxylate transport protein	VP &PS
4476051	Forward	TTTTAAAGGTTTTTAAA AAA*CTGGCACGGGTCCT GCT*ATATCTCTG	Psyr_3759	Hypothetical protein	VP &PS
4651008	Forward	TTGGCACGACTCATGCT	Psyr_3908	Extracellular solute-binding protein, family 3	PS
4763262	Forward	CTGGCACAGCTCCTGCT	Psyr_3999	General substrate transporter, Major facilitator superfamily	PS
4967149	Reverse	TTGGCACACCGCTTGCG	Psyr_4175	Transport associated	PS
5021579	Forward	GCAGCCGATTGGACGCA TGC*GTGGCACCGTCCTTG CT*AATTGCTAT	Psyr_4216	Regulatory protein, LuxR	VP & PS
5317172	Forward	TTTCCCTACAAGCTGCA CTG*TTGGCAAGCCCCTTG CT*CTGATTTGA	Psyr_4451	UreE urease accessory, N-terminal: UreE urease accessory, C-terminal	VP & PS

Table 2.2 Continued

5713977	Forward	AAAAAACCCAACACGG GGCA*CTGGCATGCAAATT GCT*CCCTTGTGA	Psyr_4817	Glutamine synthetase type I	VP & PS
5739514	Reverse	GTGGCACGCACCGTGCT		No nearby genes downstream of promoter site	PS
5791936	Forward	CTGGCACGCCCCCTGCA	Psyr_4881	RNA methyltransferase TrmH, group 2	PS

[a] Resulting promoter sequence identified by promoter scan program. Italic letters identify the sequence given by PromScan when the region was also identified by Virtual Footprint. [b] Program used to identify promoter region recognized by RpoN: Virtual footprint (VP) or PromScan (PS).

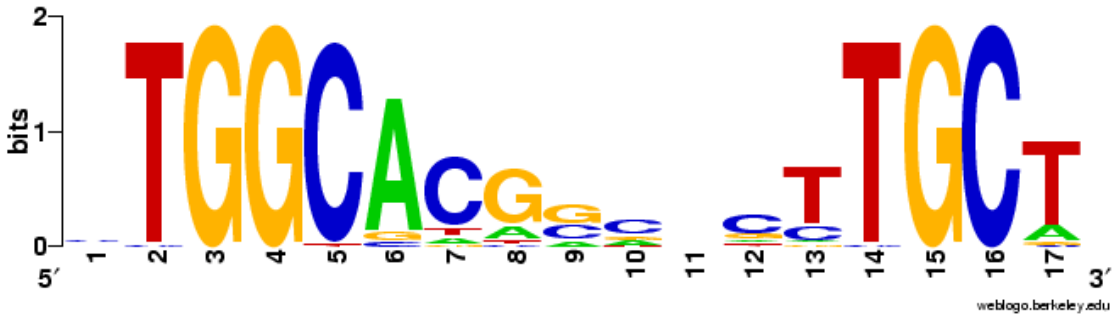
of the gene transcriptional initiation site. The promoter binding site sequences from each search were compiled in order to develop sequence logos (Fig 2.2).

Cloning and construction of an *rpoN* mutant in *P.s.* pv. *syringae* B728a

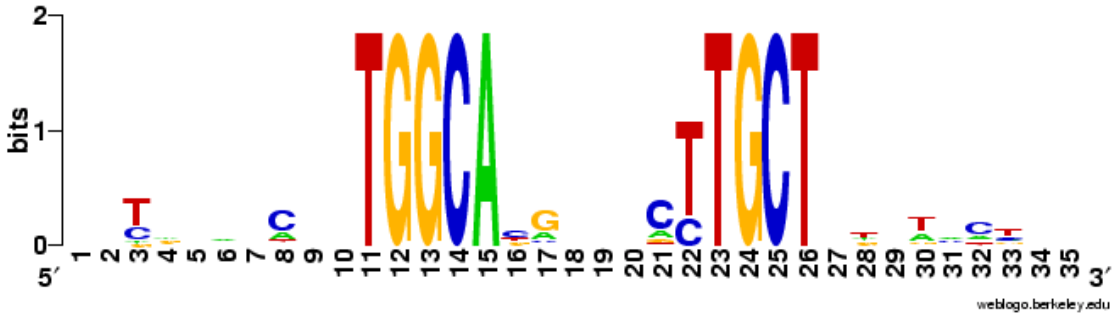
As described in the Materials & Methods section, the *rpoN* gene of *P.s.* pv. *syringae* B728a was replaced by a Km^r gene using the Red Recombinase system. Using this method, *rpoN* along with approximately 4 kb of flanking DNA was cloned in pENTr/D-TOPO using topoisomerase. After plasmid confirmation in *E. coli* Mach1 using digest and sequence analysis, the plasmid was named pE*rpoN*. After a gateway reaction into pLVC-D (producing: pL*rpoN*), the plasmid was cloned into *E. coli* SW101, which contained the heat shock activated Red Recombinase genes used to 'flip' out *rpoN* and replace it with Km^r. This plasmid was named pL*rpoN*-Km. Triparental mating was set up between *P.s.* pv. *syringae* B728a and *E. coli* (pLC*rpoN*-Km) and the replacement of *rpoN* within the genome with selection for Km^r. The *rpoN* mutant was confirmed by colony PCR and Southern blot analysis using both wild type and mutant DNA. Colony PCR of mutant *rpoN* yields a PCR product approximately 400 bp shorter than wild type (Fig 2.3). Southern blot analysis was also used to confirm that the *rpoN* mutant (Fig 2.4) lacked the gene and to confirm the presence of the Km^r cassette. Sequencing of the *rpoN* mutant was also conducted to verify the exact location of the mutation (Fig 2.5). The *rpoN* mutant was named B728aAL01.

Growth of *P.s.* pv. *syringae* B728a effected by a mutation to *rpoN*

A growth curved analysis was conducted to look into the growth rate of B728aAL01 compared to B728a, since it was observed that B728aAL01 seemed to grow



a. sequence logo developed from PromScan results



b. sequence logo developed from Virtual footprint results

Fig 2.2. Sequence logos of RpoN binding regions. Date obtained from both PromScan (a) and Virtual footprint (b) was individually compiled and used to develop sequence logos of the hypothesized promoter binding regions (larger letters represent high occurrence of that nucleotide). In both logos, the basic known sequence of TGGCAC-N5-TTGC is present with varying frequency.

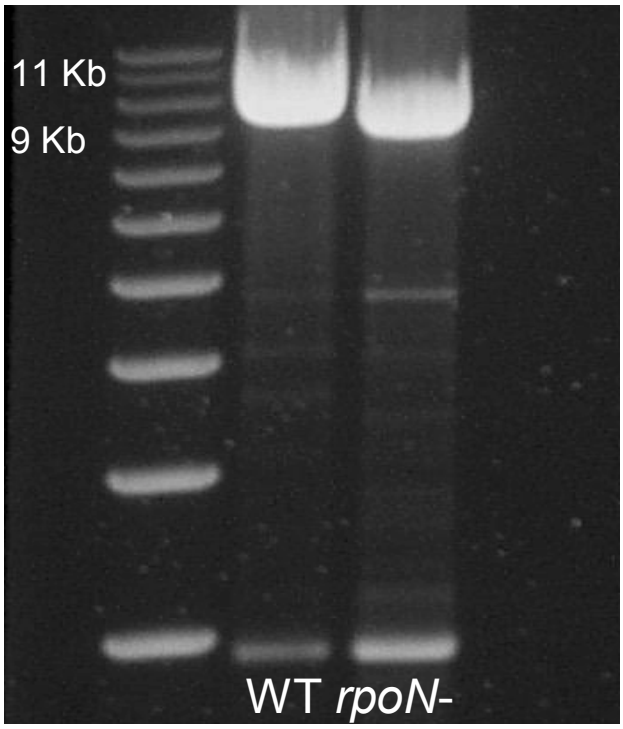


Fig 2.3. Mutational confirmation in *rpoN* of *P.s.* pv. *syringae* B728a using PCR. Primers were design to amplify outside of the genomic region used in the mutational process of *rpoN* that contained the gene of interest. A 9.6 kb PCR product bands was expected for wild type, while a 9.2 kb band was expected in the *rpoN* mutant. The 1 kb Plus DNA ladder (Invitrogen, Carlsbad, Calif) can bee seen in the left-hand lane.

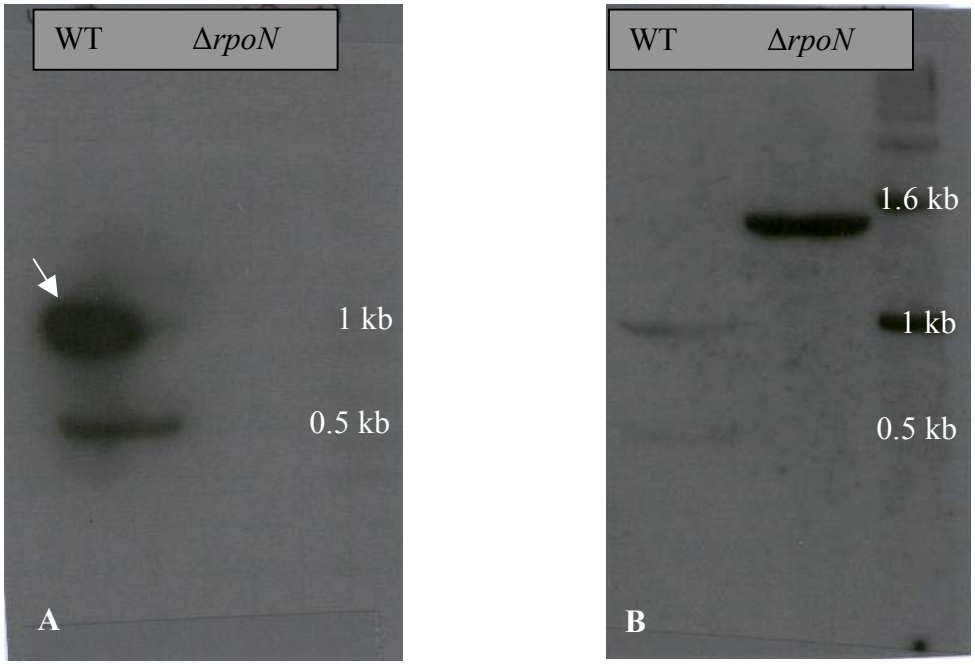


Fig 2.4. Confirmation of the *P.s.* pv. *syringae* B728a *rpoN* mutant using Southern Blot analysis. The membrane used for the southern blot contained genomic DNA of wild and mutant *rpoN* DNA digested with XhoI. Initially, the membrane was probed with a PCR segment of *rpoN* (A). A band approximately 1 kb was expected and indicated by an arrow in the figure. A band was not expected for the *rpoN* mutant. In order to show that genomic DNA was present in the mutant lane, the membrane was stripped and re-probed with the Km cassette used in the mutation process to replace the *rpoN* gene (B). In the second blot a 1.7 kb band was expected in the mutant lane and not in the wild type lane. Faint bands in the second blot are residual bands left after stripping the blot. Faint bands on the right side of both blots are remnants of the ladder used while running the gel.

cactgaccacaacgtccgggaaacgctggatatctgcgaaatggcctacatcgtcaacgatggacagctgattgcggaagg
cgactccgagaccattctggcgaatcagctggtgaaagaggtctacctcggccacgagttccgcctttaatcgactgccctttg
cgcttcgcagccga**ttgatgcctatgcagtgtattgacaacattttatttgtcatatctctctaggcaaacgcacaggtttc**
aggcatataatttgcttaagttggcgccacggcgcctgtagtagatggcgcatgcgcgccggcgaataaggtgtttag
cccctgccatgaaaccatcgctagtcttgagaatgggccagcagctgacgatgacaccgcagctg

a. The upstream region and 5' end of rpoN showing location of the mutation.

actggttgcggcggaaaatcagaaaaagccgttgagtgatagcaagatcgctggtttactggaggcacaaggcattc
aagtagcccgtcgcacagtcgccaagtaccgtgagtctctcgggatcgccccttccagtgagcgtaagcggctgatgt
gatgcaggccgagccacagcgtcccagaggcatgcgccatgcctgcctctttatgcactggcaaaggagaaagctgt
atgcaagtcaacatcagtggacaccaactggaagtgaccaaacccttcgtgaatacgttgagctcaagctcaagaagctc
gaggggcattttgacaagattaccaacgtgcaggtcacgatgacggtcgaaaagctcaagcagaagatcgaagccacgttg
cacatc

b. The downstream region and 3' end of rpoN showing location of the mutation.

Fig 2.5. Location of the *rpoN* mutation with in *P.s.* pv. *syringae* B728a. Sequence analysis was conducted on B7AL01 to identify the exact location of the mutation (insertion of the Km^r cassette). (A) The gene coding region of *rpoN* begins at base-pair 4934535 within the genome of *P.s.* pv. *sryingae* B782a and is underlined in the figure. Homology to this region using sequence analysis of the mutant ended at base-pair 4934414. This indicated the beginning of the mutation with in the genome (bold type face letters) and that 121 bp upstream of *rpoN* was also removed during the mutation process. (B) The gene coding region of *rpoN* ends at base-pair 4936070 within the genome of *P.s.* pv. *sryingae* B782a and is underlined in the figure. Homology to this region using sequence analysis of the mutant ended at base-pair 4936165. This indicated the end of the mutation with in the genome (bold type face letters) and that 95 bp upstream of *rpoN* was also removed during the mutation process.

slower then B728a. It was clear, over the course of 9 hours that B728aAL01 grew much slower then the wild type strain (Fig. 2.6). This data shows that, although RpoN is not required for normal growth of the bacteria, it does play an effect on growth and development at some level.

rpoN mutant of _P.s._ pv. _syringae_ B728a exhibited defects in nitrogen utilization

In order to test the *rpoN* mutants' ability to grow on various sole nitrogen sources, about 100 cell were plated on minimal media plates containing 5 mM of an individual nitrogen source. All plates were observed daily, for a maximum of 7days, to look for the presence of colony formation. Results of this assay are presented in Table 2.3. B728aAL01 grew best in media supplemented with glutamate, most similar to B728a growth. The mutant was also able to utilized media supplemented with arginine, asparagine. Although is did seem to be able to grow on leucine and praline, growth was very poor.

Loss of motility was observed in mutant *rpoN*

A swarming assay was conducted on both B728a and B728aAl01 cultures to test motility. 10^7 and 10^8 CFU/ml of each culture was spotted into the middle of a 0.4% agar PDA plate and incubated in a moist chamber at 25˚C. After overnight incubation, only B728a showed an ability to swarm at either concentration (Fig. 2.7). B728aAL01 plates were further incubated for an addition four days, but showed to change in motility. This data indicates B728aAL01 is non-motile.

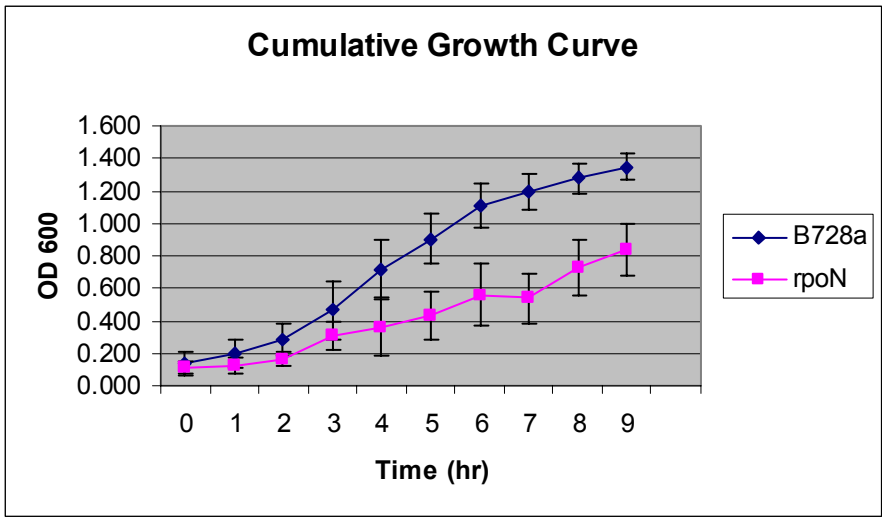


Fig 2.6. Growth of *P.s.* pv. *syringae* B728a affected by a mutation to *rpoN*. Optical density (OD) values were taken over the course of 9 hours on B728a and B278aAL01 liquid cultures. Data obtained from this assay show that B728aAL01 has a decreased growth rate when compared to wild type B728a. The data was obtained in replicate on two different days. Error bars represent the standard deviation at that location.

Table 2.3. Nitrogen utilization of *P.s.* pv. *syringae* B728a strains

Nitrogen source	**Nitrogen utilization of strain[a]:**	
	Wild type	***rpoN* mutant**
Without nitrogen	+	-
Alanine	+	-
Ammonia	+	-
Arginine	+	+
Asparagine	+	+
Glutamate	+	++
Glycine	+	-
Histidine	+	-
Isoleucine	+	-
Lysine	+	-
Leucine	+	-
Methionine	+	-
Phenylalanine	+	-
Proline	+	-
Serine	+	-
Threonine	+	-

[a] Growth (+) or lack of growth (-) on plates containing specified nitrogen source at a final concentration of 5 mM to at least 1 mm by 7 days. Presence of ++ indicates wild-type like growth of *rpoN* mutant.

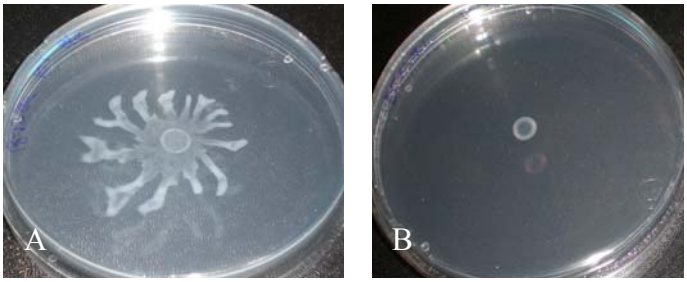


Fig 2.7. Motility assay of *P.s.* pv. *syringae* B728a strains. 10 µl drops of 10^7 CFU of either B728a (A) and B728aAL01 (B) was placed in the center of 0.4% PDA plates (10^8 data not shown). Plants were incubated 24 hr at 25°C in a moist chamber before being photographed. As expected, mutant *rpoN* was non-motile.

Mutant *rpoN* of *P.s.* pv. *syringae* B728a results in inability to form biofilm on a glass surface

A slide adhesion assay was conducted on both B728a and B728aAL01 to test if a mutation to *rpoN* has an effect on biofilm formation. The standard laboratory protocol assay used used liquid SRM_{AF} as the medium of choice. But, it was observed that B728aAL01 was unable to grow due to limited nitrogen sources. This mutant was able to grow in HMM, so it was decided to use this medium since it was also a minimal media. It was clear in this assay that B728aAL01 is defective in adhesion ability (Fig 2.8). Even after 72 hours of growth the formation of a biofilm did not occur (data not shown).

Bioassay for toxin production

P.s. pv. *syringae* B728a is known to produce toxins syringomycin and syringopeptin. Mutant strain, B728aAL01, was tested for production of these toxins and compared to control strains B728a and B728a*gacS* (Fig 2.9). Toxin production for strain B782aAL01 was slightly increased on PDA against *Geotrichum*, but the zone of inhibition was significantly larger against *Bacillus* when compared to B728a. B728a*gacS*, which contains a mutation in toxin regulation, only produced zones of inhibition against *Bacillus* when grown on PDA. Toxin production on modified HMM was poor, therefore the data is not shown.

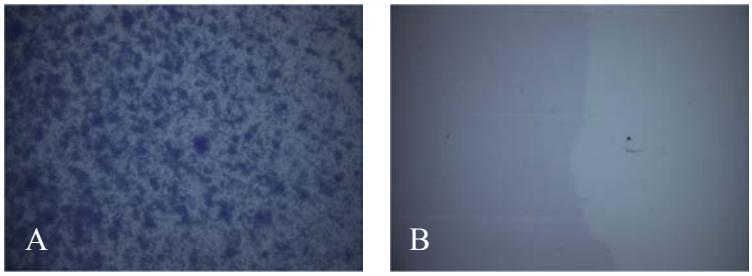


Fig 2.8. Slide adhesion of B728a and B728aAL01. Cultures were grown 3 days in HMM liquid media stationary at 25˚C. Post incubation, slides were stained with crystal violet before microscopic observation and photographing. As expected, B728a (A) was able to adhere to a glass surface, while an *rpoN* mutant (B) was unable to form a similar biofilm on a glass surface after incubation for 3 days.

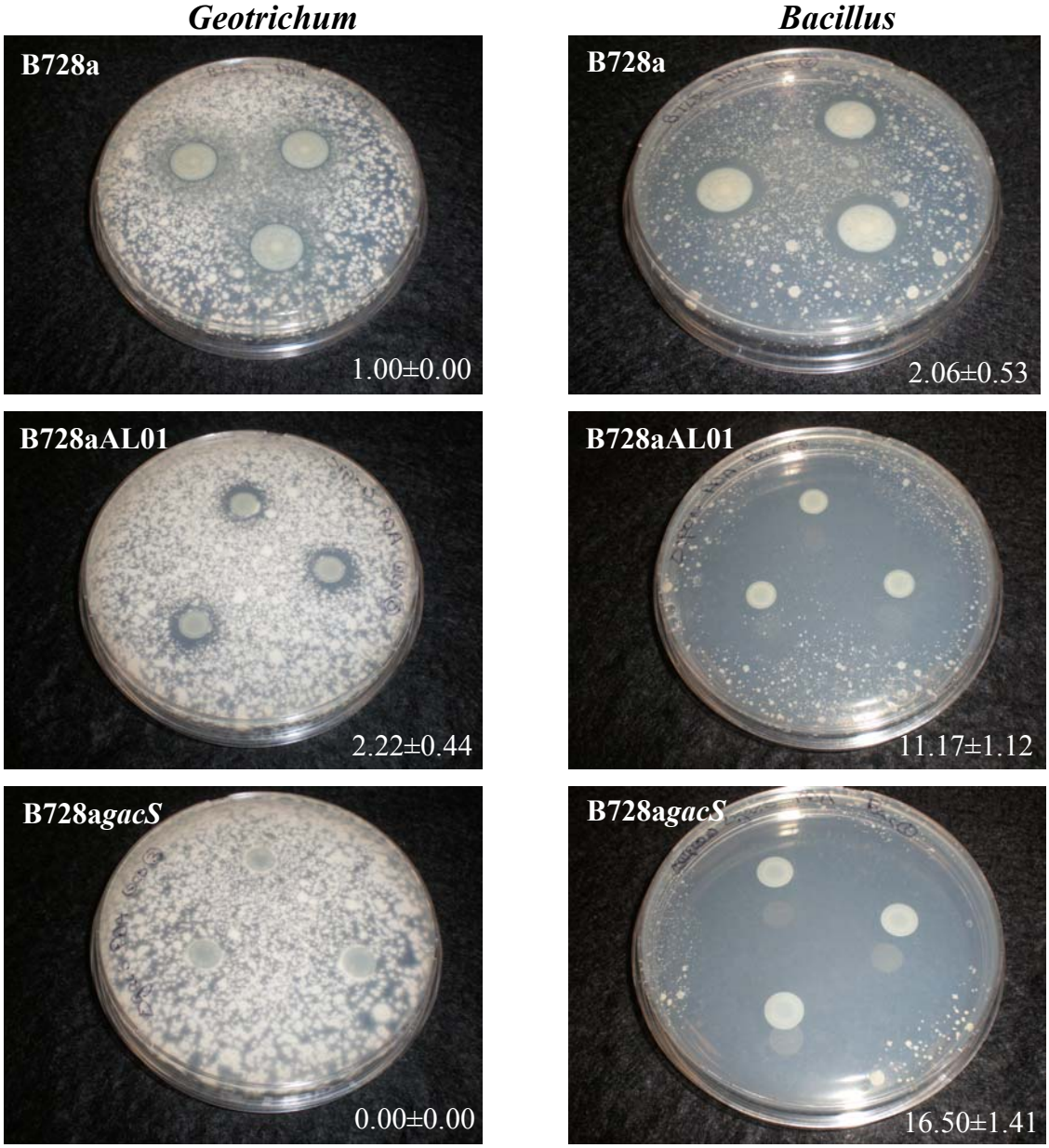


Fig. 2.9. RpoN is a negative regulator of *P.s.* pv. *syringae* B728a syringomycin and syringopeptin toxin production. PDA plates spotted with B728a, B728aALl01, and B728a*gacS* were lightly oversprayed with suspensions of *Geotrichum* and *Bacillus* after four days of incubation to analyze differences in development of zones of inhibition (measurments in millimeters). After overnight incubation, a noticeable difference was observed between B728a and B728aAL01. This results show that RpoN is a negative regulator of toxin expression in *P.s.* pv. *syringae* B728a.

Pathogenicity and ability to elicit HR affected by *rpoN* mutation in *P.s.* pv. *syringae* B728a

Pathogenicity assays were first conducted on *Nicotiana tabacum* to test for the ability of B728aAL01 to cause a hypersensitive (HR) on leaves. Post inoculation and incubation overnight, only B728a was able to cause HR (Fig 2.10). Since it was earlier observed that this mutant strain grows at a slower rate then B728a, incubation was increased, but no changes were observed.

Pathogenicity assays were also conducted in 3 week old bean seedlings using vacuum infiltration of bacterial cultures. After 3-4 days of plant incubation, disease symptoms began to develop on wild type plants. Symptoms never developed for B728aAL01 or B728a*gacS* (Fig 2.11). Population analysis was also conducted as part of this assay. Bacterial levels in the plant leaf were analyzed at day zero and at sign of disease development on B728a. At day 0, the bacterial levels in the plant leaf was approximately $6.1X10^{3}\pm5.2X10^{2}$ for B728a and $4.2X10^{0}\pm9.6X10^{-1}$ CFU/cm^2 of leaf tissue for B728aAL01. At sign of disease development, generally by day 4, bacterial levels were approximately $6.4X10^{7}\pm1.0X10^{8}$ for B728a and 0 CFU/cm^2 of leaf tissue for B728aAL01. These results indicate that B728aAL01 is unable to survive once introduced into the plant leaf.

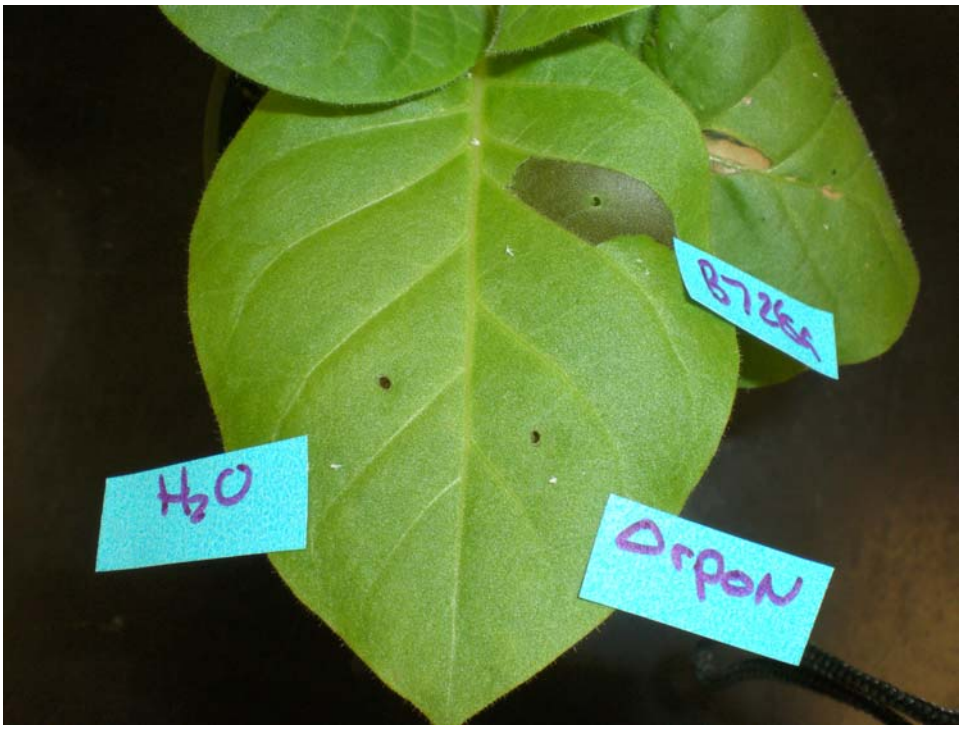


Fig 2.10. *P.s.* pv. *syringae* mutant *rpoN* defective in eliciting HR in *Nicotiana tobacum*. A *N. tobacum* plant was infiltrated with B728a, B728aAL01, and water after being punctured with a sterile needle. To ensure that the leaf was being infiltrated with approximately the same amount of bacterial cultures, each culture was set to and OD_{600} of 0.3 (equivalent to $5X10^8$ CFU/mL). Plants were incubated overnight at room temperature.

Fig 2.11. Disease development in bean requires RpoN. Vacuum infiltration studies were conducted with B728aAL01, and compared to B728a and B728a*gacS*. Plants were infiltrated with a bacterial culture containing approximately $1X10^5$ CFU/mL and incubated until sign of disease development (3-4 days). Development of disease symptoms only occurred on plants infiltrated with B728a, while B728aAL01 and B728a*gacS* appeared healthy.

MATERIALS AND METHODS

Bacterial strains and media

All bacterial strains used in this study are listed in Table 2.4. General cloning was conducted in *Escherichia coli* Mach1 T1 cells (Invitrogen, Carlsbad, Calif) and was cultured at 37˚C in Luria-Bertani (LB) liquid or agar medium. *E. col* SW105 was used in mutant formation to replace *rpoN* with the Km^r cassette, utilizing Red Recombinase genes. This strain was cultured on LB liquid or agar medium and incubated at 30˚C. *P.s.* pv. *syringae* B728a strains were grown at 25°C in nutrient broth-yeast extract (NBY) liquid or agar (58) or on King's B agar medium (KB) (28). Swarming assays were conducted on 0.4% potato-dextrose agar (PDA) medium. Nitrogen assays were conducted on minimal salts media (10% glucose, 1 M $MgSO_4$ (anhydrous), & 10X salts [30 g/L KH_2PO_4 and 60 g/L Na_2HPO_4]) supplemented with 5mM of each nitrogen source. Toxin assay were conducted on HMM (26) modified media (5 mL of 20% fructose, 5 mL of 20% mannitol, 5 mL of 20% succinate, 100 µL of 50 mM glutamate, and 250 µL of 20 mM $FeCl_3$) and PDA. Antibiotics were added at the following concentrations (µg /ml): rifampin, 100; kanamycin, 75; and tetracycline, 20.

General DNA manipulations

Restriction enzymes and Phusion high-fidelity DNA polymerase were purchased from New England Biolabs (Beverly, Mass.). Integrated DNA Technologies (Coralville, Iowa) sourced all oligonucleotides used in this study. When cloning using Gateway technology (32), genes were first amplified by PCR and cloned into vector pENTR/D-TOPO (Invitrogen). LR clonase (Invitrogen) was used for recombination between

Table 2.4 Strains and plasmids

Designation	Relevant Characteristics	Source
Bacterial Strains		
Escherichia coli		
Mach1 T1	*ΔrecA1398 endA1 tonA Φ80ΔlacM15 ΔlacX74 hsdR($r_k^- m_k^+$)*	Invitrogen
SW105	DY380 (*cro-bioA*)<>*araC*-P_{BAD}*Cre* Δ*galK*	National Cancer Institute
P.s. pv. *syringae*		
B728a	Wild type, bean pathogen, Rif^r	(36)
B728aAL01	*rpoN*-Km^r derivative of B728a, Rif^r Km^r	This Study
B728a*gacS*	*P.s.* pv. *syringae* B728a containing an insertion in *gacS*, Km^r	Steve Lindow
Plasmids		
pENTER/D-TOPO	Cloning vector, Km^r	Invitrogen
pE*rpoN*	pENTr/D-TOPO carrying *rpoN,* Km^r	This study
pKD13	Vector containing *nptII* cassette flanked by FRT sites, Ap^r, Km^r	Bruce Wanner
pLVCD	Gateway destination vector for mating with *P. syringae*, pBR322 derivative with *mob* genes from RSF1010, Tc^r Ap^r Cm^r	Steve Lindow
pL*rpoN*	pLVCD carrying *rpoN*, Tc^r Ap^r Cm^r	This Study
pL*rpoN*-Km	pLVCD carrying homology upstream & downstream to *rpoN*, Km^r, Tc^r Ap^r Cm^r	This Study
pRH*rpoN*	pRH002 carrying *rpoN* with its putative promoter region, Cm^r	This Study
pRK2073	Helper plasmid, Sp^r Trm^r	(34)

pENTR and the gateway destination vector, per manufacturer's instruction. Chemical transformation and electroporation was used to introduce plasmids into *E. coli*. For introduction of plasmids into *P.s.* pv. *syringae* B278s strains, tri-parental mating was set up using helper plasmid pRK2073 (34). Standard cycling conditions were used for PCR based on manufacturer's instruction provided with the polymerase and primer sequences are listed in Table 2.5.

Construction of *P.s.* pv. *syringae rpoN* knockout mutant

Knockout mutation to *rpoN* was constructed using the Red Recombinase to form the mating plasmid (9). Using this method (summary Fig 2.12), the gene of interest was replaced by an antibiotic resistance gene contained in a plasmid while in *E. coli*. The DNA sequence of the *rpoN* gene in B728a was obtained from NCBI, gene accession number YP_237215. PCR was set up with P049 and P050 to amplify an 8.781 kb fragment of genomic DNA containing the *rpoN* with about 3-4 kb homology on each side (5'- 3797 bp & 3'- 3458bp). The product was TOPO cloned into a gateway entry vector pENTR (Invitrogen) forming pE*rpoN*. This was followed by Gateway reaction into pLVC-D forming pL*rpoN*, using LR clonase (Invitrogen). Electroporation then occurred to place this plasmid into competent cells of *E. coli* SW105, which contain the genes necessary for the flip recombinase system to function. After confirmation, SW105 *E. coli* containing *rpoN*:pLVC-D was electroporated with a short linear piece of DNA containing homology to both the 5' and 3' end of *rpoN*, along with a Km^r cassette obtained from pKd13. This linear segment was formed by using P047 and P048 to PCR pKD13, which contains *nptII* (Km^r). These primers were designed with tags at the end

Table 2.5. Primers used for PCR amplification

Name	Sequence
P047	GTTCCGCCTTTAATCGACTGCCCTTTGCGCTTCGCAGTGTAGGCTGGAGCTGCTTCG
P048	TTTCTCCTTTGCCAGTGCATAAAGAGGCAGGCATGGATTCCGGGGATCCGTCGACC
P049	CACCCGATACAGCTTCCGCCACAA
P050	GGGGTCTGTTCTGCACGAGTTGTC

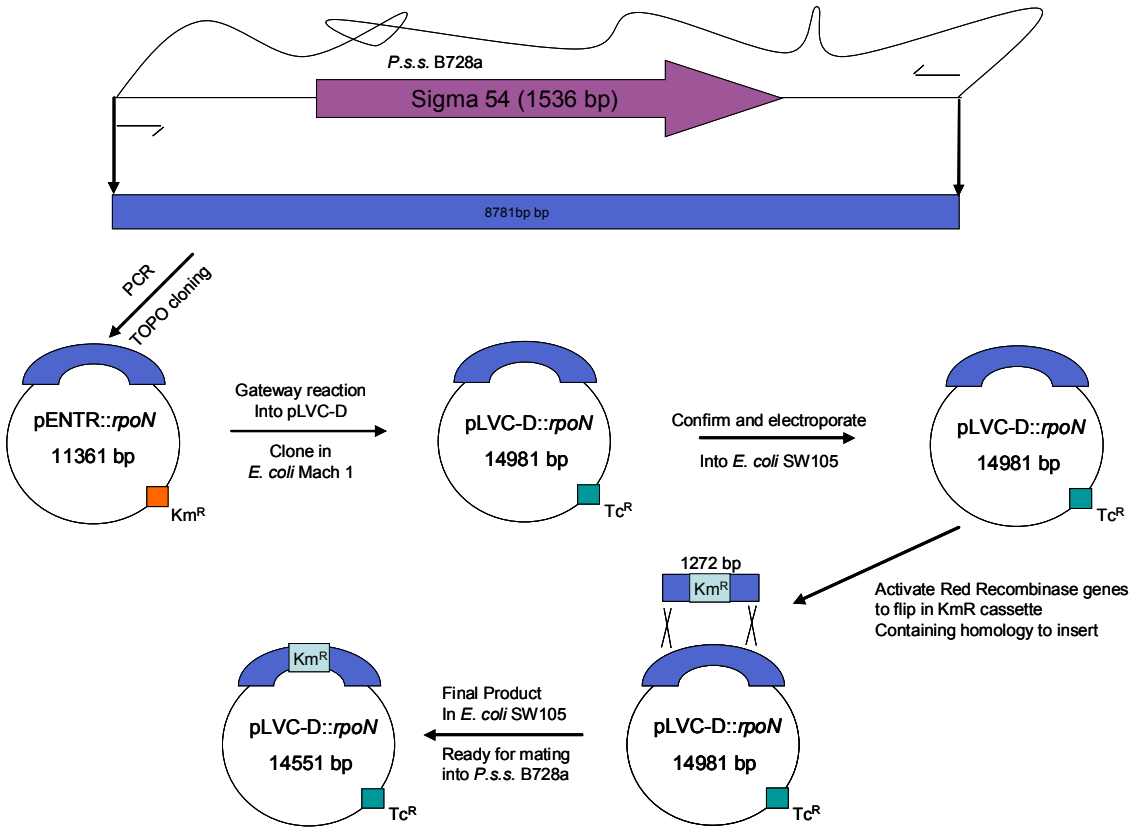
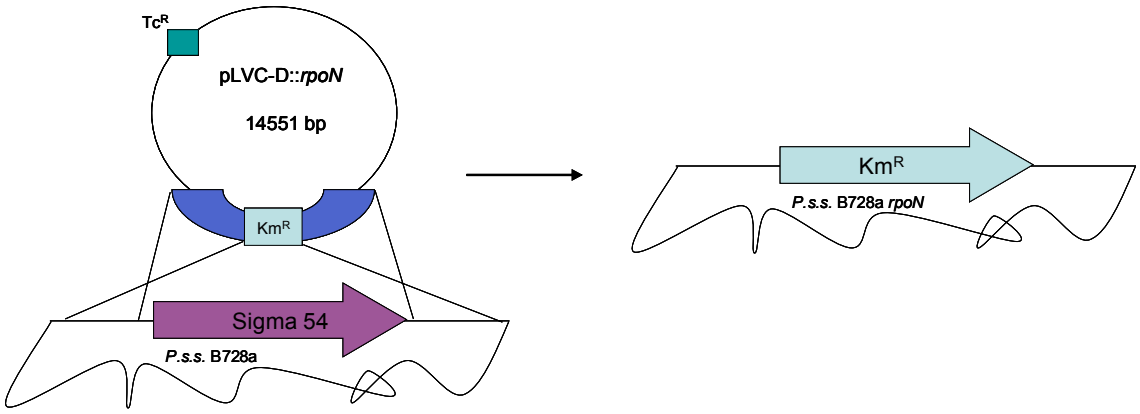


Fig 2.12. Cloning and mutational process of *rpoN* gene of *P.s.* pv. *syringae* B728a in *E. coli*. After PCR, ligation occurred in pENTR and was cloned into *E. coli* MachI, followed by gateway transformation into pLVCD. After plasmid confirmation, pL*rpoN* was electroporated into *E. coli* SW105. Red Recombinase genes were then activated, via heat shock, in order to replace *rpoN* with a non-polar Kanamycin resistance gene by double cross over of a linear piece of DNA, utilizing the Red Recombinase proteins located in *E. coli* SW105. The linear piece of DNA was electroporated into *E. coli* SW105 and 'flipped' into the pLVC-D construct after activation of recombinase genes.

which contain homology near *rpoN* in pL*rpoN*. After activation of Red Recombinase genes located in the *E. coli* SW105 genome, this segment will recombine into pL*rpoN* resulting in the removal of *rpoN* and formation of pL*rpoN*-Km. These genes are activated via heat shock at 42°C and result in leaving only the 3' and 5' homologous ends needed for integration back into B728a. Within the genome, *rpoN* is located at position 4934535 to 4936070. After completion of the Red recombination, the Km cassette replaced the segment located at 4934417-4936165. This resulted in the removal of 121 bp upstream and 95 bp downstream of the gene, and insertion of the Km^r gene (Fig 2.13). Once confirmed, *rpoN*:Km^r:pLVC-D in SW105 *E. coli* was used, along with helper *E. coli* pRK2073, for triparental mating into B728a. All bacterial colonies which grow during the mating process were analyzed for a double crossover of the vector, resulting in the loss of Tc^r and the replacement of a functional *rpoN* with a Km^r gene. The mutation was confirmed using PCR and Southern blot analysis. Sequence analysis was also conducted to confirm the exact location of the mutation. The *rpoN* mutant was named B728aAL01.

Promoter scans

Sigma 54 recognizes a -24/-12 consensus sequence which is different from Sigma 70. Two online programs were employed to search for potential promoter regions recognized by this sigma factor within the genome: PromScan (http://molbiol-tools.ca/promscan/) (55) and Virtual footprint (http://www.prodoric.de/vfp/) (42). Data received from PromScan was discarded unless it received a score of at least 90 out of 100. All data was retained from Virtual footprint analysis, as the program contained a



Note: Crossover removes 118 bp upstream and 35 bp downstream of the *rpoN* gene

Fig 2.13. Mating in *P.s.* pv. *syringae* B728a to result in the removal of *rpoN* and insertion of a non-polar kanamycin resistance gene. Potential mutants were screened for a double crossover event of the plasmid, indicating that the wild type *rpoN* gene was removed from the genome.

preset score cutoff at 0.8 out of 1.0. Although similarity existed between data sets, variation in data yield for both programs is the result of differences in programmed logarithms, with PromScan performing the most thorough search.

Growth curve analysis

A growth curved analysis was conducted to look at the growth rate of B728aAL01 compared to B728a. Cultures were initially grown overnight at 25˚C in liquid NBY with appropriate antibiotics. They were then added to 50 mL of fresh media and incubated at 25˚C, shacking (150 rpm). An OD_{600} was taken at 1 hour intervals (including time zero), over the course of 9 hours. This assay was conducted in replicate on two separate days. Obtained values were graphed after calculating their average value and standard deviations.

Phenotypic analysis

Nitrogen utilization was tested by growing cultures on minimal salts media agar plates (10% glucose, 1 M $MgSO_4$ (anhydrous), & 10X salts [30 g/L KH_2PO_4 and 60 g/L Na_2HPO_4]) containing a 5 mM nitrogen source. This is the standard level of nitrogen source shown to be sufficient in other studies. Nitrogen sources may include: NH_4Cl, L-alanine, arginine, asparagine, aspartate, cysteine, glutamine, glycine, histamine, isoleucine, leucine, lycine, methionine, phenylalanine, proline, serine, or threonine. Overnight cultures of B728a and B728aAL01 were initially diluted to and OD_{600} of 0.3 (equivalent to $5X10^8$ CFU/ml), then diluted to $1X10^4$ CFU/ml, followed by plating of 10 µl (equivalent to 10 CFU/ul). Plates were incubated for a maximum of 10 days and observed daily for the presence of colonies measuring approximately 1mm.

Motility and quorum sensing was conducted by growing cultures overnight on low percent agar (0.4%) potato dextrose plates and observing spreading ability. Plates were set up in duplicate at concentrations of $1X10^8$ and $1X10^7$, incubated overnight at 25°C, and photographed. In such a case in which swarming does not occur overnight, incubation was continued for 5 days.

A qualitative approach was taken to test adhesion ability of B728aAL01. Cultures (B728a and B728aAL01) were initially set up in 2 mL of HMM liquid medium and incubated overnight, shaking, at 25˚C. 200 µL of the fresh overnight cultures was then added to 25 mL fresh HMM liquid medium in a 50 mL plastic tube. A clean, sterile glass slide was placed into each tube. Cultures were then incubated stagnant at 25˚C. Multiple replicates were set up per culture and observed at 24 and 72 hours. Slides were stained with crystal violet and observed under a microscope to look for the presence of a biofilm at the liquid interface. Microscopic photographs were taken for each culture.

All the above phenotypic assays were conducted three times.

Bioassays for toxin production

P.s. pv. *syringae* strains B728a, B728aAL01, and B728agacS (control) were tested for the production of toxins syringopeptin and syringomycin (16, 33) on two different types of media. Briefly, strains were grown overnight in 5 mL of NBY liquid medium. Bacterial cells were collected by centrifugation, washed once in sterile distilled water (SDW), and resuspended in SDW to approximately OD_{600} 0.3 (equivalent to $5X10^8$ CFU/ml). Both PDA and modified HMM plates were spotted with 5 µL aliquots and incubated for 4 days at 25˚C. Plates were set up in triplicate with three

spots per plate. After incubating, plates were lightly oversprayed with a suspension of *Geotrichum candidum* spores or *Bacillus megaterium*. *Geotrichum* is sensitive to syringomycin (14), while *Bacillus* is sensitive to syringopeptin (33). After overnight incubation at 25˚C, zones of exclusion, in which the sprayed culture was not able to grow around the test strain, were measured and compared to the wild type strain. One measurement was taken on each plate in millimeters, choosing one of the three colonies at random. After repeating the assay three times, the nine obtained values for each scenario were averaged and standard deviations were calculated.

Pathogenicity assays

The model plant organism *Nicotiana tabacum* is also a host for *P.s.* pv. *syringae* B782a. To test the role of B728aAL01 to cause disease symptoms (presence of a hypersensitive response) in mature *N. tabaccum*, spot infiltration tests was conducted on mature leaves with the inoculum. Cultures (B728aAL01, water (positive control), and B728a (negative control)) were grown and incubated overnight in NBY with appropriate antibiotics (75 ug/µl kanamycin for the *rpoN* mutant). Leaves were infiltrated with each culture (approximately $5X10^8$ CFU/ml) after puncturing with a sterile needle. A designated leaf was infected with each culture, spaced between the veins, by forcing the bacteria into the plant puncture wound site using a syringe. Plants are incubated overnight at appropriate temperature and humidity levels. Photographs were taken of infected leafs after about 18 hours post infection.

Pathogenicity was conducted on 2-3 week old Blue Lake 274 bean seedlings (*Phaseolus vulgaris*) using standard laboratory vacuum infiltration. After overnight

growth in 5 mL liquid media (NBY) with the appropriate media, the cultures (B728aAL01, B728agacS (positive control), and B728a (negative control)) were used to inoculate 200 mL fresh media, and grown to an OD_{600} of 0.6, followed by dilution to an OD_{600} of 0.3. This OD value is equivalent to $5X10^8$ CFU/ml. The inoculum was prepared by adding 2 ml culture to 1,998 ml distilled water to obtain approximately $1X10^5$ CFU/ml. After addition of Silwet L-77, plants were vacuum infiltrated by placing under 20 in. Hg. for 1.5 min twice, to force the plants to take up the inoculum. All plants were incubated at appropriate temperature and humidity levels, and monitored daily for signs of disease development. Photographs of all plants were taken at the sign of disease development in the wild type.

Population analysis was conducted at Day 0 and at sign of symptom development (brown lesions and chlorosis) on B728a control plants to analyze the population differences between WT bacterial levels and the *rpoN* mutant. In order to analyze bacterial populations within the leaf, one trifoliate leaf was removed from each of the three plants inoculated per bacterial strain. A 2 ml screw cap microcentrifuge tube (Bio Plas Inc., San Francisco, Calif.) was used to punch out 20 leaf discs (8 mm diameter). The discs where homogenized using a mortar and pestle in Silwet Phosphate Magnesium Buffer (SPM, 0.7% K_2HPO_4, 0.4% KH_2PO_4, 0.024% $MgSO_4 \cdot 7\ H_2O$), and 0.004% Silwet L-77). Serial dilutions were set up in water and spread on KB plates with appropriate antibiotics. Colonies were counted after incubation for 48-72 hours at 25˚C.

For both the formation of HR in tobacco and bean infiltration assay, three replicates were conducted on different days.

CHAPTER III

CONCLUSIONS

Gene regulation is very important in any cellular system and multiple sigma factors exist which are involved in gene transcription. The regulatory gene Sigma 54, which is encoded by *rpoN*, has been extensively studied in many organisms including: *Rhizobium* (39), *Vibrio* (60), and several *Pseudomonas* species (27). General characteristics of its regulatory network, basic phenotype, and pathogenicity in the animal system have been previously described in these and other bacterial organisms (27, 44, 48). Unfortunately, minimal work has been conducted on *P.s.* pv. *syringae* B728a *rpoN* and how this sigma factor is involved in pathogenicity in the plant system.

O'Toole (44) showed that an *rpoN* mutant in *Vibrio anguillarum* was not only deficient in flagellum function and motility, but also did not grow when nitrogen availability was low. Research has shown that pathogenicity is affected in the human system due to lose of motility and adhesion ability (27). It was expected that a mutation to this gene would both inhibit disease development and prevent growth of the bacteria *in planta*. The ability to grow in the presence of minimal nutrients has also been shown to not occur in *rpoN* mutants. Although past studies (24) have looked into utilization of sole carbon and nitrogen utilization, nitrogen utilization was the main focus of this study. These and other known characteristics may identify the possible roles the Sigma 54 plays in activating genes involved in plant pathogenicity.

Results of the promoter scans yielded much expected data. It was not surprising to identify nitrogen or flagella related genes. In the promoter scan at least one result was not located at a -12/-24 position. Instead, it was located in the reverse orientation near an ORF. It is possible that in this case, Sigma 54 may be blocking gene transcription. Previous studies have shown that RpoN is able to negatively control itself (8, 30). It was also proposed that the presence of putative oppositely oriented RpoN binding site may be overlapping the -10 promoter region and transcription start site of Sigma 70 (39).

It is important to note, that overall, RpoN of *P.s.* pv. *syringae* B278a seems to behave in a manner very similar to other pseudomonads. Growth curve analysis of B728aAL01 showed that it grows much slower then wild type, which has been previously observed in other studies (20). This phenotype may play a role in several aspects of bacterial development. Numerous phenotypic assays were conducted as part of this project. Results of the nitrogen utilization, adhesion, and motility assay were as expected, when compared to other studies. The mutant showed a decreased ability to utilize several nitrogen sources, was not able to from a biofilm on a glass surface, or swarm on soft agar plates. It was interesting to observe that B728aAL01 appears to fluoresce less then wild type on King's B agar. This may tie in a role of Sigma 54 in iron transport, especially since promoter scans identified TonB as potentially being regulating by Sigma 54. To date, a correlation between iron regulation and Sigma 54 has not been identified.

Based on previous studies, it is expected the *P.s.* pv. *syringae* B728a RpoN will play a role in pathogenicity in both bean and *N. tabacum*. It was anticipated that disease

and hypersensitive response formation would not occur in the mutant. This may indirectly be due to several factors, such as, defects in adhesion and motility. It was not unexpected to find that B728aAL01 was not able to elicit disease symptoms in bean. A previous study in *Arabidopsis* (20) yielded similar results, and further showed that this phenotype was only partially due to lack of *hrp* gene expression.

It was interesting to find that Sigma 54 may function as a negative regulator of toxin production in *P.s.* pv. *syringae* B728a. As stated earlier, this bacterium is known to produce both syringopeptin and syringomycin. Studies have shown that RpoN is involved in production of coronatine, and that an RpoN mutant is unable to produce this toxin and elicit plant disease (20). A similar finding was not observed in B728aAL01. In this case, it appeared that toxin production was increased in the mutant strain. Upon further literature search, it was found that similar results have been reported for *P. fluorescens* CHA0 (46). This pathogen is known to produce metabolites which have been shown to posses broad range antibacterial and antifungal activity: 2,4-diacetylphloroglucinol (DAPG) and pyoluteorin (PLT) (47). In Keel's study (46), it was shown that Sigma 54 negatively regulated toxin production and may act as a control to balance toxin production. This was also reported by Heurlier (22) in *P. aeruginosa,*, in which the biocide hydrogen cyanide was expressed in greater levels in an RpoN mutant line. The mechanism for the regulatory pathway of these and many other toxins in still unclear, but this shows that Sigma 54 can act as an up regulator of toxin production in some species, and a suppressor in others. In the case of this study, Sigma 54 acts as a

suppressor of syringopeptin and syringomycin production, although likely through an indirect pathway.

This study shows that the involvement of any regulatory gene in a bacterial species is very elaborate and complex. To my knowledge, this is the first analysis of Sigma 54 in *P.s.* pv. *syringae* B728a. In general, results of this study show that the role of Sigma 54 in *P.s.* pv. *syringae* B728a is similar to other *Pseudomonas* pathovars. However, it is likely that further analysis would reveal differences among the different pathovars by use of RT-PCR and microarray analysis of the *rpoN* regulon. Through the use of current technology, over the next few years the mysteries surrounding gene regulation and the impact of Sigma 54 in the plant-pathogen interaction will be resolved.

REFERENCES

1. **Ausubel, F. M.** 1984. Regulation of nitrogen fixation genes. Cell **37:**5-6.

2. **Beynon, J., M. Cannon, V. Buchanan-Wollaston, and F. Cannon.** 1983. The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. Cell **34:**665-71.

3. **Boucher, J. C., M. J. Schurr, and V. Deretic.** 2000. Dual regulation of mucoidy in *Pseudomonas aeruginosa* and sigma factor antagonism. Mol Microbiol **36:**341-51.

4. **Buck, M., and W. Cannon.** 1992. Specific binding of the transcription factor sigma-54 to promoter DNA. Nature **358:**422-4.

5. **Burrows, P. C., K. Severinov, A. Ishihama, M. Buck, and S. R. Wigneshweraraj.** 2003. Mapping sigma 54-RNA polymerase interactions at the -24 consensus promoter element. J Biol Chem **278:**29728-43.

6. **Cannon, W., S. Missailidis, C. Smith, A. Cottier, S. Austin, M. Moore, and M. Buck.** 1995. Core RNA polymerase and promoter DNA interactions of purified domains of sigma N: bipartite functions. J Mol Biol **248:**781-803.

7. **Chi, E., T. Mehl, D. Nunn, and S. Lory.** 1991. Interaction of *Pseudomonas aeruginosa* with A549 pneumocyte cells. Infect Immun **59:**822-8.

8. **Clark, S. R., I. J. Oresnik, and M. F. Hynes.** 2001. RpoN of *Rhizobium leguminosarum* bv. *viciae* strain VF39SM plays a central role in FnrN-dependent microaerobic regulation of genes involved in nitrogen fixation. Mol Gen Genet **264:**623-33.

9. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.

10. **Dombrecht, B., K. Marchal, J. Vanderleyden, and J. Michiels.** 2002. Prediction and overview of the RpoN-regulon in closely related species of the *Rhizobiales*. Genome Biol **3:**RESEARCH0076.

11. **Feil, H., W. S. Feil, P. Chain, F. Larimer, G. DiBartolo, A. Copeland, A. Lykidis, S. Trong, M. Nolan, E. Goltsman, J. Thiel, S. Malfatti, J. E. Loper, A. Lapidus, J. C. Detter, M. Land, P. M. Richardson, N. C. Kyrpides, N. Ivanova, and S. E. Lindow.** 2005. Comparison of the complete genome

sequences of *Pseudomonas syringae* pv. *syringae* B728a and pv. *tomato* DC3000. Proc Natl Acad Sci U S A **102:**11064-9.

12. **Fellay, R., L. Rahme, M. Mindrinos, R. Frederick, A. Pisi, and N. Panopoulus.** 1991. Genes and signals controlling *Pseudomonas syringae* pv. *phaseolicola*-plant interactions. Molecular genetics of plant-microbe interactions**:**45-52.

13. **Fullone, M. R., A. Paiardini, D. C. Gross, S. E. Lu, A. Fiore, and I. Grgurina.** 2007. Mutational analysis and homology modelling of SyrC, the aminoacyltransferase in the biosynthesis of syringomycin. Biochem Biophys Res Commun **364:**201-7.

14. **Grgurina, I., D. C. Gross, N. S. Iacobellis, and M. Benincasa.** 1996. Phytotoxin production by *Pseudomonas syringae* pv. *syringae*: Syringopeptin production by *syr* mutants defective in biosynthesis or secretion of syringomycin. FEMS Microbiol Lett **138:**35-39.

15. **Grimm, C., and N. J. Panopoulos.** 1989. The predicted protein product of a pathogenicity locus from *Pseudomonas syringae* pv. *phaseolicola* is homologous to a highly conserved domain of several procaryotic regulatory proteins. J Bacteriol **171:**5031-8.

16. **Gross, D. C., and J. E. DeVay.** 1977. Population dynamics and pathogenesis of *Pseudomonas syringae* in maize and cowpea in relation to the *in vitro* production of syringomycin. Phytopathology **67:**475-483.

17. **Guenzi, E., G. Galli, I. Grgurina, D. C. Gross, and G. Grandi.** 1998. Characterization of the syringomycin synthetase gene cluster. A link between prokaryotic and eukaryotic peptide synthetases. J Biol Chem **273:**32857-63.

18. **Hanson, P., and S. Whiteheart.** 2005. AAA+ Proteins: have engine, will work. Nat Rev Mole Cell Biol. **6:**519-529.

19. **Hendrickson, E. L., P. Guevera, and F. M. Ausubel.** 2000. The alternative sigma factor RpoN is required for hrp activity in *Pseudomonas syringae* pv. *maculicola* and acts at the level of *hrpL* transcription. J Bacteriol **182:**3508-16.

20. **Hendrickson, E. L., P. Guevera, A. Penaloza-Vazquez, J. Shao, C. Bender, and F. M. Ausubel.** 2000. Virulence of the phytopathogen *Pseudomonas syringae* pv. *maculicola* is *rpoN* dependent. J Bacteriol **182:**3498-507.

21. **Hendrickson, E. L., J. Plotnikova, S. Mahajan-Miklos, L. G. Rahme, and F. M. Ausubel.** 2001. Differential roles of the *Pseudomonas aeruginosa* PA14

rpoN gene in pathogenicity in plants, nematodes, insects, and mice. J Bacteriol **183:**7126-34.

22. **Heurlier, K., V. Denervaud, G. Pessi, C. Reimmann, and D. Haas.** 2003. Negative control of quorum sensing by RpoN (sigma54) in Pseudomonas aeruginosa PAO1. J Bacteriol **185:**2227-35.

23. **Hirouuki, S., F. Suzuki, I. Matsuda, and N. Saitou.** 1999. Phylogenetic analysis of *Pseudomonas syringae* pathovars suggests the horizontal gene transfer of *argK* and the evolutionary stability of *hrp* gene cluster. J Mol Evol **49:**627-653.

24. **Hirschman, J., P. K. Wong, K. Sei, J. Keener, and S. Kustu.** 1985. Products of nitrogen regulatory genes *ntrA* and *ntrC* of enteric bacteria activate *glnA* transcription *in vitro*: evidence that the *ntrA* product is a sigma factor. Proc Natl Acad Sci U S A **82:**7525-9.

25. **Hutchison, M. L., and D. C. Gross.** 1997. Lipopeptide phytotoxins produced by *Pseudomonas syringae* pv. *syringae*: comparison of the biosurfactant and ion channel-forming activities of syringopeptin and syringomycin. Mol Plant Microbe Interact **10:**347-54.

26. **Huynh, T. V., D. Dahlbeck, and B. Staskawicz.** 1989. Bacterial Blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science **245:**1374-1377.

27. **Kazmierczak, M. J., M. Wiedmann, and K. J. Boor.** 2005. Alternative sigma factors and their roles in bacterial virulence. Microbiol Mol Biol Rev **69:**527-43.

28. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Med. **44:**301-307.

29. **Kohler, T., S. Harayama, J. L. Ramos, and K. N. Timmis.** 1989. Involvement of *Pseudomonas putida* RpoN sigma factor in regulation of various metabolic functions. J Bacteriol **171:**4326-33.

30. **Kullik, I., S. Fritsche, H. Knobel, J. Sanjuan, H. Hennecke, and H. M. Fischer.** 1991. *Bradyrhizobium japonicum* has two differentially regulated, functional homologs of the sigma 54 gene (*rpoN*). J Bacteriol **173:**1125-38.

31. **Kustu, S., E. Santero, J. Keener, D. Popham, and D. Weiss.** 1989. Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. Microbiol Rev **53:**367-76.

32. **Landy, A.** 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu Rev Biochem **58:**913-49.

33. **Lavermicocca, P., N. S. Iacobellis, M. Simmaco, and A. Graniti.** 1997. Biological properties and spectrum of activity of *Pseudomonas syringae* pv. *syringae* toxins. Physiol. Mol. Plant Pathol **50:**129-140.

34. **Leong, S. A., G. S. Ditta, and D. R. Helinski.** 1982. Heme biosynthesis in Rhizobium. Identification of a cloned gene coding for delta-aminolevulinic acid synthetase from Rhizobium meliloti. J Biol Chem **257:**8724-30.

35. **Lindgren, P. B., R. C. Peet, and N. J. Panopoulos.** 1986. Gene cluster of *Pseudomonas syringae* pv. "*phaseolicola*" controls pathogenicity of bean plants and hypersensitivity of nonhost plants. J Bacteriol **168:**512-22.

36. **Loper, J. E., and S. E. Lindow.** 1987. Lack of evidence for *in situ* fluorescent pigment production by *Pseudomonas syringae* pv. *syringae* on bean leaf surfaces. Phytopathology **77:**1449-1454.

37. **Lu, S. E., B. K. Scholz-Schroeder, and D. C. Gross.** 2002. Characterization of the *salA*, *syrF*, and *syrG* regulatory genes located at the right border of the syringomycin gene cluster of Pseudomonas syringae pv. syringae. Mol Plant Microbe Interact **15:**43-53.

38. **Lu, S. E., N. Wang, J. Wang, Z. J. Chen, and D. C. Gross.** 2005. Oligonucleotide microarray analysis of the *salA* regulon controlling phytotoxin production by *Pseudomonas syringae* pv. *syringae*. Mol Plant Microbe Interact **18:**324-33.

39. **Michiels, J., T. Van Soom, I. D'Hooghe, B. Dombrecht, T. Benhassine, P. de Wilde, and J. Vanderleyden.** 1998. The *Rhizobium etli rpoN* locus: DNA sequence analysis and phenotypical characterization of *rpoN, ptsN*, and *ptsA* mutants. J Bacteriol **180:**1729-40.

40. **Mooney, R. A., S. A. Darst, and R. Landick.** 2005. Sigma and RNA polymerase: an on-again, off-again relationship? Mol Cell **20:**335-45.

41. **Morett, E., and L. Segovia.** 1993. The sigma 54 bacterial enhancer-binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J Bacteriol **175:**6067-74.

42. **Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert, and D. Jahn.** 2005. Virtual footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. Bioinformatics **21:**4187-9.

43. **Neto, J., T. Koide, C. Abe, S. Gomes, and M. Marques.** 2008. Role of Sigma 54 in the regulation of genes involved in type I and type IV pili biogenesis in *Xylella fastidiosa*. Arch Microbiol **189:**249-261.

44. **O'Toole, R., D. L. Milton, and H. Wolf-Watz.** 1996. Chemotactic motility is required for invasion of the host by the fish pathogen *Vibrio anguillarum*. Mol Microbiol **19:**625-37.

45. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu Rev Microbiol **57:**677-701.

46. **Pechy-Tarr, M., M. Bottiglieri, S. Mathys, K. B. Lejbolle, U. Schnider-Keel, M. Maurhofer, and C. Keel.** 2005. RpoN (sigma54) controls production of antifungal compounds and biocontrol activity in Pseudomonas fluorescens CHA0. Mol Plant Microbe Interact **18:**260-72.

47. **Raaijmakers, J. M., M. LVlami, and J. de Souza.** 2002. Antibiotic production by bacterial biocontrol agents. Int. J. Gen. Mol. Microbiol. **81:**537-547.

48. **Reitzer, L., and B. L. Schneider.** 2001. Metabolic context and possible physiological themes of sigma(54)-dependent genes in *Escherichia coli*. Microbiol Mol Biol Rev **65:**422-44.

49. **Rodrigue, S., R. Provvedi, P. E. Jacques, L. Gaudreau, and R. Manganelli.** 2006. The sigma factors of *Mycobacterium tuberculosis*. FEMS Microbiol Rev **30:**926-41.

50. **Scholz-Schroeder, B. K., M. L. Hutchison, I. Grgurina, and D. C. Gross.** 2001. The contribution of syringopeptin and syringomycin to virulence of *Pseudomonas syringae* pv. *syringae* strain B301D on the basis of *sypA* and *syrB1* biosynthesis mutant analysis. Mol Plant Microbe Interact **14:**336-48.

51. **Scholz-Schroeder, B. K., J. D. Soule, and D. C. Gross.** 2003. The *sypA*, *sypS*, and *sypC* synthetase genes encode twenty-two modules involved in the nonribosomal peptide synthesis of syringopeptin by *Pseudomonas syringae* pv. *syringae* B301D. Mol Plant Microbe Interact **16:**271-80.

52. **Scholz-Schroeder, B. K., J. D. Soule, S. E. Lu, I. Grgurina, and D. C. Gross.** 2001. A physical map of the syringomycin and syringopeptin gene clusters localized to an approximately 145-kb DNA region of *Pseudomonas syringae* pv. *syringae* strain B301D. Mol Plant Microbe Interact **14:**1426-35.

53. **Shingler, V.** 1996. Signal sensing by sigma 54-dependent regulators: derepression as a control mechanism. Mol Microbiol **19:**409-16.

54. **Studholme, D. J., and M. Buck.** 2000. The biology of enhancer-dependent transcriptional regulation in bacteria: insights from genome sequences. FEMS Microbiol Lett **186:**1-9.

55. **Studholme, D. J., and R. Dixon.** 2003. Domain architectures of sigma 54-dependent transcriptional activators. J Bacteriol **185:**1757-67.

56. **Totten, P. A., J. C. Lara, and S. Lory.** 1990. The *rpoN* gene product of *Pseudomonas aeruginosa* is required for expression of diverse genes, including the flagellin gene. J Bacteriol **172:**389-96.

57. **Travers, A. A., and Burgessrr.** 1969. Cyclic re-use of the RNA polymerase sigma factor. Nature **222:**537-40.

58. **Vidaver, A. K.** 1967. Synthetic and complex media for rapid detection of fluorescence of phytopathogenic pseudomonads: effect of carbon source. Appl. Microbiol. **15:**1523-1524.

59. **Villain-Guillot, P., L. Bastide, M. Gualtieri, and J. P. Leonetti.** 2007. Progress in targeting bacterial transcription. Drug Discov Today **12:**200-8.

60. **Visick, K. L., and E. G. Ruby.** 1998. The periplasmic, group III catalase of *Vibrio fischeri* is required for normal symbiotic competence and is induced both by oxidative stress and by approach to stationary phase. J Bacteriol **180:**2087-92.

61. **Wang, L., Y. Guo, and J. D. Gralla.** 1999. Regulation of sigma 54-dependent transcription by core promoter sequences: role of -12 region nucleotides. J Bacteriol **181:**7558-65.

62. **Wang, N., S. E. Lu, A. R. Records, and D. C. Gross.** 2006. Characterization of the transcriptional activators SalA and SyrF, Which are required for syringomycin and syringopeptin production by *Pseudomonas syringae* pv. *syringae*. J Bacteriol **188:**3290-8.

63. **Wang, N., S. E. Lu, J. Wang, Z. J. Chen, and D. C. Gross.** 2006. The expression of genes encoding lipodepsipeptide phytotoxins by *Pseudomonas syringae* pv. *syringae* is coordinated in response to plant signal molecules. Mol Plant Microbe Interact **19:**257-69.

64. **Wang, N., S. E. Lu, Q. Yang, S. H. Sze, and D. C. Gross.** 2006. Identification of the *syr-syp* box in the promoter regions of genes dedicated to syringomycin

and syringopeptin production by *Pseudomonas syringae* pv. *syringae* B301D. J Bacteriol **188:**160-8.

65. **Weiss, D. S., J. Batut, K. E. Klose, J. Keener, and S. Kustu.** 1991. The phosphorylated form of the enhancer-binding protein NTRC has an ATPase activity that is essential for activation of transcription. Cell **67:**155-67.

66. **Wigneshweraraj, S. R., M. Channey, and A. Ishihama.** 2000. Regulatory sequences in Sigma 54 localised near the start of DNA melting. J Microbiol **306:**681-701.

67. **Yan, Q., X. G. Wu, H. L. Wei, H. M. Wang, and L. Q. Zhang.** 2008. Differential control of the PcoI/PcoR quorum-sensing system in *Pseudomonas fluorescens* 2P24 by sigma factor RpoS and the GacS/GacA two-component regulatory system. Microbiol Res. *In Press.*

68. **Zhang, J. H., N. B. Quigley, and D. C. Gross.** 1997. Analysis of the *syrP* gene, which regulates syringomycin synthesis by *Pseudomonas syringae* pv. *syringae*. Appl Environ Microbiol **63:**2771-8.

VITA

Name:	Amber Lorge
Address:	Texas A&M University 2132 TAMU Dept. Plant Pathology College Station, TX 77843-2132
Email Address:	amber_lorge@hotmail.com
Education:	B.S., Biology, Marian University, 2005
	M.S., Plant Pathology, Texas A&M University, 2009