CHARACTERIZATION OF THE PSEUDOMONAS SYRINGAE PATHOVAR TOMATO DC3000 RETS HYBRID TWO COMPONENT SENSOR FOR INDUCTION OF THE TYPE THREE SECRETION SYSTEM AND MOTILITY

by Alan Hale Chambers

CHARACTERIZATION OF THE PSEUDOMONAS SYRINGAE PATHOVAR TOMATO DC3000 RETS HYBRID TWO COMPONENT SENSOR FOR INDUCTION OF THE TYPE THREE SECRETION SYSTEM AND MOTILITY

A Thesis

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Masters of Science

by

Alan Hale Chambers

August 2010

ABSTRACT

RetS is a unique, hybrid two component sensor highly conserved among sequenced *Pseudomonas* species. RetS is best characterized in *P. aeruginosa* where it modulates chronic versus acute infection with its antagonist LadS via signaling through GacS/A and small regulatory RNAs. We conducted a transposon mutagenesis screen of *P. syringae* DC3000 carrying a *hrp* box promoter fused to a GUS reporter plasmid to investigate regulation of the type three secretion system. We isolated several transposon insertion events in PSPTO_4868 that showed reduced reporter activity in *hrp* inducing medium. The predicted coding sequence of PSPTO_4868 shows 58.6% identity over 889 residues to *P. aeruginosa* RetS (PA4856) and 94.0% identity over 929 residues to *P. syringae* B728a RetS (Psyr_4408). A DC3000 PSPTO_4868 pKnockout strain (hereafter Δ*retS*) showed reduced type three secretion (T3SS) activity *in vitro* as measured by an avrPtoB::Lux reporter plasmid in *hrp* minimal medium supplemented with fructose or mannitol, but not when supplemented with sucrose, succinate, glutamate, or GABA as a carbon source. qRT-PCR confirmed that the Δ*retS* is unable to fully activate the T3SS, and this response is modulated at least in part through RsmZ similarly to *P. aeruginosa*. The mutant was able to swarm in the presence of NaCl where WT is immotile. Δ*retS* had no HR defect in tobacco. These results indicate that RetS in DC3000 is potentially involved in pathogenesis, but its actual role *in planta* remains to be determined.

BIOGRAPHICAL SKETCH

Alan Hale Chambers was born in North Ogden, Utah to Craig and Rosemary Chambers. He was raised primarily in Northern Virginia, which is where he graduated from West Springfield High School in 2000. Alan attended Brigham Young University (BYU) from August 2000 until July 2007 graduating with a BS in Genetics and Biotechnology. He took a short hiatus from his undergraduate work to serve a full-time mission for the Church of Jesus Christ of Latter-day Saints in Phoenix, Arizona from January 2001 until February 2003.

In August 2003 Alan had the paramount opportunity of his life, which was to marry Sandra McQuade in the Salt Lake City Temple of the aforementioned church. Before leaving BYU, Alan had accumulated over 1,000 hours of undergraduate research, obtained internships at both Monsanto (8 months) and the University of Utah (3 months), and been blessed with three of the most wonderful children on the planet (Sheila '04, Aaron '05, and Kiara '07). Alan started his graduate work at Cornell University August 2007 in the Department of Plant Pathology and Plant-Microbe Biology. He found a research home in the Sam Cartinhour laboratory working with the plant pathogen *Pseudomonas syringae* pv. *tomato* DC3000. Finally, in 2009, Alan and Sandi welcomed their latest addition, Alila, to their family.

In August 2010 Alan will start his PhD at the University of Florida with Dr. Kevin Folta and Dr. Vance Whitaker conducting molecular breeding research to improve flavor in cultivated strawberry.

ACKNOWLEDGMENTS

I would obviously like to first thank Sam for taking me into his lab as a graduate student in 2008, and for all of his support since then. I have received so much from the entire Cartinhour group (Bryan Swingle, Bronwyn Butcher, Charlene Maciak, Chris Myers, Dave Schneider, Eric Markel, Irene Park, James Bolton, Johnson Zhang, Magdalen Lindeberg, Melanie Filiatrault, Paul Stodghill, Phil Bronstein, Simon Moll, and Zhongmeng Bao) and thank them for research advice, support, and for the many conversations that lightened gloomy days. I wish to acknowledge my gratitude to Alan Collmer for serving on my committee, and for the insights that he provided from his encyclopedic mind to help my project move forward.

This work would not have been possible without the financial support of the USDA National Needs Fellowship and from USDA ARS.

Finally, thanks to my family for their unwavering support during this last three years especially during weekends, evenings, and other odd, long hours as I worked on this and other research projects. Coming home to them everyday made life sweet.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Pseudomonas syringae pv. *tomato* DC3000 (hereafter DC3000) is an ideal candidate for studying molecular pathogenesis and plant-microbe interactions. It causes disease on both tomato (*Lycopersicon esculentum*) (Cuppels & Ainsworth, 1995) and *Arabidopsis thaliana* (Whalen *et al.*, 1991), and is used in studies with the non-host *Nicotiana benthamiana* (Abramovitch & Martin, 2004). Both DC3000 (Buell *et al.*, 2003) and Arabidopsis (The Arabidopsis Genome Initiative, 2000) have been sequenced and are genetically tractable. This greatly facilitates in depth research for characterizing DC3000 pathogenicity at the molecular level. Many DC3000 regulatory elements of pathogenesis are conserved among other pathogenic *Pseudomonas* species including environmental sensing by two-component systems (TCS), signal transduction via GacS/GacA/RsmA/regulatory small RNA molecules, and alternative sigma factors. This signal transduction relay ultimately leads to the expression of the Type Three Secretion System (T3SS) forming a syringe-like organelle that translocates effectors from the bacterium into the host cell compromising host immunity.

Two-Component Systems

Bacteria use TCS to sense and respond to environmental stimuli. The classical TCS contains a sensor histidine kinase that transduces a signal from the periplasm to the bacterial cytoplasm when activated by a specific ligand. This information is then relayed to a cognate response regulator, usually by phosphorylation from conserved His-to-Asp residues, which induces a physiological response. RetS (regulator of exopolysaccharide and Type III Secretion) is a hybrid TCS that contains both sensory and response regulatory

domains. It has also been described as a unique TCS because it has two C-terminal receiver domains. RetS is especially interesting because it is involved in *P. aeruginosa* pathogenicity (Goodman *et al.*, 2004).

Δ*retS* phenotypes

RetS was independently discovered, characterized, and published in *P. aeruginosa* by two different groups. In the first published study, RetS-dependent induction of T3SS activity was shown by systematic replacement of TCSs with a gentamicin antibiotic cassette (Goodman et al., 2004). In the alternate case, RetS was discovered by mapping transposon insertions responsible for reduced virulence and virulence-associated phenotypes (Laskowski *et al.*, 2004, Zolfaghar *et al.*, 2005). These initial studies demonstrated the role of RetS in various regulatory cascades linking an environmental sensor with known components of T3SS regulation. RetS is also involved in biofilm development, adhesion to mammalian cells, chronic versus acute persistence in a murine mouse model, the expression of toxin genes, and it was hypothesized to work through the GacS/GacA/RsmZ pathway (Goodman et al., 2004). RetS (under the name RtsM) was further shown to affect the expression of the T3SS regulators ExsA and ExsD, and secretion of effectors ExoU and ExoT, which could be compensated for by overexpressing *vfr* or *exsA* (Laskowski et al., 2004). The *retS* mutant also displayed a defect in twitching motility, which relies on Type IV pili, attenuated virulence in mouse corneal infection assays at 12 hours post inoculation (yet increased colonization after 7 days), and reduced invasion of epithelial cells showing effects both within and outside of the T3SS (Zolfaghar et al., 2005, Zolfaghar *et al.*, 2006). RetS is required for sliding motility (Murray &

Kazmierczak, 2008) and swarming (Goodman et al., 2004). Later studies that built upon the regulatory model also showed the antagonistic responses between RetS and LadS (lost adherence sensor) on biofilm formation (chronic infection) or T3SS induction (acute infection) through the small regulatory RNA RsmZ as shown in Figure 1 (Ventre *et al.*, 2006). For comparison, the *P. syringae* pv. *syringae* B728a (hereafter B728a) RetS model is shown in Figure 2 (Records & Gross, 2010). From our experiments, the DC3000 RetS model (Figure 3) more closely resembles the *P. aeruginosa* RetS model than the B728a model.

RetS and LadS also reciprocally regulate the Type Six Secretion System (T6SS) and exopolysaccharide production with RetS repressing and LadS activating these systems in acute and chronic infections respectively (Goodman et al., 2004, Mougous *et al.*, 2006, Brencic & Lory, 2009). Research documenting the effects of RetS and LadS orthologs in B728a were recently published (Records & Gross, 2010). The RetS ortholog in B728a is a negative regulator of the T6SS and mucoidy (alginate production), and positive regulator of T3SS. The *retS* mutant also grew poorly compared to wild type (WT) on bean leaves in low relative humidity, but similarly to WT with high relative humidity. The *retS* mutant was able to cause disease symptoms when infiltrated into a host plant similar to WT.

T3SS regulation via RetS

Mutational studies of RetS in *P. aeruginosa* reveal interesting functions and domain architecture. Removal of the periplasmic sensory domain resulted in slightly increased virulence in one study, which also showed that receiver domain 2, but not receiver domain 1, is required for virulence in mouse

(Laskowski & Kazmierczak, 2006). RetS directly binds to GacS, preventing GacA phosphorylation, which inhibits biofilm formation through the GacA/RsmZ/RsmA pathway (Goodman *et al.*, 2009). The direct binding and inhibition of GacS by RetS does not require the predicted phosphorelay residues of the RetS REC domains (Goodman et al., 2009). RetS does not appear to be capable of autophosphorylation (Jing *et al.*, 2010, Goodman et al., 2009), but can phosphorylate HptB which is involved in swarming and biofilm phenotypes (Hsu *et al.*, 2008).

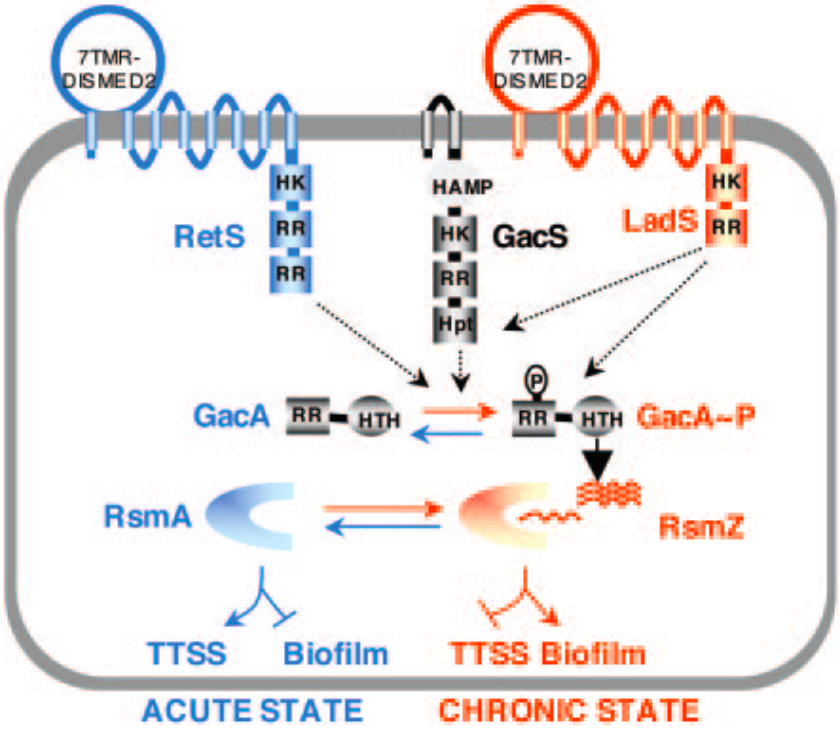

Figure 1. *P. aeruginosa* RetS model.
Model of RetS in P. aeruginosa showing antagonistic effects of RetS and LadS (Ventre et al., 2006). RetS represses GacS from activating GacA. This leads to reduced levels of RsmZ leaving RsmA free to repress biofilm. Biofilm and T3SS (or TTSS in Figure 1) are inversely regulated. The T3SS is induced when biofilm is repressed and vice versa.

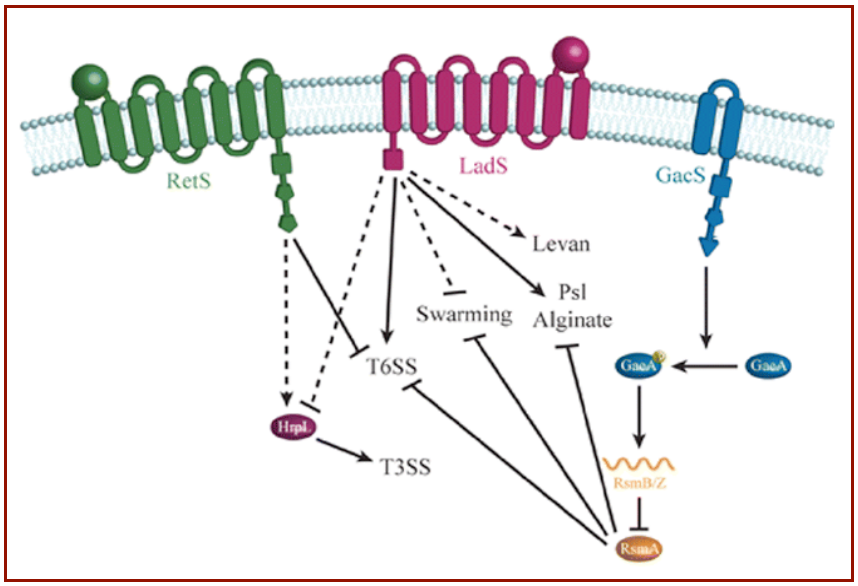

Figure 2. B728a RetS model.
Model showing GacS-independent role of RetS for inducing the T3SS in B728a (Records & Gross, 2010).

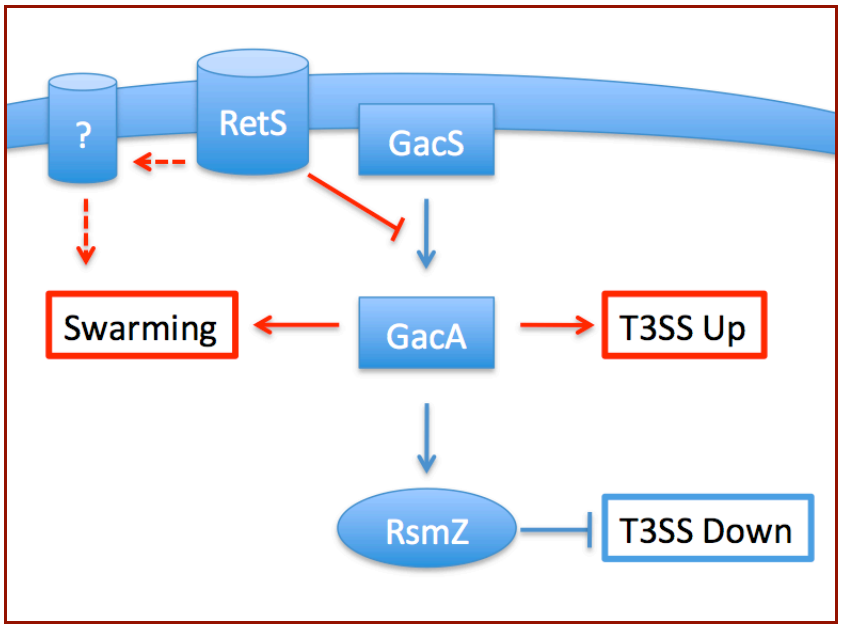

Figure 3. Proposed DC3000 RetS model.
Model of RetS at the top of DC3000 T3SS regulation. Red colors show RetS-dependent T3SS induction or swarming, blue lines show inverse regulation. Dashed lines indicate predicted alternate associations relevant to swarming.

In *P. aeruginosa*, RetS also represses the T6SS in an HptB-independent manner through the activity of RsmA and the small RNAs RsmY and RsmZ (Bordi *et al.*, 2010, Brencic & Lory, 2009). The current model suggests that RetS is found as a dimer in the cell membrane, dissociates after binding an unidentified ligand, and directly interacts with GacS, interfering with GacS signaling leading to decreased RsmZ levels and induction of the T3SS (Jing et al., 2010). The specific environmental signal that binds to the RetS periplasmic domain is not known, but the N-terminal region contains an amino acid sequence that is predicted to have a carbohydrate binding domain.

T3SS in DC3000

Induction of the T3SS in *P. syringae* is environmentally regulated and induced in a host plant or in minimal medium that may mimic the host environment. Specific elicitors of T3SS include carbon and nitrogen sources, osmolarity, pH, and plant specific molecules (Huynh *et al.*, 1989, Rahme *et al.*, 1992, Grimm *et al.*, 1995, van Dijk *et al.*, 1999). *P. syringae* mirrors *P. aeruginosa* in the activity of GacA, in that GacA disruption had pleiotropic effects in both species including effects on virulence-associated phenotypes. In *P. aeruginosa*, GacA signaling works through the two regulatory small RNAs, RsmY and RsmZ. GacA mutants show upregulation of the T3SS and type IV pili genes, and downregulation of biofilm and T6SS genes (Brencic *et al.*, 2009). In DC3000, GacA is a master regulator responsible for induction of the T3SS and the small regulatory RNAs RsmB and RsmZ, and a GacA mutant shows reduced swarming motility and quorum sensing signal production (Chatterjee *et al.*, 2003). Downstream of GacA, HrpR and HrpS induce the expression of *hrpL*, an alternative sigma factor (Hutcheson *et al.*,

2001, Grimm et al., 1995, Xiao *et al.*, 1994). *hrpL* activation by HrpR and HrpS requires RpoN (Hendrickson *et al.*, 2000). HrpR is antagonized by Lon-associated degradation in nutritionally rich medium (Bretz *et al.*, 2002). HrpL induces the expression of the *hrp* structural genes and effectors (Xiao et al., 1994). For an excellent review on host-microbe interactions and comparative gene regulation see (Brencic & Winans, 2005).

Genomics based T3SS studies in *P. syringae*

Previous genomics-based mutagenic studies have attempted to uncover additional upstream regulatory elements of *P. syringae* T3SS. One used a phrpZ::uidA reporter construct in *P. syringae* pv. *maculicola*, mutagenized with nitrosoguanidine, and then plated transformants on minimal medium (Hendrickson et al., 2000). They screened 14,000 colonies and found 297 putative *hrp* regulatory mutants, but were unable to identify any regulatory genes outside of the *hrp* gene cluster. Another study used a pavrPto::Luc reporter plasmid and transposon mutagenesis to screen for inhibition of reporter activity in *Arabidopsis* leaves for 6,000 *P. syringae* pv. *phaseolicola* NPS3121 transformants (Xiao *et al.*, 2007). They identified two Tn5 insertion mutants in *rhpS*, as part of the RhpS/R TCS that reduced T3SS induction *in planta* and in minimal medium. *rhpS* mutants also had a reduced HR phenotype in the non-host *Nicotiana tabacum* and reduced disease in the Red Kidney bean host. A recent genomics-based mutagenesis study used two constructs, pavrPto::Luc and phrpL::Luc, and screened a total of 27,872 transposon insertion mutants for repressed reporter activity in liquid cultures of inducing minimal medium (Deng *et al.*, 2009). Insertion events were mapped from 92 mutants with decreased reporter activity to *rhpS*, *hrpS*, *aefR*, and

many genes involved in basic cellular metabolism. Finally, though not a mutagenic study, a genomics-based *in vivo* expression technology study successfully identified *P. syringae* pv. *tomato* gene promoters that are expressed in the Arabidopsis host but not when grown on rich medium (Boch *et al.*, 2002). They characterized 79 genes from their experiment and found both known and unknown virulence genes.

We conducted a transposon mutagenesis screen of DC3000 to identify upstream regulatory elements influencing reporter activity from a phrpJ::uidA reporter plasmid. The strengths of this study are the large number of colonies that we were able to screen (~120,000) and the potential for saturation mutagenesis as indicated by the successful identification of multiple insertion events in *hrpR*, *hrpS*, *hrpL*, *rhpS*, and *tvrR* which are all known DC3000 T3SS regulatory elements. One gene, PSPTO_4868, had 17 independent insertion events that displayed a reduction in reporter activity. The predicted amino acid sequence of PSPTO_4868 shows conservation and synteny with *P. aeruginosa* RetS (58.6% identity, 889 residues) and B728a RetS (94.0% identity, 929 residues) (http://expasy.ch/tools/sim.html), and is hereafter referred to as RetS. RetS completely repressed swarming in medium supplemented with NaCl. The Δ*retS* strain also had reduced T3SS reporter activity in *hrp* minimal medium supplemented with fructose or mannitol, but not when supplemented with citrate, GABA, L-glutamate, succinate, or sucrose as carbon sources. qRT-PCR additionally showed that RetS induces the T3SS through the RsmZ regulatory cascade similarly to in *P. aeruginosa*.

MATERIALS AND METHODS

Strains, plasmids, and primers

The strains, plasmids, and primers used in this study are listed in Table 1. The strain nomenclature from Table 1 will be used throughout this report.

Table 1. Strains, plasmids, and primers

Strains	**Antibiotic Markers**	**Functional Characteristics**
WT DC3000	Rif	Parental WT DC3000 strain
WT pBS45	Tet, Kan	WT DC3000 with pavrptoB::LuxCDABE plasmid reporter
WT pBS63	Tet, Kan	WT DC3000 with phrpJ::LuxCDABE plasmid reporter
WT RecTE	Rif, Gent	Integrates dsDNA into chromosome with specificity
WT pAC1	Rif, Tet	phrpJ::iucD expresses GUS under T3SS inducing conditions
Δ*retS*-RecTE	Rif, Kan	PSPTO_4868 mutant derived by RecTE system
Δ*retS*	Rif, Spec	PSPTO_4868 mutant derived by pKnockout mutagenesis
Δ*retS* Comp	Rif, Spec, Gent	PSPTO_4868 pKO mutant complemented on plasmid
Δ*retS* pBS45	Tet, Kan	Δ*retS* with pavrptoB::LuxCDABE plasmid reporter
Δ*vfr* pBS63	Tet, Kan	Δ*vfr* with phrpJ::LuxCDABE plasmid reporter
Plasmids	**Antibiotic Markers**	**Functional Characteristics**
pBS45	Tet, Kan	pavrptoB::LuxCDABE
pBS46	Gent	Complementation and overexpression plasmid
pBS63	Tet, Kan	phrpJ::LuxCDABE
pBS181	Tet, Kan	phrpJ::iucD expresses GUS under T3SS inducing conditions
pAC1	Tet	pBS181 except with KanR gene excised via PstI digest
pENTR/SD/D	Kan	Gateway entry vector
pKnockout Ω plasmid	Spec	Used to generate gene knockouts via plasmid integration
pUCP24/RecTE	Gent	RecTE overexpression plasmid
Primer ID	**Primer Sequence 5' to 3'**	
oSWC02330	CCTTTGCCATGTTTCAGAAACAACT	
oSWC02332	CCGTTGAATATGGCTCATAACACCCCT	
oSWC02210	GCAATGTAACATCAGAGATTTTGAG	
oSWC01139	GTAACACTGGCAGAGCATTACGCTG	
oSWC02331	GGATCAGATCACGCATCTTCCCGACA	
oSWC02209	ACCTACAACAAAGCTCTCATCAACC	
oSWC00141	GGCCACGCGTCGACTAGTACNNNNNNNNNNGAACG	
oSWC00142	GGCCACGCGTCGACTAGTAC	
oSWC02728	CACCGTGCGCTGGCTCAGGATTGCCA	
0SWC02729	TCAGTTTTGCCGATAGTGCTCGCCAT	
oSWC02724	AGTAGTGGATCCCTGCTGCTCAACCTGCTTCGCCCTA	
oSWC02725	AGTAGTGGATCCAAGCGCAACGCCGAGCAGGATTT	
oSWC00379	AGGCAAGTATTTCCTGTCGCC	
oSWC00380	CTGGTACTCACCCAGCAGTTTTT	
oSWC02464	AACCGCATTCACGAGTTGTCCA	
oSWC02465	TTGACCACACCGAATAACTGGCTC	
oSWC02466	GGATGACATTCTCCAGTGCGTGTT	
oSWC02467	TTCAGCGCGATGCCACACAG	
oSWC02468	GATCAGGACACCGGGCTGGATA	
oSWC02469	CCGTCGGTGTAATCCAGAACCTGT	

Antibiotics Key: Tet tetracycline, Kan kanamycin, Gent gentamicin, Spec spectinomycin.

Growth conditions

Strains were routinely grown on KB medium (King *et al.*, 1954). Strains were grown on *hrp* minimal medium supplemented with Fructose (10mM) as a carbon source to induce expression of the T3SS *in vitro* (Huynh et al., 1989). Antibiotics and other supplements were used as appropriate at the following concentrations (*μ*g/ml): kanamycin (50), tetracycline (10), spectinomycin (50), gentamicin (10), and X-Gluc (80). Strains were grown at room temperature (RT) when on plates, and at 28˚C at 225 rpm when in liquid cultures except for Lux assay plates which were incubated at RT and at ~1100 rpm.

Transposon mutagenesis

Freezer stocks of electrocompetent DC3000 pAC1 were prepared as follows. Cells were grown overnight in 50 ml Kings B (KB) medium at 28˚C and 225 rpm. The overnight culture was diluted the next morning into 130 ml KB at starting OD600=0.1. This culture was grown to OD600=0.8, and cells were harvested by centrifugation at 6,000 x g for 5 minutes at RT in a Beckman Coulter Avanti J-E centrifuge. The supernatant was discarded, and cells were washed twice in an equal volume of 300 mM sucrose followed by two 10% glycerol washes with centrifugation as above. Cells were resuspended in 1/60th volume 10% glycerol after the final wash, and 100 *μ*l aliquots were apportioned into 0.6 ml microcentrifuge tubes. A 1 ml pipette box insert with sides removed was used to freeze cells in the -80˚C freezer. Cells were transferred to storage at -80˚C for future electroporations after they were completely frozen in the modified pipette rack. No changes in efficiency were observed as cells were stored over time.

Electroporations

Frozen, electrocompetent DC3000 pAC1 aliquots were thawed on ice for 8 minutes and allowed to equilibrate to RT for 8 minutes before electroporation. 1 μl EZ-Tn5 Kan^R transposome (Epicentre Biotechnologies) was used per electroporation. Warmed DC3000 pAC1 electrocompetent aliquot and transposome were mixed briefly by pipette and immediately electroporated (Voltage 2500, Capacitance μF 25, Resistance 200) in a 2 mm cuvette. 900 μl SOC or KB medium was added, and cells recovered for 2 hours at 28˚C and 225 rpm. Recovered cells were then diluted 4 fold (total 4 ml) with SOC or KB and 100 μl was plated on *hrp* minimal medium plates supplemented with 10 mM fructose, kanamycin (50 μg/ml), tetracycline (10 μg/ml), and X-Gluc (80 μg/ml).

Selection and mapping of transformants

Cells were grown on *hrp* minimal medium for 5-7 days at 28˚C to allow for color development. In general, ~1/1000 colonies showed a decrease in GUS expression compared to average expression levels. Gus-deficient colonies were patched onto the same *hrp* minimal medium as above, and cells from patches that continued to exhibit decreased GUS phenotype were archived in 20% glycerol at -80˚C.

Colony PCR was used to map insertions from stains with reduced reporter activity. A small sample of cells (~2 mm^2) was taken from the patched plates and resuspended in 50 μl ddH_2O. These were then boiled for 10 minutes at 99˚C in a thermocycler, placed on ice for 5 minutes, spun down for 5 minutes, and the supernatant was used as a DNA template.

Arbitrary PCR

Transposon insertions were mapped via arbitrary PCR adapted from a previously published method (Caetano-Anolles, 1993, Peters & Craig, 2000). Ex Taq (Takara) polymerase was used to amplify DNA for sequencing.

1st Round PCR. Arbitrary genomic primer oSWC00141 and oSWC02330 or oSWC001139 were used for 1st round PCR. 3 µl of boiled template was used in a 20 µl PCR reaction.

PCR1 – 10 µl Ex Taq, 1 µl oSWC00141, 1 µl oSWC02330 or oSWC001139, 3 µl DNA template, 5 µl ddH2O. Themocycler Protocol PCR1. 94˚ 4 min, (94˚ 30 sec, 42˚ 30 sec, 72˚ 3 minutes) 6 cycles, (94˚ 30 sec, 52˚ 30 sec, 72˚ 3 minutes) 25 cycles.

2nd Round PCR. Nested genomic primer oSWC00142 and transposon primer oSWC02332 or oSWC02331 were used for 2nd round PCR.

PCR2 – 10 µl Ex Taq, 1 µl oSWC00142, 1 µl oSWC02332 or oSWC02331, 1.5 µl 1st round PCR reaction, 6.5 µl ddH2O. Themocycler Protocol PCR2. 95˚ 5 min, (94˚ 30 sec, 45˚ 30 sec, 72˚ 3 minutes) 40 cycles.

ExoSAP-IT. 10 µl of the PCR product was cleaned with 4 µl ExoSAP-IT (USB) according to the manufacturer's instructions in a thermocycler. Sequencing was performed at Cornell University Life Sciences Core Laboratories Center. Sequencing Reaction: 14 µl ExoSAP-IT reaction, 1.5 µl Sequencing Primer oSWC02210 or oSWC02209, 2.5 µl ddH2O.

pKnockout mutagenesis

The *retS* pKnockout strain was created using previously described method (Windgassen *et al.*, 2000). pKnockout primers oSWC02724 and oSWC02725 were designed with BamHI (NEB) restriction sites. Ω pKO

plasmid was isolated from lab strain G1707 Ω. Ex Taq was used to amplify PCR templates from WT DC3000, and then plasmid and template were digested with BamHI according to manufacturer's instructions. Plasmid was treated with antarctic phosphatase (NEB) according to manufacturer's instructions. Digested PCR fragment and digested, phosphatase treated plasmid were cleaned with Qiaquick PCR purification kit (Qiagen). Cleaned PCR fragments and plasmid were then ligated for 10 minutes at RT with T4 DNA ligase (NEB) in a 3:1 ratio (insert:vector) according to manufacturer's instructions. 1 μl ligation reaction was then transformed into chemically competent One Shot Top Ten (Invitrogen) *E. coli* cells. Spectinomycin resistant colonies were isolated, verified for *retS* fragment via PCR, and grown overnight in 5 ml LB plus spectinomycin at 37˚C and 225 rpm. Plasmid preps were done early the following morning with QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. Frozen WT DC3000 electrocompentent cells were prepared as described above for WT DC3000 pAC1 cells. 1 μl plasmid (~100 ng) was used for electroporation reactions. Spectinomycin resistant DC3000 was verified for plasmid integration by PCR.

RecTE-mediated recombination

Electrocompetent freezer stock RecTE cells were prepared as WT pAC1 electrocompetant freezer stock cells described above, with some modifications. An overnight 50 ml KB culture with gentamicin was inoculated at OD600>0.5. The following morning, the culture was diluted to OD600=0.4 and grown to OD600=0.8 with 0.5x gentamicin in 120 ml KB. Cells were washed and frozen as described earlier in this study for WT pAC1 cells.

Mutant alleles from the Tn5 screen were transferred to a fresh WT background via RecTE-mediated recombination (Bryan Swingle, *in press*). Primers oSWC02729 and oSWC02729 were used to amplify the mutant allele with Ex Taq. The PCR product was cleaned with the QIAquick PCR purification Kit (Qiagen). 1.5 μg of cleaned PCR product (~1.5 μg total) was electroporated into WT RecTE electrocompetent freezer stock cells, recovered in KB for 4 hours, and plated on KB Kan selective medium.

Strains with the mutant allele were verified by PCR and cured of the RecTE plasmid by subculturing overnight without gentamicin. Plasmid curing was verified by replica patching single colonies onto KB and KB plus gentamicin and screening for gentamicin sensitivity.

Growth curves

Overnight cultures were established in 50 ml KB liquid medium. Strains were diluted in 50 ml KB liquid medium to OD600=0.15. All growth curve experiments were run in triplicate at 28˚C and with shaking at 225 rpm in a Barnstead Lab-Line MaxQ 5000 floor shaker. Multiple time points were taken at least half an hour apart during logarithmic growth (~OD600=0.2-0.9).

Motility assays

Swarming motility assays were conducted on traditional swimming medium (Rashid & Kornberg, 2000). The basic formulation is as follows: 10 g/l Bacto Tryptone (BD), 5 g/l NaCl (MPBio), and 0.3% agar (wt/vol) (USB). Overnight 5 ml KB cultures of WT DC3000 and mutants were diluted to OD600=0.3 in liquid KB medium, and 5 μl was spotted onto swimming medium and incubated at RT. Swarming was scored after 24-30 hours.

Traditional swarming medium was used for regular swarming assays (Rashid & Kornberg, 2000). The formulation is as follows: 8 g/l Nutrient Broth powder (BD), 0.5% agar (wt/vol) (USB). Cells were grown as for swimming assays, and 5 μl of inoculum at an OD600=0.3 was spotted onto the agar surface and incubated at RT for 24-30 hours.

Twitching assays were performed by increasing the swarming medium agar percentage to 1.0% (wt/vol), and inoculating 1 μl through a thinly poured layer of medium to the Petri dish-nutrient agar interface. Twitching assays were incubated for 3 days at RT.

Cloning PCR

Expand High Fidelity PCR System (Roche) was used for cloning reactions. The standard reaction follows manufacturer's instructions: 39.25 μl ddH_2O, 5 μl Roche 10x buffer with $MgCl_2$, 1 μl dNTPs, 1 μl DNA template (50 ng/ul), 1.5 μl primer #1, 1.5 μl primer #2, 0.75 μl Roche HF polymerase. The standard Roche HF polymerase thermocycler program is as follows: 94˚C 4 minutes, (94˚C 30 seconds, 55˚C 30 seconds, 72˚C 1 minutes) 30 cycles, 72˚C 4-7 minutes, 4˚C hold indefinitely. This can be adjusted based on primer melting temperatures and length of fragment to be amplified.

Lux assays

Lux assays were conducted in black, clear bottom 96-well assay plates (BD) with lux activity and cell density measured using a Tecan GENios Pro with Magellan 6.3 software (Method_Multilabel_Plate_Def.mth method on machine). Strains were grown overnight in 50 ml KB plus tetracycline. The

following morning, 10ml of culture was washed twice in an equal volume of *hrp* minimal wash (*hrp* minimal medium without a carbon source). The final resuspension was usually concentrated 2-3x. The concentrated inoculum was then diluted into 1 ml *hrp* minimal wash at OD600=10.0. 20 µl of this was added to 180 µl medium supplemented with tetracycline to maintain the reporter plasmid. Reporter activity was monitored over 12 hours, but the most contrasting data between strains was usually obtained at 10 hours post inoculation (hpi).

RNA Work

RNA extractions were accomplished using the RNeasy Mini Kit (Qiagen). The protocol is essentially the same as the RNeasy Mini protocol for isolation of total RNA from bacteria with an on-column DNase (Qiagen) digestion and two 30-minute DNase I (Ambion) treatments before final RNA cleanup. qRT-PCR was performed using iQ SYBR Green Supermix (Bio Rad). Plates were read on an iQ5 multicolor real-time PCR detection system with Bio Rad iQ5 optical system software 2.0. All qRT-PCR reactions were run with three technical replicates per strain and condition. *gyrA* was routinely used as the housekeeping gene. The following formula was used for calculating fold changes using cycle threshold (CT) values:

2^-ΔΔCT = 2^-((Target Gene 1 – Housekeeping 1) – (Target Gene 2 – Housekeeping 2))

qScript (Quanta Biosciences) cDNA supermix. 4ul qScript supermix, 1 µl RNA (100 ng/µl), 15 µl RNase free H_2O. Cycler Program. 25˚C 5min, 42˚C 30min, 85˚C 5min, 4˚C hold

Primer check. 10 μl iQ SYBR Green Supermix (Bio Rad), 5 μl H2O, 4 μl Primers (mixed and at 2 pmol/μl each), 1 μl template.

qRT-PCR primer dilutions. Primers were diluted to 100 mM as usual but with nuclease free water. 8 μl of each primer (8 + 8) plus 384 μl nuclease free water yielded 2 pM/μl of each primer in the final reaction.

RESULTS

Transposon mutagenesis identifies components of T3SS regulon

Transposon mutagenesis proved to be a powerful technique to identify T3SS-inducing genes. The EZ-Tn5 transposon is 1221 base pairs long and flanked by 19 nucleotide mosaic end sequences that are recognized by transposase. The EZ-Tn5 transposon can either disrupt the expression of a gene, or in some cases promote the expression of a gene. The transposon is driven by two strong nptII promoters and has the potential for read-though induced gene expression. This should be taken into consideration when analyzing the mutagenesis results. The total number of insertions screened was ~120,000 from four independent electroporation reactions. 120 total colonies showed reproducibly reduced reporter activity on *hrp* minimal medium after patching. Figure 4 shows the workflow of mutant isolation .

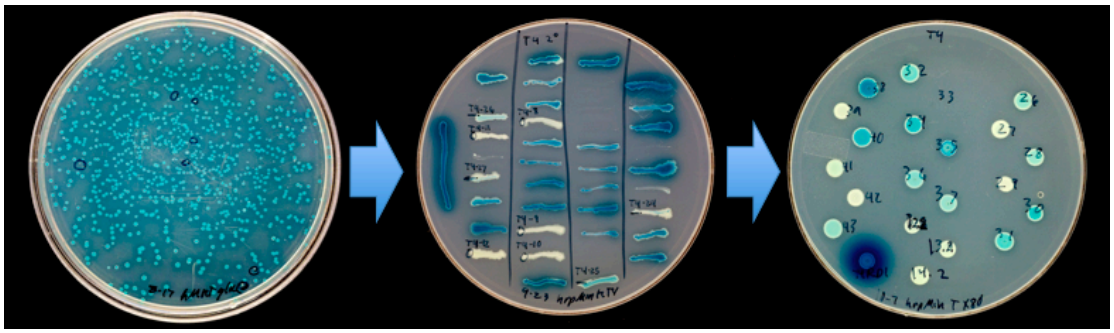

Figure 4. Transposon insertion mutant isolation and phenotyping. Far left, isolation of single colonies after electroporation. Middle, secondary patch to confirm colony phenotype. Far right, colony plating for visually comparing reduced reporter activity.

Our methodology eliminated auxotrophs that were prevalent in a previous study (Deng et al., 2009). This was advantageous because the focus of this investigation was to discover regulatory factors acting upstream of HrpL

while avoiding metabolic mutants that affected T3SS. Only mutants that showed an obvious deviation from average blueness after patching were subjected to further analysis. There is a formal possibility that a colony arises which has more than one transposon insertion in the same clonal population, or that two independent insertion mutants grew closely enough to be mistaken for a single colony. These situations would most likely provide overlapping sequencing reactions and be unmappable using our strategy. A high percentage of isolated colonies (86%) were successfully mapped suggesting that the transposon mutagenesis reaction worked as anticipated in the majority of cases.

Mutants that met stringent screening criteria were sequenced, and some were further characterized. 104 out of 120 (86%) colonies selected from the screen were successfully mapped to the DC3000 chromosome (or reporter plasmid) and are briefly described in Table 2 (for an expanded list see Appendix A). These isolates showed reproducibly reduced reporter activity when patched onto *hrp* minimal medium. We also attempted to minimize sibs from arising by shortening the post-electroporation recovery time to 2 hours. This is too short of a time for DC3000 to complete multiple rounds of replication.

Colonies were grown on *hrp* minimal medium supplemented with fructose, kanamycin, tetracycline, and X-Gluc for five days to allow for color development. We developed four categories for scoring colony phenotypes. The vast majority of colonies showed an identical blueness. These colonies were characterized as “average” blue and ignored. The other three categories are as follows: white, no blue color; pale, below average blue color; and dark blue, exceptionally dark blue color.

Table 2. Insertions with reduced reporter activity.

Count	Unique	PSPTO_	Name	Pheno	Note
25	23	PSPTO_2222	rhpS	White	Sensor histidine kinase
17	17	PSPTO_4868	retS	Pale	Sensor histidine kinase/response regulator RetS
15	15	Vector - Gus		White	
11	8	PSPTO_3576	tvrR	Pale	TetR-like virulence regulator
9	9	PSPTO_1380	hrpS	White	Type III transcriptional regulator HrpS
3	3	PSPTO_0362		Pale	DeoR family transcriptional regulator
2	1	PSPTO_1379	hrpR	Pale	Type III transcriptional regulator HrpR
2	2	PSPTO_0080	pyrE	White	Orotate phosphoribosyltransferase
2	2	PSPTO_1404	hrpL	White	RNA polymerase sigma factor HrpL
2	2	PSPTO_3836		Pale	Hypothetical protein, conserved in Pseudomonads
1	1	intergenic		Pale	Intergenic PSPTO_4434 PSPTO_4433
1	1	PSPT0_4943	miaA	Pale	tRNA modification, tRNA delta(2)-isopentenylpyrophosphate
1	1	PSPTO_0211	iucD	White	L-lysine 6-monooxygenase
1	1	intergenic		Pale	Intergenic shcF T3SS chaperone PSPTO_0503
1	1	PSPTO_0963	pcnB	White	PcnB is required for the rapid degradation of RNA
1	1	PSPTO_1751	ihfB	White	Integration host factor subunit Beta
1	1	PSPTO_2362	map-2	White	Methionine aminopeptidase, type I
1	1	PSPTO_2621	polB	White	DNA polymerase II
1	1	PSPTO_2663		Pale	LysR family transcriptional regulator
1	1	PSPTO_2705		Pale	Mannitol ABC transporter, permease protein
1	1	PSPTO_3369	nuoF	White	NADH dehydrogenase I subunit F
1	1	PSPTO_4505	dnaK	White	Molecular chaperone DnaK
1	1	PSPTO_4735	hrpB	White	ATP-dependent helicase HrpB
1	1	PSPTO_4817	hopAJ2	White	Lytic murein transglycosylase
1	1	PSPTO_5341		Pale	Involved in the assembly of outer membrane proteins in *E. coli*
1	1	PSPTO_5502		White	Hypothetical protein, conserved in Pseudomonads

Count is the total number of times that a transposon insertion was mapped to the gene listed. Unique is the number of putative independent insertion events. Insertion events were conservatively considered to be unique if the insertion location was greater than 10 base pairs from all other insertion locations from a single electroporation reaction. Gene names are provided in the table if annotated. Phenotype is the reporter activity level of the secondary patch. Note provides additional information for each gene.

Table 2 shows that this screen successfully identified many known components of DC3000 T3SS regulation including *hrpR*, *hrpS*, *hrpL* (Brencic & Winans, 2005), *rhpS* (Xiao et al., 2007), and *tvrR* (Preiter *et al.*, 2005). There

are also hits homologous to pathogenesis-related or host-responsive genes from other species including *ihfB* (Stonehouse *et al.*, 2008), *iucD* (Boch et al., 2002), and *nuoF* (Hernandez-Morales *et al.*, 2009). It was surprising to us that we identified insertions in the reporter plasmid itself. This suggests that the reporter plasmid may be present in low copy numbers. It could also be explained as a rare event that arises due to observer bias (white colonies stand out on a plate of average blue colonies). There are also a large number of genes with a single insertion event recorded. Multiple independent hits into a gene build confidence that the gene is involved in T3SS under the conditions tested, and those with lower counts necessarily require increased confirmation to solidify the gene-phenotype relationship. The gene-phenotype relationship should be further confirmed by various approaches including an independently generated mutant strain.

An interesting finding is the large number of insertions in *retS* (PSPTO_4868). RetS has not been reported to play a role in DC3000 T3SS regulation. RetS does have characterized homologs in both *P. aeruginosa* and B728a that have been implicated in activation of the T3SS (Goodman et al., 2004, Records & Gross, 2010). This gene was therefore selected for further characterization in DC3000.

Darker than average colonies also obtained in screen

A small number of transposon mutants displayed a dark blue phenotype. These colonies were easily distinguished from the average blue colonies. Often, the dark blue colonies were smaller and seemed to hypersecrete blue pigment into the medium, forming a blue halo. These dark

blue colonies were isolated and 29 were successfully mapped. Table 3 shows the results for mapped dark blue insertion mutants.

Table 3. Insertions with increased reporter activity.

Count	Gene	Name	Notes
6	PSPTO_3724	lon-1	ATP-dependent protease Lon-1
4	PSPTO_0829	clpB	ATP-dependent chaperone
4	PSPTO_3353	clpA	ATP-dependent Clp protease
2	PSPTO_3024	gacA	Master regulator gacA
2	PSPTO_1691	gacS	Master regulator gacS
1	PSPTO_4897	cbpA	Curved-DNA-binding protein
1	PSPTO_5141	hslU	Heat shock protein, degradation of misfolded proteins
1	PSPTO_5308		Putative aminotransferase
1	PSPTO_A0024		No conserved domains
1	PSPTO_2712		Sensor histidine kinase/response regulator
1	PSPTO_3127	malQ	4-alpha-glucanotransferase, glycogen synthase operon
1	PSPTO_0606	anmK	Murein recycling
1	PSPTO_1038		Conserved hypothetical
1	PSPTO_1067		Glycosyl transferase, group 2 family protein
1	PSPTO_2299	oprF	Outer membrane porin OprF
1	PSPTO_4175		Sensor histidine kinase

Count refers to the number of colonies with an insertion in a gene. The PSPTO and gene names are provided with a note about putative function.

It is also important to note that some of the dark blue colonies also had insertions in the reporter plasmid itself. In these cases, the transposon integrated into the pAC1 reporter plasmid very near the 5' end of the *iucD* reporter gene. Theoretically, this result could occur if the transposon inserted into the correct orientation to the *iucD* gene for significant read-through from

the transposon's strong promoters. This situation would potentially produce an *iucD* overexpression strain. The possibility that promoters internal to the transposon are driving expression outside of the insertion should be taken into consideration when analyzing the data from this screen.

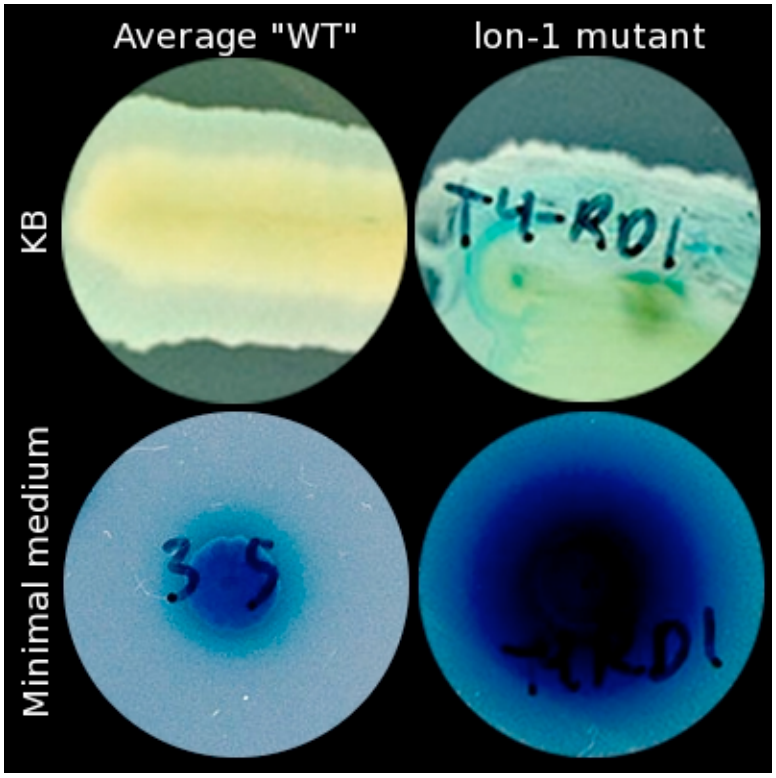

Figure 5. *lon*-1 mutant on rich and minimal media.
WT and the Δ*lon*-1 mutant were grown on KB rich or *hrp* minimal media each supplemented with X-Gluc for 5 days to allow for color development.

Lon-1 is known to repress the T3SS especially in rich medium. A *lon*-1 mutant shows increased HrpR stability and hypersecretion of effector proteins (Bretz et al., 2002). Lon-1 could also be active on *hrp* minimal medium, so that a *lon*-1 mutant displays the dark blue phenotypes observed. A few *lon*-1 insertion mutants were tested on KB rich medium with antibiotics and X-Gluc.

These colonies continued to show reporter activity while WT showed none. This is represented in Figure 5.

Many other mutants in Table 3 are chaperones and proteases. Proteases have been shown to play a role in pathogenicity as described for Lon-1 above, and chaperones can protect effectors from degradation (Losada & Hutcheson, 2005).

Two insertion mutants listed in Table 3 have an unexpected phenotype. These are insertions in *gacS* and *gacA*. The insertion locations in *gacS* map ~1.4kb apart and are therefore not siblings. One *gacA* insertion has only 26 base pairs of sequence overlap with the DC3000 chromosome, which is uniquely mappable, but the sequencing reaction should be repeated to verify the insertion with a much longer read. The second *gacA* insertion is 21 bases from the annotated start site and is in the correct orientation to drive expression of *gacA* from the transposon's strong promoters. A *gacA* mutant in *P. aeruginosa* shows increased T3SS activity in inducing conditions versus wild type (Brencic et al., 2009). This phenotype is the due to the reciprocal regulation of biofilm/chronic infection (*gacS*/*A* activation via LadS) and T3SS induction/acute infection (GacS/A inhibition via RetS) (Goodman et al., 2004, Brencic et al., 2009). In contrast, a DC3000 *gacA* mutant was reported to have decreased *hrpL* abundance, a swarming defect, and also a HR defect in tobacco (Chatterjee et al., 2003). The *gacA* mutant from our screen showed the same reduction in swarming and a similar HR defect (at the same inoculum concentration) as previously reported (data not shown). The discrepancy between high reporter activity and reduced HR for our *gacA* mutant, and the differences between *gacA* and T3SS in *P. aeruginosa* versus

DC3000 cannot be explained at present. It would be interesting to further investigate the mechanisms behind these conflicting responses.

DC3000 RetS domain architecture

RetS has a unique domain architecture. It has two receiver domains as shown in Figure 6 (Marchler-Bauer & Bryant, 2004, Marchler-Bauer *et al.*, 2009).

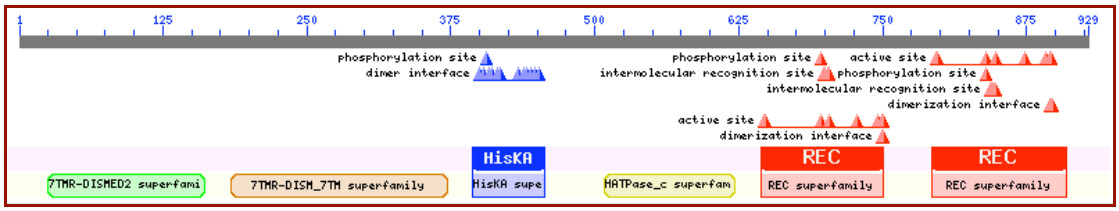

Figure 6. DC3000 RetS domains.
The domains most relevant to this study are the putative carbohydrate binding domain in the N-terminal region, and the REC domains with conserved, putative phosphorylation sites.

The N-terminal region has a predicted periplasmic carbohydrate binding domain. The ligand for the putative carbohydrate binding domain is not currently known, but it, and most of the following seven transmembrane-spanning domain, is not required for T3SS activation in *P. aeruginosa* (Laskowski & Kazmierczak, 2006). The dual receiver domains make this hybrid TCS protein unique. Each has a putative, conserved aspartic acid residue that may be used for phosphorylation relay signaling events.

Δ*retS in vitro* growth curve

It is important to characterize any mutant for *in vitro* growth rates to identify any substantial growth defects. The Δ*retS* mutant growth curve is

shown in Figure 7. The growth rate defect is not specific to the Δ*retS* mutant as the original transposon insertion mutant and Δ*retS*-RecTE also showed similar, slight growth defects when compared to WT DC3000 growing in rich medium (data not shown).

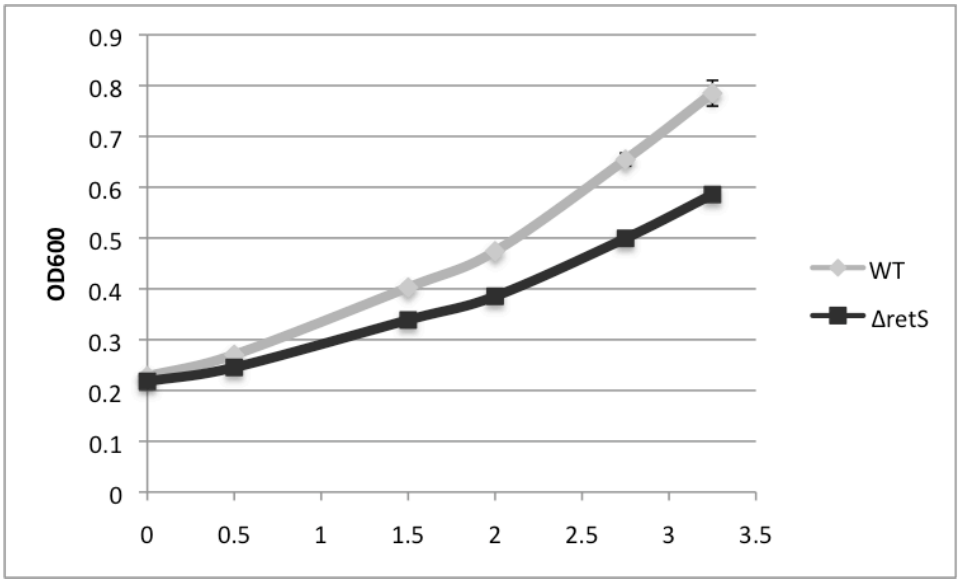

Figure 7. Δ*retS* and WT DC3000 growth rates.
Representative graph of exponential growth phase is shown for three technical replicates of WT and Δ*retS*. Liquid cultures were diluted to OD600=0.15 in 50 ml KB rich medium and grown at 225 rpm and at 28˚C. Error bars are shown.

This growth defect was not anticipated because a mutation in a membrane sensor is not predicted to influence growth. The moderate growth rate decrease for Δ*retS* may be related to the slightly smaller colonies observed when growing on solid medium plates. The growth rate data at 1-3 hours post inoculation (hpi) shows that Δ*retS* grows at ~66% of the WT rate in exponential growth phase. *In vitro* growth rate data has not been reported for the B728a *retS* mutant (Records & Gross, 2010), and *retS* in *P. aeruginosa* was only reported as non-essential in both rich and *hrp* minimal media

(Goodman et al., 2004). The finding that a *retS* mutant shows an *in vitro* growth rate defect makes it challenging to use the DC3000 *retS* mutant for *in planta* growth rates, as the mutant may show a decrease in growth. This could masquerade as a plant-microbe interaction effect.

Δ*retS* hyperswarming phenotype in medium with NaCl

RetS is reported to have an effect on motility (Hsu et al., 2008, Records & Gross, 2010). We used the Δ*retS*-RecTE mutant to investigate swarming motility on traditional NB-based swarming medium as shown in Figure 8. Δ*retS*-RecTE swarms similarly to WT on this medium.

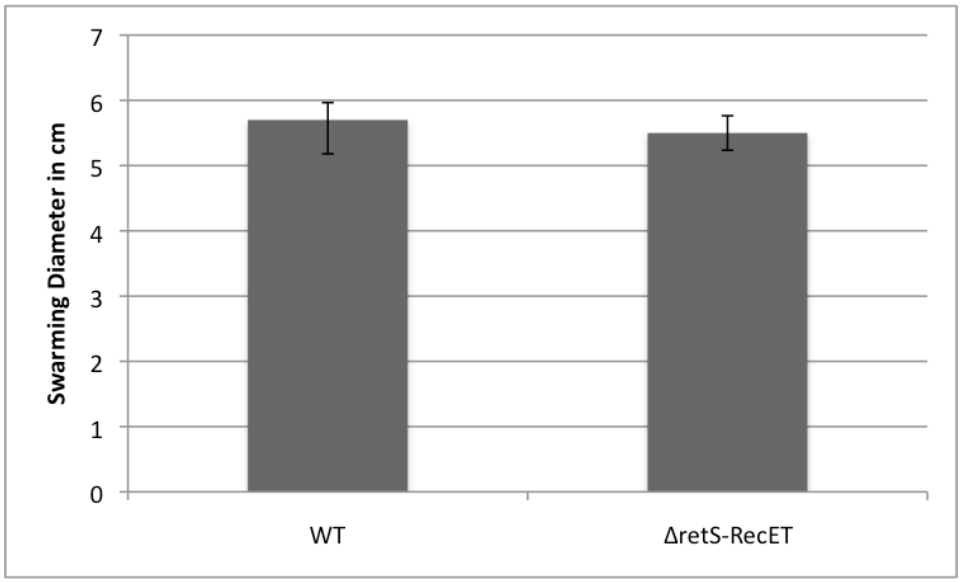

Figure 8. WT and Δ*retS*-RecTE swarming.
Swarming on NB medium solidified with 0.5% agar. Plates were inoculated with 5 μl of culture at OD600=0.3. WT and ΔretS-RecTE mutant show similar swarming activity after 24 hours. A representative data set from three technical replicates is shown.

We next investigated swimming motility with Δ*retS* on traditional tryptone-based swimming medium (Rashid & Kornberg, 2000). It if difficult to

report whether Δ*retS* shows reduced swimming motility, because the mutant displays a hyperswarming phenotype on swimming medium (Figure 9). The WT strain will swim through the medium when stab inoculated into swimming medium. In contrast, Δ*retS* and Δ*retS*-RecTE exhibit a hyperswarming phenotype without swimming much, if at all, through the medium. Note that the dendritic motility pattern is most easily obtained on medium with a high amount of surface water. Drying the plates for 15 minutes in the hood after pouring will result in more uniform motility that is easier to quantify as shown in Figure 10. The assessment of motility is not affected.

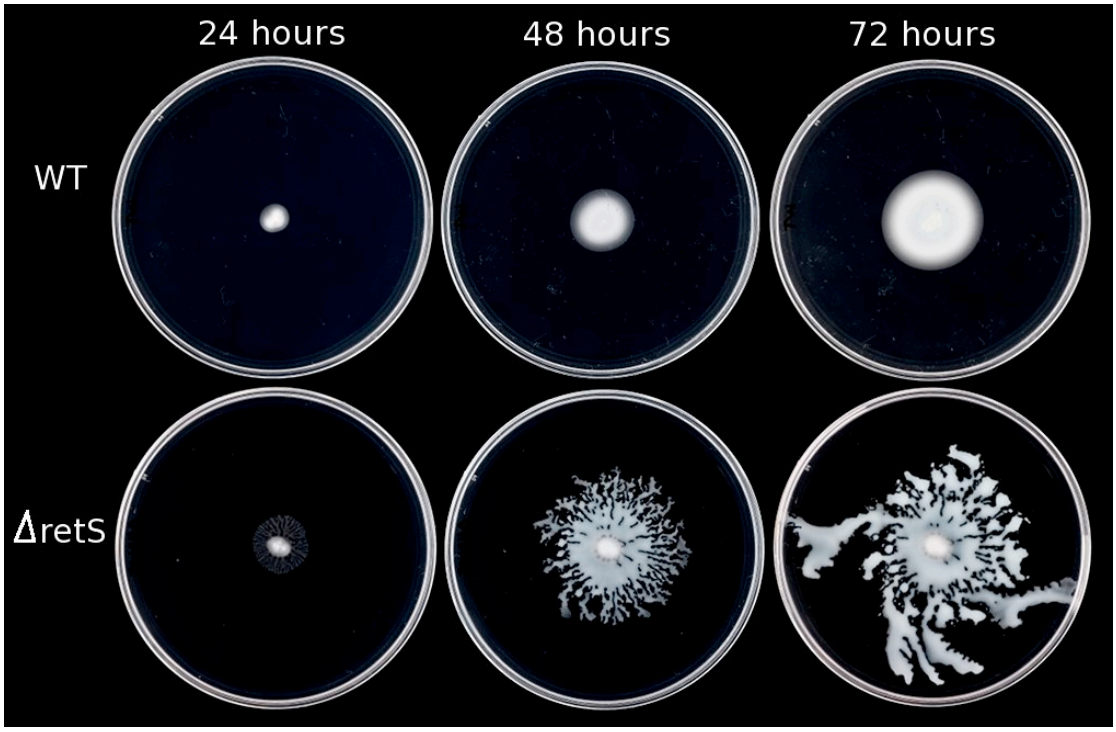

Figure 9. WT and Δ*retS* swarming on swimming medium.
Plates were inoculated with 1 μl of culture at OD600=0.3 into the agar, and incubated at RT for 72 hours. Plates were not dried prior to inoculation which produces the dendritic pattern. Representative plates are shown from three technical replicates.

The Δ*retS* swarming on swimming medium phenotype was unique to all the transposon insertion mutant strains examined (~10 strains). As a result, the swimming assay was altered to detect swarming on swimming medium by inoculating onto the surface of the agar instead of into the agar (this prevents WT DC3000 from swimming through the agar). Factors involved in motility were revealed in more detail when NaCl was excluded from the medium. Figure 10 shows the RetS-dependent on/off swarming motility response with and without NaCl (0.086 mM NaCl is in the standard swimming medium).

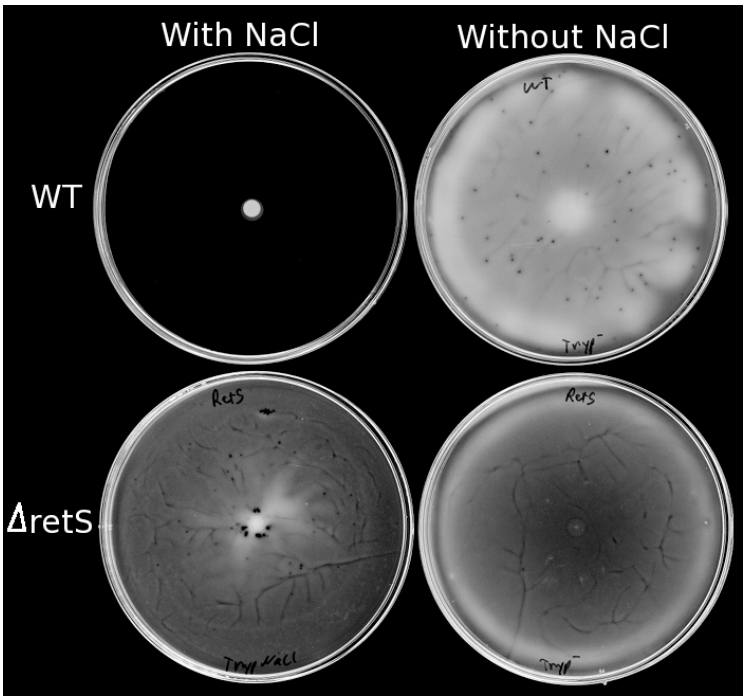

Figure 10. WT and Δ*retS* swarming with and without NaCl.
Swarming either with or without 0.086 mM NaCl on tryptone-based medium. 5 μl OD600=0.3 inoculated onto plates and incubated for 48 hours at RT. Representative photos from three independent experiments are shown.

The white haze on the plates results from bacteria covering the entire plate, having spread from the center where the plates were inoculated. This is

a typical 48-hour response. The pictures in Figure 10 were taken after quantitative data collection at 24-30 hpi, but illustrate how WT DC3000 swarming is repressed on medium supplemented with 0.086 mM NaCl and that the Δ*retS* mutant displays a hyperswarming phenotype under the same conditions.

We investigated this response further to see if osmotic stress was responsible for the phenotypes observed. Polyethylene glycol MW 200 (PEG200) is membrane permeable and commonly used to produce osmotic stress as a NaCl substitute. Using both NaCl and PEG200 supplements can show that a phenotype is osmotic stress related and not Na^+ or Cl^- ion specific. Both WT DC3000 and Δ*retS* were inoculated onto tryptone-based swarming medium with NaCl or PEG200 at -0.25 MPa, NaCl or PEG200 at -0.5 MPa, or without supplements. Figure 11 shows the results from a representative sample of these experiments.

Figure 12 similarly contains both NaCl and PEG200 supplemented medium at -0.25 MPa, and also included the Δ*retS* Comp strain (*retS* complemented on a plasmid with the nptII promoter). The results can be also be seen in the pictures in Figure 13 taken at 48 hpi.

Both the swarming diameter data at 24 hpi and the pictures at 48 hpi suggest that RetS elicits a general response to NaCl and PEG200 supplemented medium with repressed swarming as one output. WT DC3000 can swarm on PEG200 farther than on NaCl at the same MPa stress level, though it is greatly reduced. WT DC3000 was never observed to swarm on NaCl supplemented medium even after 7-10 days of incubation. The PEG200 phenotype is not as obvious as the NaCl phenotype, but this data suggests that RetS is part of the signaling pathway for osmotic stress.

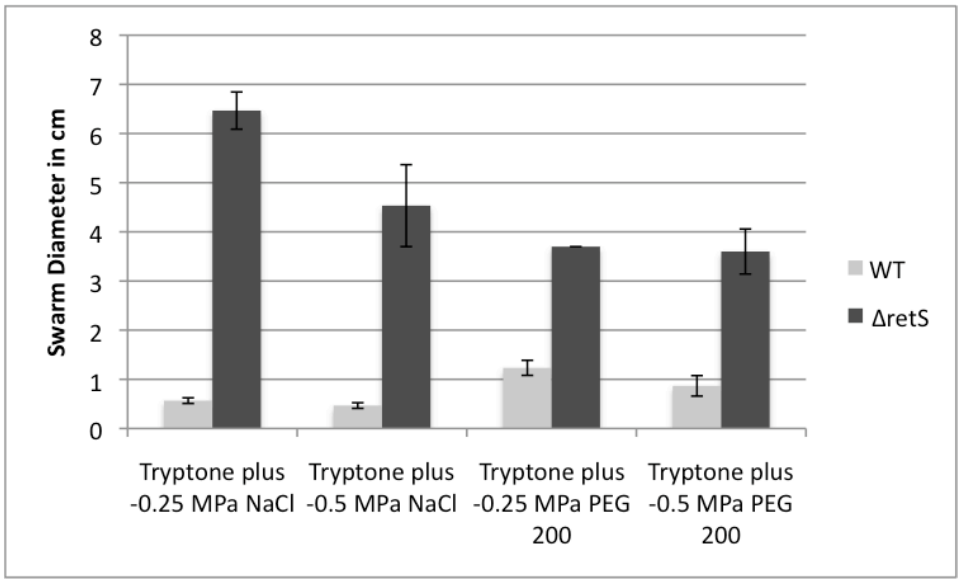

Figure 11. Swarming with NaCl and PEG200 supplements.
WT and Δ*retS* mutant strains were inoculated with 5 *µ*l of culture at OD600=0.3 on tryptone-based swarming medium and incubated at RT for 24 hours. Representative data from three technical replicates are shown.

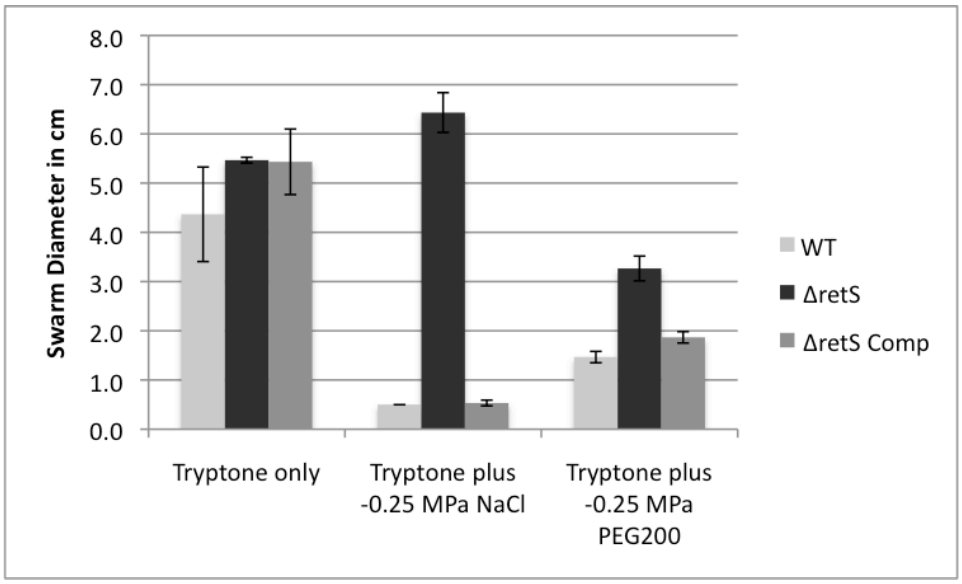

Figure 12. WT, ΔretS, and ΔretS complemented swarming.
WT, Δ*retS*, and ΔretS Comp strains were inoculated with 5 *µ*l of culture at OD600=0.3 on tryptone-based swarming medium and incubated at RT for 24 hours. Representative data from three technical replicates are shown.

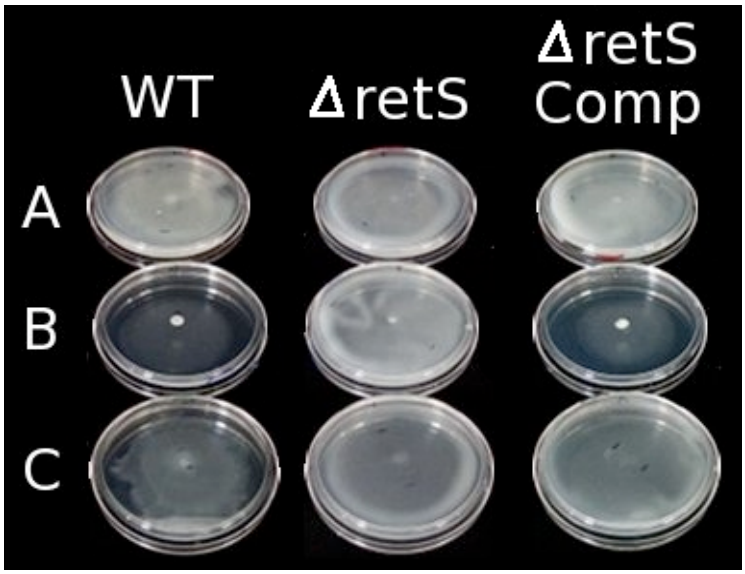

Figure 13. Pictures of swarming with and without NaCl and PEG200. 5 μl of WT, Δ*retS*, and Δ*retS* Comp strains at OD600=0.3 were incubated on tryptone-based swarming medium for 48 hours at RT with the following supplements: A, none; B, -0.25 MPa NaCl; C, -0.25 MPa PEG 200.

There was no twitching defect for Δ*retS* when compared to WT DC3000 (data not shown) similarly to the B728a *retS* mutant (Records & Gross, 2010). This contrasts with the twitching defect reported for the *P. aeruginosa retS* mutant (Zolfaghar et al., 2005).

Monitoring T3SS activity in Δ*retS*

WT DC3000 and Δ*retS* were transformed with pBS45 to monitor HrpL activity in response to various supplements in different media. pBS45 contains the avrPtoB promoter positioned 5' to the LuxCDABE operon (pavrPtoB::LuxCDABE). This construct allows for simplified monitoring of reporter activity over an extended period of time.

Our first experiments investigated reporter activity in liquid medium similar to the hyperswarming assays. Figure 14 shows Lux data at 10 hpi in

hrp minimal medium (supplemented with 10 mM fructose) and tryptone-based medium both with and without NaCl or PEG200.

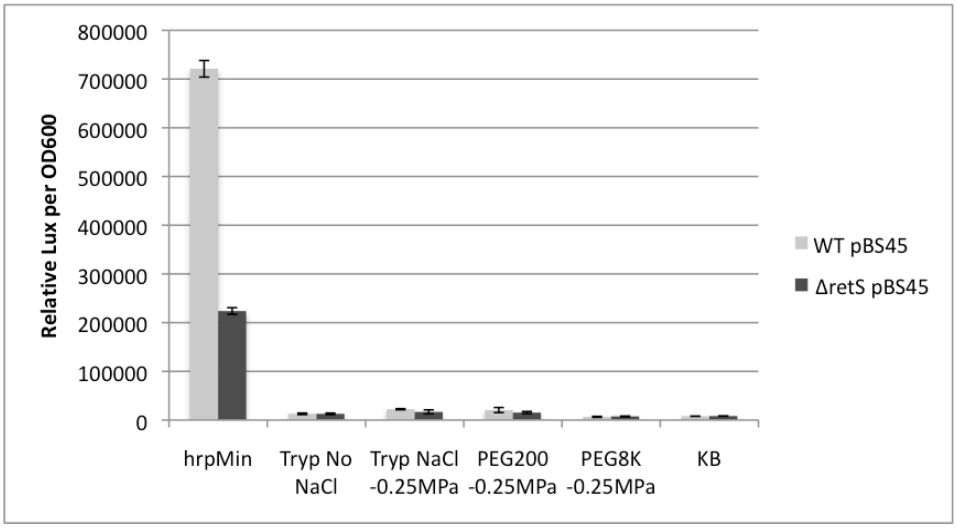

Figure 14. Lux reporter activity with NaCl, PEG200, or PEG8000. Liquid medium cultures were inoculated to OD600=1.0 and incubated at RT with shaking at 1100 rpm. Lux activity was recorded at 10 hpi. Results are shown for three technical replicates.

The first condition in this graph shows again that a mutation in *retS* prevents a WT level of T3SS activity as measured by a different reporter construct, but in similar inducing conditions (this is the same minimal medium formulation from the original screen). The other five conditions illustrate the RetS-independent suppression of T3SS activity in nutritionally rich medium that is also not dependent on NaCl or PEG supplements. We cannot conclude that osmotic stress from NaCl or PEG plays no role in induction of the T3SS in DC3000, but nutritionally rich medium is indeed a very potent inhibitor of T3SS which would mask any T3SS inducing effect. Minimal medium could be more suitable for future osmotic stress assays.

The next series of experiments on this theme investigated the relationship between carbon source and induction of the T3SS, to determine if the responses are RetS-dependent. Prior work demonstrated the effect of carbon sources on T3SS activity in *P. syringae* pv. *glycinea* and connected the results to how sugars feed into the TCA cycle (Huynh et al., 1989) (for relevant data from this paper see Appendix B). We set up an experiment in DC3000 investigating T3SS induction with different carbon sources in *hrp* minimal medium. The results of this experiment are shown in Figure 15.

Nutritionally rich medium (KB) represses reporter activity. Citrate, GABA, L-glutamate, succinate, and sucrose carbon sources all show similar effects comparing WT and the Δ*retS* mutant. These results concur with the T3SS induction findings of a previous study with *P. syringae* pv. *glycinea* (Huynh et al., 1989). The two most interesting carbon sources from our experiments are fructose and mannitol. Our data suggests that RetS is linked to an ~3 fold increase in reporter activity only in the presence of fructose and mannitol hinting at a possible mechanism. This RetS-dependent phenotype is constant over many hours as shown in Figure 16 for fructose, Figure 17 for mannitol, and Figure 18 for sucrose (as a control).

We noted that, in general, reporter activity increased over time even when normalizing for cell density. This was not surprising as it has been hypothesized that nutrient starvation can lead to the induction of the T3SS (Deng et al., 2009). Also, the half-life of the LuxCDABE proteins in DC3000 is not known and may accumulate over time. These experiments provide evidence that RetS plays a role in T3SS induction, with fructose and mannitol as specific elicitors, after a relatively short period of time in inducing medium.

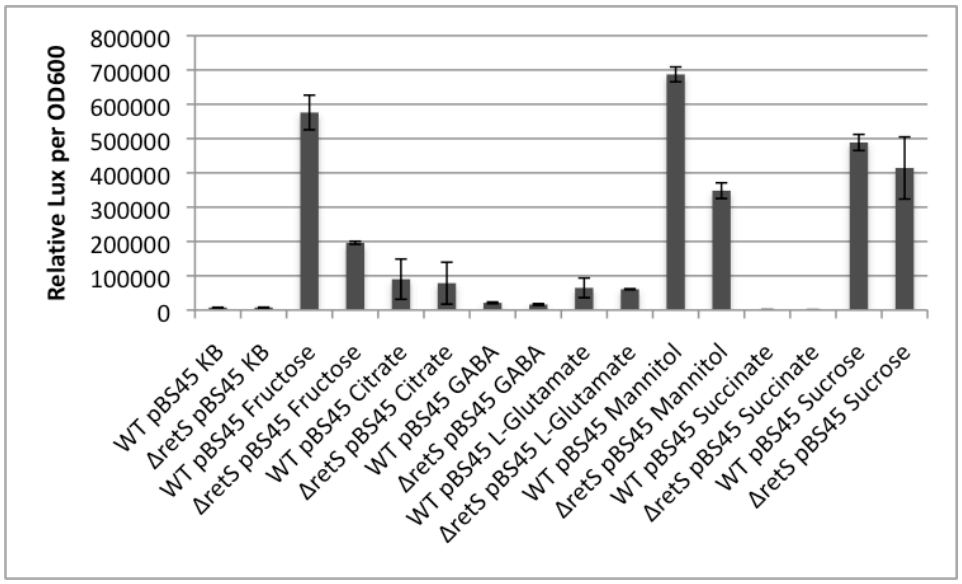

Figure 15. Carbohydrate effects on Lux reporter activity.
WT pBS45 and Δ*retS* pBS45 were used to measure carbohydrate effects on T3SS activity. KB medium was used as a negative control. All other media are based on *hrp* minimal medium with the various sole carbon sources listed. Results are reported for three technical replicates.

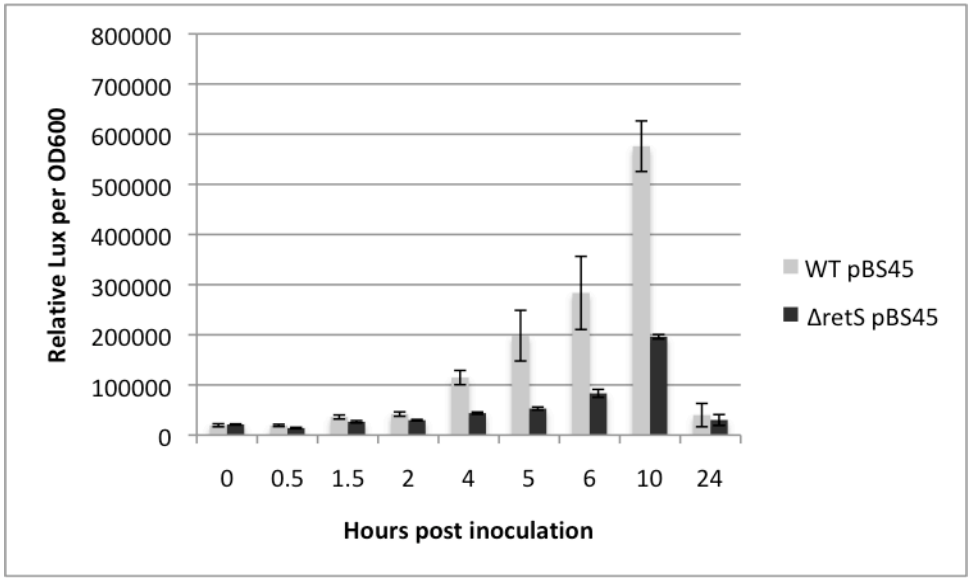

Figure 16. Lux reporter activity with fructose as a function of time.
200 µl cultures were started in *hrp* minimal medium with fructose at starting OD600=1.0 and incubated at RT with shaking at 1100 rpm. Results are shown for three technical replicates with time points indicated over 24 hours.

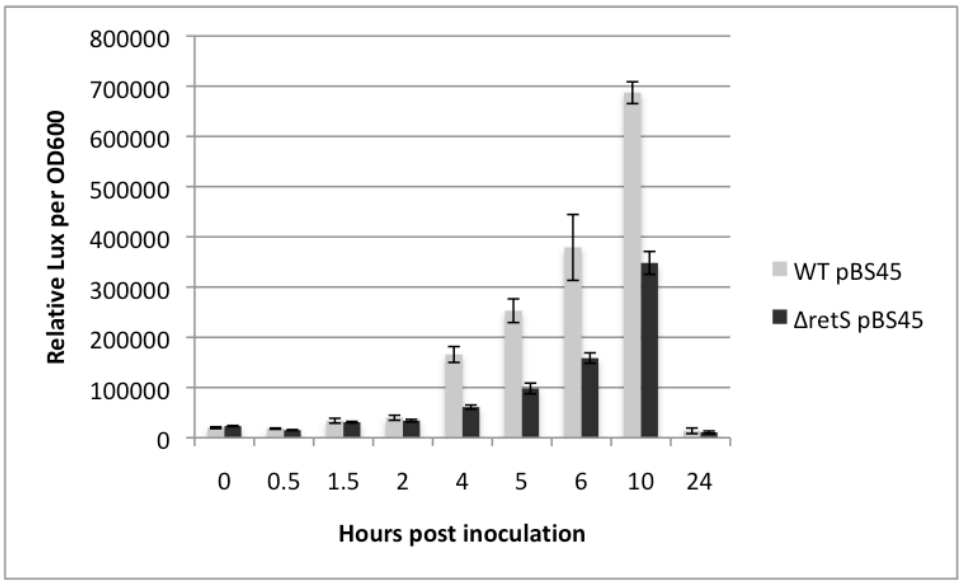

Figure 17. Lux reporter activity with mannitol as a function of time. 200 µl cultures were started in *hrp* minimal medium with mannitol at starting OD600=1.0 and incubated at RT with shaking at 1100 rpm. Results are shown for three technical replicates with time points indicated over 24 hours.

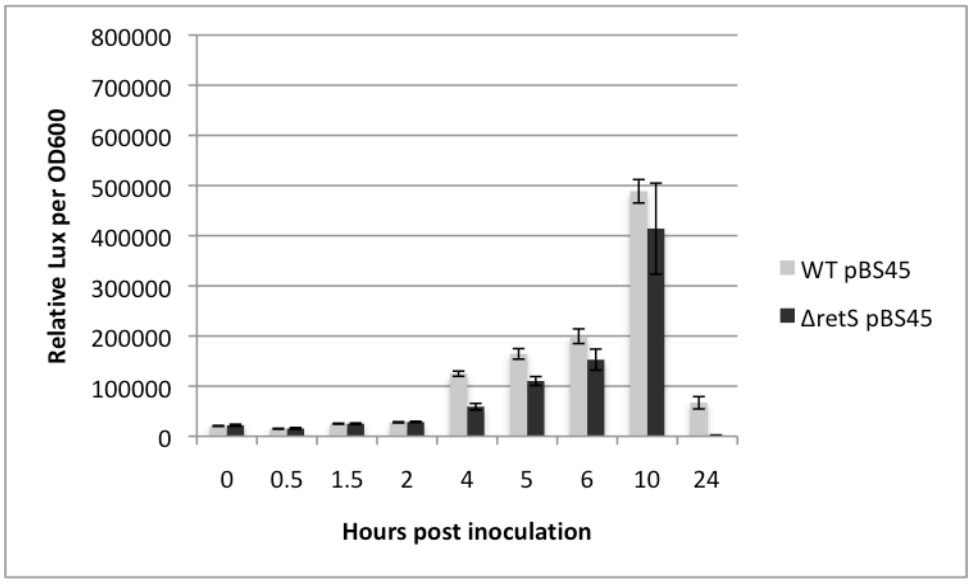

Figure 18. Lux reporter activity with sucrose as a function of time. 200 µl cultures were started in *hrp* minimal medium with sucrose at starting OD600=1.0 and incubated at RT with shaking at 1100 rpm. Results are shown for three technical replicates with time points indicated over 24 hours.

The swarming experiments above show that RetS is involved in the hyperswarming phenotype with NaCl and PEG200 supplements. We thought that it would be interesting to investigate the role of NaCl and PEG200 in minimal medium to look for additive or RetS-dependent activation of T3SS. The results of this experiment are shown in Figure 19.

Once again, reporter activity is repressed in nutritionally rich medium, and the Δ*retS* mutant shows a reduction in reporter activity in both fructose and mannitol, and not in sucrose or L-glutamate. Otherwise, sucrose represses reporter activity in both WT and Δ*retS* to a similar proportion, and PEG200 decreases reporter activity in a similar pattern to sucrose yet more intensely. No obvious RetS-dependent phenotype was observed under these conditions.

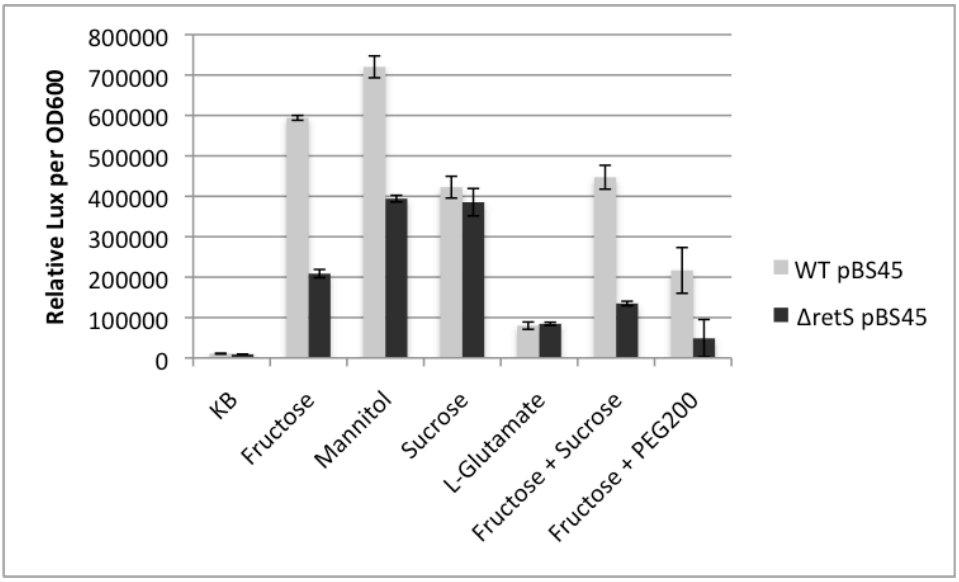

Figure 19. Lux reporter activity in mixed media.
WT pBS45 and Δ*retS* pBS45 were used to measure mixed carbohydrate effects on T3SS activity in 200 μl cultures. KB medium was used as a negative control. All other media are based on *hrp* minimal medium with the supplements listed. Results are reported for three technical replicates.

Δ*retS* qRT-PCR

Quantitative real time PCR (qRT-PCR) was used to verify reporter activity (see Appendix C) and investigate T3SS pathway intermediates in the RetS-dependent signaling cascade. Cells were prepared in the same *hrp* minimal medium used in the screen. The Δ*retS* mutant strain was compared with WT for T3SS-related gene expression. qRT-PCR targets included *hrpL*, *hrpJ*, and *rsmZ*. The WT strain has an almost 23-fold increase in *hrpL* and a 10-fold increase in *hrpJ* transcript levels when compared to the Δ*retS* mutant after 4 hours. This data confirms that Δ*retS* is unable to fully activate the T3SS in inducing conditions when compared to WT, and suggests an analogous mechanism to the *P. aeruginosa* model.

In *P. aeruginosa*, RetS works through the GacS/A regulatory pathway that includes small regulatory molecules including *rsmZ*. The RetS model in *P. aeruginosa* shows that high *rsmZ* levels titrate "free" RsmA and lead to the expression of biofilm related genes. RetS is an antagonist to this pathway by reducing the level of RsmZ, leaving RsmA to inhibit biofilm-related gene expression. *rsmZ* was therefore selected as a qRT-PCR target in DC3000. Δ*retS* had a 31-fold increase in *rsmZ* transcript levels compared to WT in inducing conditions after 4 hours. This suggests that in DC3000, like in *P. aeruginosa*, RetS activates T3SS by reducing *rsmZ* transcripts.

The Δ*retS* hypersensitive response

The hypersensitive response (HR) assay tests for a functional T3SS. These experiments use a 2-fold dilution series to investigate subtle defects in secretion machinery. The highest concentration of cells (injected into the top of the leaf) is 2×10^7 cfu, and the lowest is 0.25×10^6 cfu (at the lowest

position on the leaf). WT DC3000 is injected into the left side of a fully expanded tobacco leaf, and the mutant under investigation is injected on the right side of the leaf. The HR results from the Δ*hrpR*, Δ*hrpL*, and Δ*retS* mutants are shown in Figure 20.

The Δ*hrpL* and Δ*hrpR* mutants do not have the ability to induce the T3SS and, as shown, do not induce the HR response in tobacco. The Δ*retS* mutant shows reduced T3SS activity *in vitro*, but shows no difference versus WT in tobacco. This could be due to redundancy in T3SS activating pathways, or because the Δ*retS* mutant is not compromised enough in T3SS to show an HR defect with this assay.

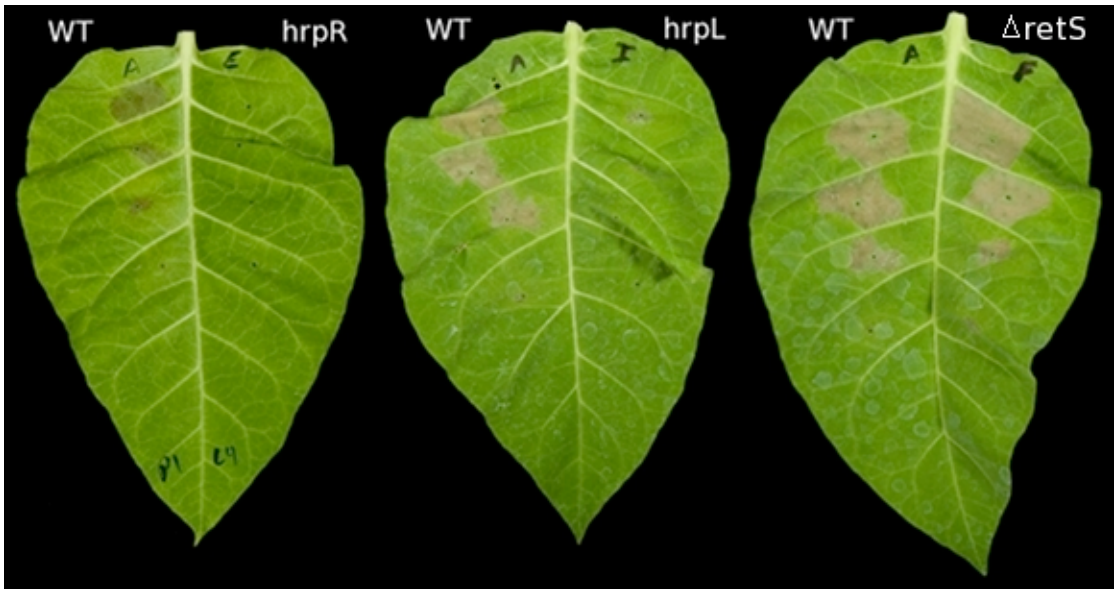

Figure 20. HR assays for Δ*hrpR*, Δ*hrpL*, and Δ*retS*.
Overnight cultures were diluted 2-fold serially and inoculated into a fully expanded *N. benthamiana* leaf from top to bottom at the following concentrations (in colony forming units): 2.0 x 10^7, 1.0 x 10^7, 0.5 x 10^6, and 0.25 x 10^6. WT is on the left side of the leaf, and mutants on the right.

Vfr and RetS

Vfr (virulence factor regulator) has been compellingly implicated in *P. aeruginosa* virulence-related gene expression in connection with RetS (Goodman et al., 2004, Laskowski et al., 2004). These reports suggest that RetS may induce T3SS expression through the transcriptional regulator Vfr. Microarray studies also showed significant overlap in a set of genes related to acute infection in Δ*retS* and Δ*vfr* mutants (Wolfgang *et al.*, 2003, Goodman et al., 2004).

We used Lux reporter plasmids to investigate T3SS activity under different *in vitro* conditions for Δ*retS* and Δ*vfr* DC3000 mutants. The plasmids used are similar but not identical. pBS45 has pavrPtoB::LuxCDABE as a reporter, and pBS63 has phrpJ::LuxCDABE as a reporter. Both promoters are *hrp* boxes. While similar, they are not identical and this should be taken into consideration when analyzing the following data.

An earlier experiment with WT pBS63 and Δ*vfr* pBS63 showed a similar pattern of reduction in reporter activity to that shown for WT pBS45 and Δ*retS* pBS45. The results of the Δ*vfr* Lux experiments are shown in Figure 21. The starting cell density of the Δ*vfr* Lux experiments was OD600=0.4, and not OD600=1.0 as for Δ*retS* assays. Once again, it appears that fructose and mannitol could be inducing the T3SS through the activity of Vfr similarly to that shown for the Δ*retS* mutant in this study. Figure 22 is also from the Δ*vfr* Lux data set, but only shows time course data for selected carbon sources to simplify the visual representation.

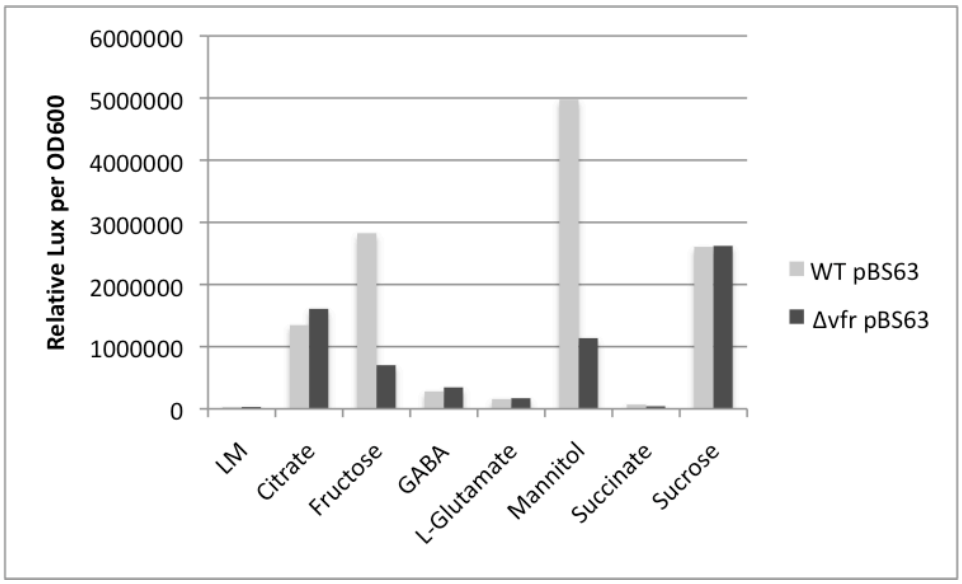

Figure 21. WT and Δ*vfr* Lux reporter with different carbon sources.
WT pBS63 and Δ*vfr* pBS63 were inoculated into 5 ml of LM or *hrp* minimal medium with specific carbon sources. Initial inoculum was OD600=0.4 and reporter activity was recorded at 12 hpi.

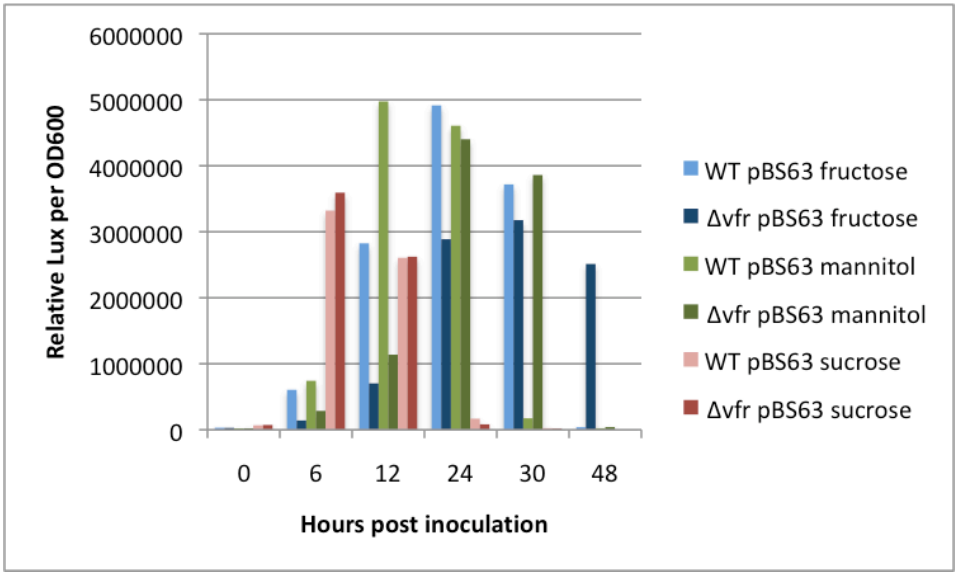

Figure 22. WT and Δ*vfr* Lux as a function of time.
WT pBS63 and Δ*vfr* pBS63 were inoculated at OD600=0.4 into 5 ml *hrp* minimal medium with supplements as identified in the graph legend. Sucrose is provided as a negative control.

The data in Figure 22 is paired by colors with WT lighter than the mutant. Sucrose is shown as a control that is not thought (from our experiments) to influence T3SS through the RetS-dependent pathways that we are studying. Vfr seems to be active only in the early stages of growth in minimal medium and not as the cells reach a higher density into stationary phase after or around 24 hours. Vfr is known to have cell density-dependent effects in *P. aeruginosa* (Bertani *et al.*, 2003).

The Δ*vfr* Lux experiments were repeated to include the Δ*retS* Lux strains. The protocol and initial cell density followed the Δ*retS* Lux experiments. Figure 23 shows the results.

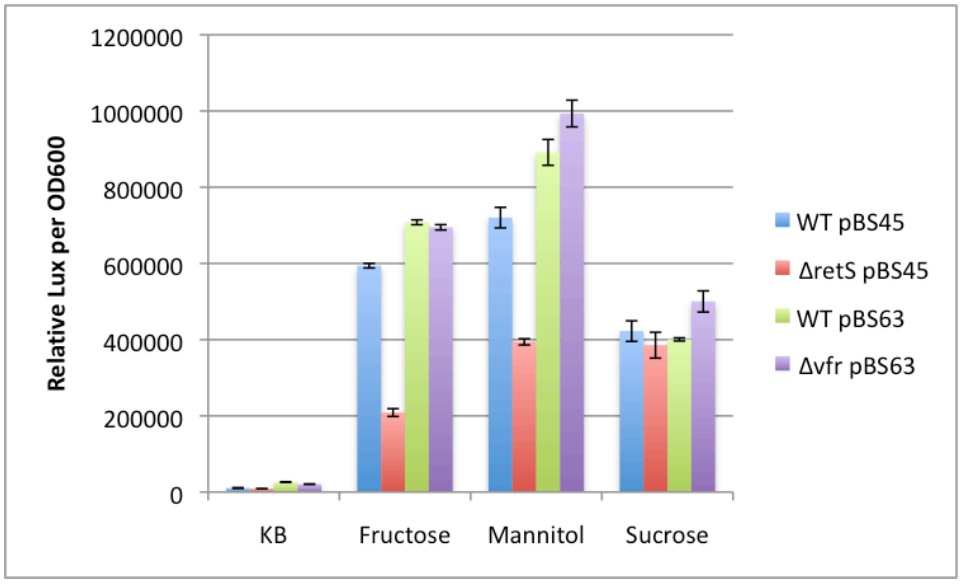

Figure 23. Δ*retS* and Δ*vfr* Lux reporter activity.
WT pBS45, Δ*retS* pBS45, WT pBS63, and Δ*vfr* pBS63 were inoculated into *hrp* minimal medium with supplements as described in the graph at initial OD600=1.0. Data is from 10 hpi. Three technical replicates are shown.

The fructose and mannitol response of the Δ*retS* pBS45 mutant was not replicated in this experiment for the Δ*vfr* pBS63 mutant strain. This response

could be due to the initial inoculum cell density of OD600=1.0. Earlier experiments with Δ*vfr* pBS63 showed differences from WT in fructose and mannitol only when cell density was less than OD600=0.8, which is lower than the initial cell density tested in Figure 23. Also, note that this response is not considered to be the result of a growth defect because the Δ*vfr* mutant strain grows at an equivalent rate to WT in rich medium (data not shown)

DISCUSSION

RetS is a unique, hybrid TCS that plays an important role in T3SS regulation in DC3000 as in *P. aeruginosa* (Goodman et al., 2004, Laskowski et al., 2004). However, in DC3000 the Δ*retS* mutant fails to show a plant phenotype. It is unlikely that DC3000 would have this conserved sensory protein purely for the *in vitro* conditions tested here. It is more likely that we have so far been unable to identify the purpose of RetS in the DC3000 lifestyle as it relates to the *in vitro* motility and T3SS regulation phenotypes that we have recorded.

We do know that a DC3000 Δ*retS* mutant is still able to induce the HR in the tobacco non-host. This finding is the same as that reported for the Δ*retS* mutant in B728a (Records & Gross, 2010). It would be informative to investigate the DC3000 Δ*retS* mutant phenotype in other plant species including the tomato host, but these assays must be done with caution because the Δ*retS* mutant had a moderate growth defect in rich medium during exponential growth. The lack of a HR phenotype suggests that RetS does not have complete control over T3SS (versus *hrpL* for example), and/or that there is redundancy for activating the T3SS in tobacco that is RetS-independent. This could indicate the regulatory complexity that a pathogen faces for integrating multiple environmental signals that feed into one master regulatory network (T3SS for example).

With growth rate aside, there are compelling Δ*retS* mutant phenotypes. The hyperswarming phenotype on NaCl supplemented medium is striking. RetS is a signaling component that represses DC3000 swarming in the presence of NaCl or PEG200, but we were unable to prove that osmotic stress alone induces the T3SS from the few experiments conducted so far. We

hypothesize that our *in vitro* study may mimic an environment where it is favorable for DC3000 to remain immotile and that RetS is a part of the signal transduction relay for this phenomenon. It would be interesting to see if the first REC domain is responsible for the motility phenotype as only the second REC domain has been functionally phenotyped for its involvement in pathogenicity (Laskowski & Kazmierczak, 2006). Our current hypothesis is that RetS is involved in regulatory networks both feeding into and outside of T3SS cascades. The RetS regulatory network outside of T3SS cascades could work through genes with histidine-containing phosphotransfer (Hpt) domains like in *P. aeruginosa* (Hsu et al., 2008). *P. aeruginosa* displayed a hyperswarming phenotype when the Hpt cascade PA3346-47 (PSPTO_1964-63 orthologs) was interrupted, and implicated RetS as one component of this signaling cascade.

We have also discovered that RetS is involved in the pathway that represses *rsmZ* and activates T3SS, in inducing conditions, similar to *P. aeruginosa* (Ventre et al., 2006). Our current hypothesis is that RetS is at the top of one T3SS activating pathway. Further, RetS induces T3SS in response to fructose and mannitol supplemented minimal medium but not the other carbon sources tested. This leads us to speculate that fructose and mannitol (and perhaps other similarly structured carbohydrates) are directly sensed by the 7TMR-DISMED2 RetS domain and activate The T3SS. It would be very interesting to discover if this pathway is involved in plant-microbe interactions enabling DC3000 to discern the appropriate moment for pathogenesis.

More data will be needed to fully compare and contrast the *P. aeruginosa*, B728a, and DC3000 RetS models. Our *rsmZ* data suggests that DC3000 is more similar to *P. aeruginosa* than B728a when inducing the T3SS.

The DC3000 *gacS* and *gacA* mutant phenotypes from our screen also concur with the *P. aeruginosa* model. Interestingly, T3SS induction in B728a is GacS-independent . It would be informative to know how the predicted RsmZ homolog in B728a is affected by the *retS* deletion to see if it is similar to *P. aeruginosa* and DC3000. Ultimately, more will have to be done on the entire RetS regulon in DC3000 to understand why it seems to be more similar to a mammalian pathogen than a related plant pathogen.

Finally, fructose, mannitol, and sucrose were the three most potent *in vitro* activators of T3SS in *P. syringae* pv. *glycinea* (Huynh et al., 1989) (see also Appendix C). The results of our study hint at the mechanism for RetS-dependent sensing of fructose and mannitol, but not sucrose, leading to T3SS induction in DC3000. Fructose and mannitol are very similar in structure, and could provide a key to unlock RetS carbohydrate binding specificity.

FUTURE WORK

Non-metabolizable fructose analog

This experiment would seek to clarify if the *hrp* system is directly activated by binding fructose, or indirectly through metabolic effects. A non-metabolizable fructose analog could easily be used as a supplement in the Lux assays developed in this study. If the mutant still shows a reduction in reporter activity in the presence of the fructose analog it would suggest that direct binding through RetS induces T3SS instead of through general metabolic effects.

Dip inoculation assay

Dip inoculation assays may provide a link between the motility phenotype for Δ*retS* and a plant host. The B728a Δ*retS* mutant shows reduced survival as an epiphyte on a leaf surface with low humidity (Records & Gross, 2010). RetS may play a role in decision making when it is favorable to be non-motile. The dip inoculation assay may help explain the role of RetS as DC3000 interacts with the plant leaf surface and attempts to obtain entry into the plant.

RetS carbohydrate binding

Finding the ligand(s) that bind(s) to the periplasmic sensory domain of RetS would be a significant contribution to understanding RetS function. It may be feasible to clone the RetS periplasmic sensor from DC3000 (~150bp), attach an epitope tag such as FLAG (built into the cloning primers), and overexpress this construct in *E. coli*. The sensor would then be purified from an *E. coli* culture and analyzed using Mass Spectrometry. Identification of

fructose or mannitol binding would be detected by comparing samples with and without the putative ligands and observing a change in peptide mass where the ligand is bound. Negative controls could include ligands used in this study that showed no difference in Lux activity between WT and Δ*retS*.

Phosphorylation assays

Mass spectrometry could also provide data on the phosphorylation state of the RetS receiver domains. The purpose of this experiment would be to determine the mechanism of RetS signal transduction. Each RetS receiver domain appears to have a conserved aspartic acid residue similar to those in *P. aeruginosa* PA01 RetS. The second REC domain was linked to T3SS induction in *P. aeruginosa* (Laskowski & Kazmierczak, 2006). I would be interested to analyze potential RetS phosphorylation changes in response to fructose and mannitol, or with NaCl and PEG200 supplements like in the swarming assays.

Create a *retS* deletion strain

One critical goal is to construct a complete *retS* deletion strain using the pK18mobsacB protocol routinely used in the lab. This strain would provide assurance that there are no remnants of functional RetS in the Δ*retS* strain.

Expand carbon sources and Lux assays

These initial studies have only looked at a handful of carbon sources. It would be simple to expand the carbon sources investigated for T3SS induction in DC3000. The most informative carbon sources would be those with a similar structure to fructose or mannitol and that do not activate the T3SS.

This could provide data on binding specificity for RetS. Related carbon sources that similarly feed into the TCA cycle could also help improve contrast between T3SS stimulating compounds versus metabolic effects on T3SS activation.

Change ions for swarming assay

This experiment would involve switching NaCl with another salt (ie KCl) and used at the same molarity for the swarming assays. The purpose would be to investigate if the swarming phenotype is specific to the Na^+ or Cl^- ions, or just general osmotic stress.

Alginate and levan assays

The RetS-dependent biofilm lifestyle of *P. aeruginosa* has been investigated (Goodman et al., 2004). Recently, RetS was also found to play a role in biofilm formation in B728a (Records & Gross, 2010). A simple experiment would be to investigate alginate and/or levan production in DC3000 and the Δ*retS* mutant using the same protocols in the reference just cited. This data could provide insight into the lifestyle choices of DC3000 and the role of RetS in that decision making process.

Complementing Δ*retS* in DC3000

Another approach to complementing RetS is to overexpress *rsmA*. In *P. aeruginosa* this shifts the acute versus chronic equilibrium to the chronic side of the equation. Overexpressing *rsmA* could potentially complement the effects of a Δ*retS* mutation leading to increased T3SS activity. Additionally, overexpressing *vfr* has also been used to complement the *retS* mutant in *P.*

aeruginosa (Laskowski & Kazmierczak, 2006). *hrpL* could also be overexpressed to complement the *retS* mutant.

Vfr and CHIP-Seq

vfr and *retS* deletions have significant overlap in microarray gene expression experiments in *P. aeruginosa* (Wolfgang et al., 2003, Goodman et al., 2004). I would very much like to see a phenotype for the DC3000 Δ*vfr* deletion strain. This data could be provided by conducting qRT-PCR for *rsmZ* with cells in both exponential and stationary phase for WT and the Δ*vfr* deletion. If this proves fruitful, I have already constructed a vfr-FLAG tagged strain that could be used for CHIP-Seq to see if Vfr is involved in the subtle T3SS DC3000 response seen in the Δ*retS* mutant.

LadS mutant

DC3000 has a predicted *ladS* gene (PSPTO_4796) orthologous to *ladS* characterized in both *P. aeruginosa* (Ventre et al., 2006) and B728a (Records & Gross, 2010). I attempted to construct a *ladS* pKnockout multiple times (side by side with successful *retS* pKnockout generation), but no *ladS* mutants were obtained. This would be an informative and essential tool for further eliciting the RetS regulon in DC3000 to compare it to *P. aeruginosa*. Perhaps a different method (like a *ladS* deletion strain) would work. A *ladS* deletion in DC3000 could also be lethal.

APPENDIX A: EXPANDED REDUCED REPORTER ACTIVITY TABLE

Table 4. Expanded reduced reporter activity table.

Colony ID identifies the number given to the isolated colony where T1-T4 are the independent electroporation reactions. Gene number and names are given. Seq Order is given to locate the original sequencing reads. Pheno and 2˚ Pheno are the color of the isolated colony after patching and after re-plating respectively. Seq Primer is the direction of sequencing off of the transposon. Start is the coordinate of the first base from the sequencing reaction with homology to the DC3000 genome, and Stop is the final base of homology. Coverage is the number of homologous base pairs from the sequencing reaction mapped to the DC3000 genome. E-value is the standard E-value from a BLAST search. Artemis contains notes from the annotated DC3000 genome from the Artemis browser.

Colony ID	T1-1	T1-2	T1-3	T1-4	T1-5
PSPTO	PSPTO_4868		PSPTO_1380	PSPTO_1380	Vector - Gus
Seq Order	10202160		10202501	10202160	10202160
Pheno	pale	pale	pale	white	pale
2˚ Pheno	Blue	Pale	White	White	White
Seq Primer	Rev		Rev	Rev	Rev
Start	5515050		1523934	1523909	In Vector
Stop	5514809		1523784	1523785	
Coverage	241		150	124	
E-value	9.00E-83		2.00E-56	9.00E-43	
Gene name	retS		hrpS	hrpS	
Artemis	sensor histidine kinase/response regulator RetS		type III transcriptional regulator HrpS	type III transcriptional regulator HrpS	

T1-6	T1-7	T1-8	T1-9	T1-10	T1-11	T1-11	T1-12
Vector - Gus	PSPTO_4868	PSPTO_2222	PSPTO_4868	PSPTO_0362	PSPTO_2222	PSPTO_2222	
10202160	10201476	10202160	10202501	10202160	10201221	10201221	
pale	pale	white	pale	pale	white	white	pale
White	Blue	White	Pale	Pale	White	White	
Rev	Rev	Rev	Rev	Rev	Fwd	Rev	
In Vector	5515368	2445773	5516470	396425	2445786	2445826	
	5515321	2445862	5516440	396525	2445652	2445878	
	47	89	30	100	134	52	
	6.00E-07	9.00E-12	4.00E-05	1.00E-26	2.00E-71	5.00E-10	
	retS	rhpR	retS		rhpR	rhpR	
	sensor histidine kinase/response regulator RetS	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	sensor histidine kinase/response regulator RetS	DeoR family transcriptional regulator, Transcriptional regulators of sugar metabolism	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	

T1-13	T1-14	T1-15	T1-15	T1-16	T1-16	T1-17	T1-18
PSPTO_4868		PSPTO_0362	PSPTO_0362	PSPTO_3369	PSPTO_3369	PSPTO_1751	PSPTO_2222
10203022		10201221	10201221	10201221	10201221	10202501	10200910
pale	pale	white	white	white	white	white	white
Blue		Pale	Pale	White	White	Pale	White
Fwd		Fwd	Rev	Fwd	Rev	Rev	Rev
5514864		396499	396440	3807938	3807929	1923296	2445650
5514544		396777	396386	3807611	3808229	1923146	2445372
320		278	54	327	300	150	278
e-175		4.00E-60	3.00E-14	0	e-159	1.00E-72	e-100
retS				nuoF	nuoF	ihfB	rhpR
sensor histidine kinase/response regulator RetS		DeoR family transcriptional regulator, Transcriptional regulators of sugar metabolism	DeoR family transcriptional regulator, Transcriptional regulators of sugar metabolism	NADH dehydrogenase I subunit F	NADH dehydrogenase I subunit F		Sensor histidine kinase, operon with 2223 - DNA-binding response regulator

T1-18	T1-18	T1-19	T1-20	T1-21	T1-22	T1-23	T1-23
PSPTO_2222	PSPTO_2222	PSPTO_2222	PSPTO_2222	PSPTO_1380	PSPTO_4817	PSPTO_1379	PSPTO_1380
10201221	10201221	10203022	10202160	10202160	10202160	10202160	10202160
white	white	white	white	white	white	white	white
White	White	White	White	White	Pale	White	White
Fwd	Fwd	Rev	Rev	Rev	Rev	Rev	Rev
2445694	2445861	2446357	2445934	1523595	5455796	1522603	1523577
2445837	2445986	2446152	2446042	1523809	5455962	1522657	1523822
143	125	205	108	214	166	54	245
7.00E-77	5.00E-50	2.00E-46	7.00E-28	2.00E-55	1.00E-41	5.00E-10	e-113
rhpR	rhpR	rhpR	rhpR		hopAJ2	hrpR	hrpS
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator		type III effector HopAJ2	type III transcriptional regulator HrpR	type III transcriptional regulator HrpS

T1-24	T1-25	T1-26	T1-27	T1-28	T1-29	T1-30	T1-31
PSPTO_2222	PSPTO_4505	PSPTO_5502	Vector - Gus	PSPTO_2222	PSPTO_4868	PSPTO_4868	Vector - Gus
10202160	10202160	10202160	10202160	10202160	10202501	10202501	10202160
white	white	white	white	white	white	white	white
White	Blue	Pale	White	White	Blue	Pale	White
Rev	Rev	Rev	Rev	Rev	Fwd	Rev	Rev
2446010	5075877	6268135	In Vector	2445755	5517004	5514690	In Vector
2445751	5076275	6268206		2446002	5517124	5514544	
259	398	71		247	120	146	
e-112	e-179	3.00E-24		e-116	3.00E-24	4.00E-57	
rhpR	dnaK			rhpR	retS	retS	
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	molecular chaperone DnaK	hypothetical, putative conserved effector locus protein [Pseudomonas viridiflava]		Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	mapped to intergenic region 3'	sensor histidine kinase/response regulator RetS	

T1-32	T1-33	T1-34	T1-35	T1-36	T1-37	T1-38	T1-39
	PSPTO_0362	PSPTO_0963	PSPTO_1379			PSPTO_4735	Vector - Gus
	10202501	10202160	10202160			10202501	10202501
white	white	white	pale	white	white	white	white
	Pale	Blue	Blue	Pale	Pale	Blue	White
	Fwd	Rev	Rev			Rev	
	396292	1045625	1522580			5363458	In Vector
	396143	1045936	1522284			5363536	
	149	311	296			78	
	1.00E-32	e-124	e-125			7.00E-28	
		pcnB	hrpR			hrpB	
	could be intergenic 0362 and 0361	PcnB is required for the rapid degradation of RNAI, the antisense RNA that controls the	type III transcriptional regulator HrpR				

T1-40	T1-41	T2-1	T2-1	T2-2	T2-3	T2-3	T2-4
PSPTO_2222	Vector - Gus	PSPTO_1404	PSPTO_1404	Vector - Gus	PSPTO_1379	PSPTO_1380	PSPTO_2222
10203022	10202501	10201476	10201771	10202160	10201476	10201476	10201476
white	white	white	white	white	white	white	white
White	Blue	Pale	Pale	White	White	White	White
Rev		Rev	Fwd	Rev	Rev	Fwd	Fwd
2446175	In Vector	1543286	1543355	In Vector	1522947	1523669	2446376
2446141		1543166	1543383		1524139	1523234	2445654
34		120	28		1192	435	722
1.00E-07		9.00E-37	2.00E-06		0	0	0
rhpR		hrpL	hrpL		hrpR	hrpS	rhpR
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator		RNA polymerase sigma factor HrpL	RNA polymerase sigma factor HrpL		type III transcriptional regulator HrpR	type III transcriptional regulator HrpS	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator

T2-4	T2-5	T2-5	T2-6	T2-6	T2-6	T2-7	T2-8
PSPTO_2222	PSPTO_1379	PSPTO_1379	PSPTO_0211	PSPTO_0211	PSPTO_0211		PSPTO_2222
10201476	10201476	10201476	10201476	10201476	10201771		10201771
white	white	white	white	white	white	white	white
White	White	White	White	White	White	no grow	White
Rev	Fwd	Rev	Fwd	Rev	Fwd		Fwd
2446367	1523184	1523062	236740	236733	236841		2446237
2446958	1523344	1522641	237067	236350	236957		2445662
591	160	421	327	383	116		575
0	9.00E-52	0	e-113	0	5.00E-44		0
rhpR	hrpR	hrpR	iucD	iucD	iucD		rhpR
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	type III transcriptional regulator HrpR	type III transcriptional regulator HrpR	L-lysine 6-monooxygenase	L-lysine 6-monooxygenase	L-lysine 6-monooxygenase		Sensor histidine kinase, operon with 2223 - DNA-binding response regulator

T2-9	T2-10	T2-11	T2-12	T2-13	T2-14	T2-15	T2-16
PSPTO_1404	PSPTO_2222	PSPTO_1380	PSPTO_4868	PSPTO_3576		PSPTO_3836	PSPTO_4868
10202160	10202160	10201771	10201771	10202160	10203022	10201771	10201771
white	white	white	pale	pale	pale	pale	pale
White	White	White	pale	pale	white	pale	blue
Rev	Rev	Fwd	Fwd	Rev	Fwd	Fwd	Fwd
1543187	2445903	1523520	5514879	4033871	Vector	4342954	5515116
1543330	2446045	1523273	5514491	4033590		4342695	5515150
143	142	247	388	281		259	34
3.00E-48	2.00E-22	3.00E-61	0	1.00E-87		e-127	9.00E-12
hrpL	rhpR	hrpS	retS	tvrR			retS
	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	type III transcriptional regulator HrpS	sensor histidine kinase/response regulator RetS			hypothetical	sensor histidine kinase/response regulator RetS

T2-17	T2-17	T2-18	T2-19	T2-20	T2-21	T2-21	T2-22
PSPT0_4943	PSPT0_4943	PSPTO_4868	PSPTO_3576	PSPTO_4868	PSPTO_3576	PSPTO_3576	PSPTO_3836
10203022	10203022	10201771	10201771	10202160	10203022	10203022	10202501
pale	pale	pale	pale	pale	pale	pale	pale
white/low grow	white/low grow			pale	pale	pale	pale
Fwd	Rev	Fwd	Fwd	Rev	Fwd	Rev	Rev
5602781	5602797	5515440	4033499	5515917	4033612	4033620	4342899
5602656	5603111	5514879	4032903	5515570	4033214	4034020	4343255
125	314	561	596	347	398	400	356
1.00E-47	e-157	0	0	e-156	e-180	e-162	4.00E-54
miaA	miaA	retS	tvrR	retS	tvrR	tvrR	
tRNA modification, tRNA delta(2)-isopentenylpyrophosphate	tRNA modification, tRNA delta(2)-isopentenylpyrophosphate	sensor histidine kinase/response regulator RetS	TetR-like virulence regulator	sensor histidine kinase/response regulator RetS	TetR-like virulence regulator	TetR-like virulence regulator	could be interfering with PSPTO_3837 maf-1

T2-23	T2-24	T3-1	T3-2	T3-3	T3-4	T3-5	T3-6
Vector - Gus					PSPTO_3576		intergenic PSPTO_4434
10202501					10203022		10203022
pale	pale	white	white	white	pale	pale	pale
white	pale						
Fwd					Rev		Rev
In Vector					4033549		5001989
					4033806		5001508
					257		481
					2.00E-74		0
					tvrR		
					TetR-like virulence regulator		sulfate adenylyltransferase subunit 2

T3-7	T3-8	T3-9	T4-1	T4-2	T4-3	T4-3	T4-4
		PSPTO_4868	PSPTO_2222	PSPTO_2222	PSPTO_2222	PSPTO_2222	
		10203022	10204507	10204507	10204049	10204049	10204049
pale	pale	pale	white	white	white	white	white
			white	white	white	white	white
		Fwd	Rev	Rev	Fwd	Rev	Rev
		5514494	2446247	2446101	2446290	2446569	658
		5514405	2446330	2446216	2446561	2446958	918
		89	83	115	271	389	260
		2.00E-40	2E-37	4E-26	3E-73	4E-180	4E-128
		retS	rhpR	rhpR	rhpR	rhpR	Vector
		sensor histidine kinase/response regulator RetS	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	

T4-5	T4-6	T4-6	T4-6	T4-7	T4-8	T4-9	T4-9
PSPTO_2222	PSPTO_2222			PSPTO_2222	PSPTO_2222	PSPTO_1380	PSPTO_1380
10204507	10204049	10204049	10204049	10204049	10204049	10204049	10204049
white	white	white	white	white	white	white	white
white/pale	white	white	white	white	white	white	white
Rev	Fwd	Fwd	Fwd	Fwd	Rev	Fwd	Rev
2446141	2446174	1	1182	2445376	2446250	1523708	1523479
2446396	2446204	21	1221	2445619	2446270	1523900	1523637
255	30	20	39	243	20	192	158
1E-143	0.000000002	0.002	0.0000005	1E-115	0.002	5E-84	2E-62
rhpR	rhpR			rhpR	rhpR	hrpS	hrpS
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator			Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	type III transcriptional regulator HrpS	type III transcriptional regulator HrpS

T4-9	T4-10	T4-11	T4-12	T4-12	T412.2	T4-13	T4-13.2
	PSPTO_1380	PSPTO_0080	PSPTO_2222	PSPTO_2222		PSPTO_0080	PSPTO_2222
10204049	10204049	10204049	10204049	10204507		10204049	10204049
white	white	white	white	white	white	white	white
white	white	white	white	white	white	white	white
Fwd	Rev	Rev	Fwd	Rev		Rev	Fwd
1184	1523972	101626	2445951	2446316		101636	2446262
1211	1524141	101695	2446308	2446681		101723	2446394
27	169	69	357	365		87	132
0.008	3E-88	0.000002	2E-99	5E-152		1E-32	1E-41
	hrpS	pyrE	rhpR	rhpR		pyrE	rhpR
	type III transcriptional regulator HrpS		Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator			Sensor histidine kinase, operon with 2223 - DNA-binding response regulator

T4-13.2	T4-14	T4-14	T4-14.2	T4-15	T4-16	T4-16	T4-16
PSPTO_2222	PSPTO_2621	PSPTO_2621	PSPTO_2222	PSPTO_1380	PSPTO_2362	PSPTO_2362	PSPTO_2362
10204049	10204049	10204507	10204049	10204049	10204049	10204049	10204507
white	white	white	white	white	white	white	white
white	white	white	white	white			
Rev	Rev	Rev	Rev	Rev	Fwd	Rev	Rev
2446465	2912631	2912633	2445752	1523590	2615876	2616035	2616035
2446935	2912887	2912887	2445951	1523904	2616043	2616359	2616362
470	256	254	199	314	167	324	327
0	4E-128	4E-143	2E-99	1E-149	2E-83	1E-174	0
rhpR	polB	polB	rhpR	hrpS	map-2	map-2	map-2
Sensor histidine kinase, operon with 2223 - DNA-binding response regulator			Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	type III transcriptional regulator HrpS			

T4-16	T4-16	T4-17	T4-18	T4-18	T4-19	T4-20	T4-21
		PSPTO_2222	PSPTO_2222	PSPTO_2222	pAC1	PSPTO_4868	PSPTO_4868
10204049	10204049	10204507	10204049	10204507	10204166	10204049	10204049
white	white	white	white	white	white	pale	pale
		white/pale	white	white	white	pale	blue
Fwd	Fwd	Rev	Rev	Rev	Fwd	Rev	Rev
1177	1	2446062	2446608	2446465	797	5514794	5516068
1221	24	2446280	2446720	2446957	6151	5515120	5516339
44	23	218	112	492	5354	326	271
3E-14	0.002	1E-75	2E-31	0		1E-133	6E-133
		rhpR	rhpR	rhpR		retS	retS
		Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator		sensor histidine kinase/response regulator RetS	sensor histidine kinase/response regulator RetS

T4-22	T4-23	T4-24	T4-25	T4-26	T4-27	T4-28	T4-28
	PSPTO_4868	PSPTO_2222	PSPTO_2705	PSPTO_3576		PSPTO_3576	
10204049	10204049	10204049	10204049	10204049		10204049	10204049
pale	pale	pale	pale	pale	white	pale	pale
white	pale	pale		pale		pale	pale
Rev	Rev	Fwd	Rev	Rev		Rev	Fwd
958	5514905	2445652	3001292	4033604		4033599	1
1321	5515221	2445931	3001585	4033922		4033937	24
363	316	279	293	318		338	23
2E-166	8E-151	2E-148	6E-142	9E-166		0	0.002
Vector	retS	rhpR		tvrR		tvrR	
	sensor histidine kinase/response regulator RetS	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Mannitol ABC transporter, permease protein	TetR-like virulence regulator		TetR-like virulence regulator	

T4-28	T4-29	T4-30	T4-31	T4-32	T4-33	T4-34	T4-35
		PSPTO_0503	PSPTO_3576	PSPTO_3576	PSPTO_2663	PSPTO_4868	PSPTO_5341
10204049	10204049	10204049	10204049	10204049	10204049	10204049	10204049
pale	pale	pale	pale	pale	pale	pale	pale
pale	white	pale	pale	pale		pale	blue?
Fwd	Rev	Fwd	Fwd	Fwd	Rev	Rev	Rev
1197	1368	551464	4033636	4033657	2959655	5516048	6072913
1221	1693	551625	4033943	4033937	2959766	5516203	6073077
24	325	161	307	280	111	155	164
0.000009	3E-122	3E-48	9E-95	1E-100	1E-23	1E-23	3E-61
	Vector	intergenic shcF TTSS chaperone	tvrR	tvrR		retS	
			TetR-like virulence regulator	TetR-like virulence regulator		sensor histidine kinase/response regulator RetS	

T4-36	T4-37	T4-38	T4-39	T4-40	T4-41	T4-41	T4-41
PSPTO_3576	PSPTO_3576	PSPTO_4868		PSPTO_4868	PSPTO_2222	PSPTO_2222	
10204049	10204049	10204049	10204049	10204049	10204049	10204049	10204049
pale	pale	pale	pale	pale	pale	pale	pale
pale	pale	blue	white	pale	pale	pale	pale
Rev	Rev	Rev	Rev	Fwd	Fwd	Rev	Fwd
4033611	4033748	5516236	1311	5516870	2446092	2445807	1
4033844	4033890	5516595	1456	5517119	2446145	2446084	24
233	142	359	145	249	53	277	23
1E-109	4E-29	0	8E-49	5E-75	3E-11	8E-123	0.002
tvrR	tvrR	retS	Vector	retS	rhpR	rhpR	
TetR-like virulence regulator	TetR-like virulence regulator	sensor histidine kinase/response regulator RetS		sensor histidine kinase/response regulator RetS	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	Sensor histidine kinase, operon with 2223 - DNA-binding response regulator	

T4-41	T4-42	T4-43	T4-43	T4-43
		PSPTO_3576		
10204049	10204049	10204507	10204049	10204049
pale	pale	pale	pale	pale
pale	white	pale	pale	pale
Fwd	Rev	Rev	Fwd	Fwd
1198	840	4033755	1	1183
1221	1190	4034047	24	1221
23	350	292	23	38
0.002	5E-164	5E-164	0.002	0.0001
	Vector	tvrR		
		TetR-like virulence regulator		

APPENDIX B: CARBON SOURCE AND T3SS ACIVITY IN *P. SYRINGAE* PV. *GLYCINEA*

Figure 25 is provided for the readers' benefit. This figure is from experiments with *P. syringae* pv. *glycinea* (Huynh et al., 1989). It relates to carbon source effects on Lux activity in DC3000 and the Δ*retS* mutant.

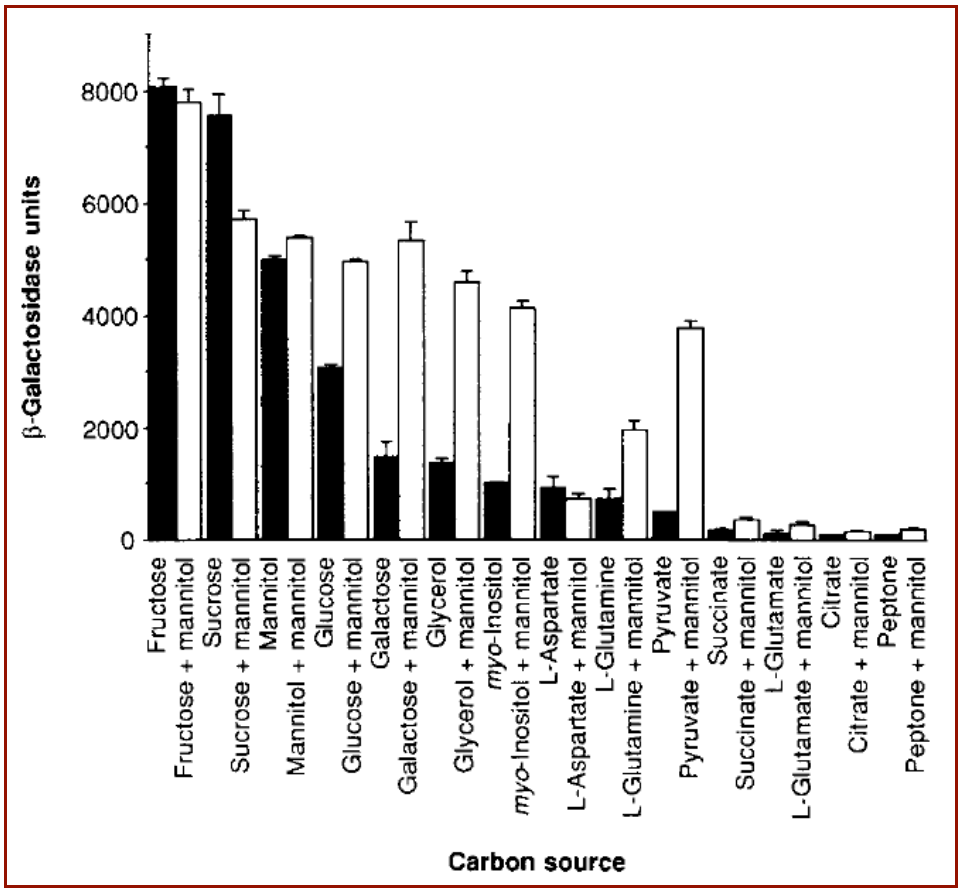

Figure 25. Effects of carbon source on T3SS activity.
pavrB::LacZ fusion reporter in *P. syringae* pv. *glycinea*. Carbon sources were added to 10 mM in *hrp* minimal medium except for glycerol and pyruvate at 20 mM each.

APPENDIX C: VERIFYING GUS REPORTER ACTIVITY

The first round of qRT-PCR was used to verify reporter activity of a number of interesting mutants from the screen. Mutants selected and qRT-PCR results are listed in Table 4 and shown in Figure 24 respectively. The qRT-PCR data shows that, in general, reduced reporter activity is accurately reflecting reduced target gene transcript abundance. The other interesting finding here is that the colonies with a dark blue phenotype (those with overactive reporter activity) were anticipated to show an increase in T3SS-related transcript levels. This data suggests that the T3SS activity is still reduced in these dark colony mutants. The mechanism for this result was not further investigated.

Table 4. Verifying GUS reporter activity with qRT-PCR.

Gene	Name	Pheno	Notes
PSPTO_0362		pale	DeoR family transcriptional regulator of sugar metabolism
PSPTO_4817	hopAJ2	pale	Lytic transglycoslyase, hrp activated
PSPTO_0211	iucD	white	L-lysine 6-monooxygenase, lysine degradation
PSPTO_3724	lon-1	Dark	ATP-dependent protease Lon-1
PSPTO_2712		Dark	Sensor histidine kinase/response regulator
PSPTO_1379	hrpR	white	Type III transcriptional regulator HrpR
PSPTO_3836		pale	Conserved in green sulfur bacteria and marine environmetal samples

Transposon insertion mutants selected to verify reporter activity.

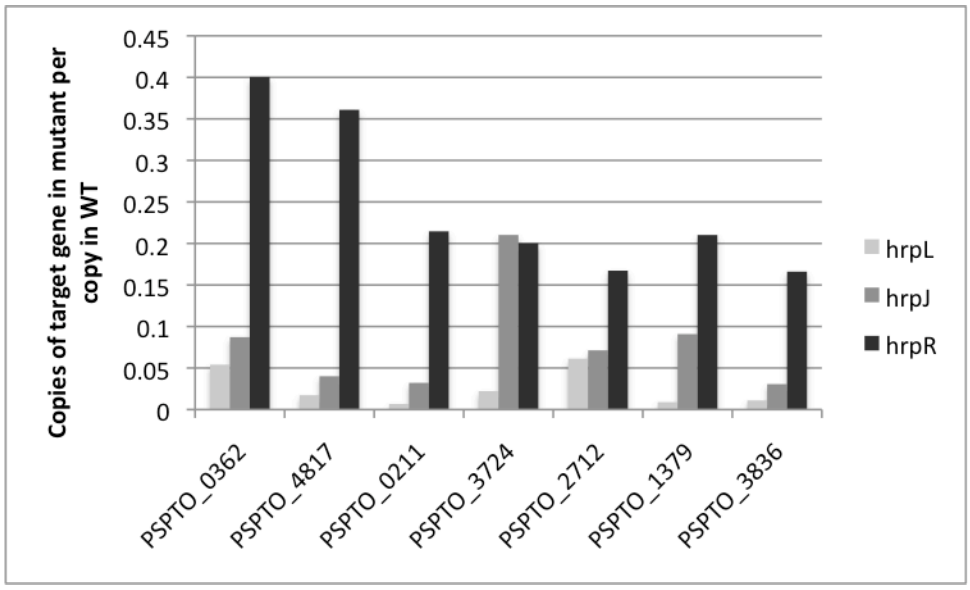

Figure 24. qRT-PCR for *hrpL, hrpJ, and hrpR*.
WT and transposon insertion strains mapped to the genes listed in the figure legend were grown in 5 ml *hrp* minimal medium for 4 hours and prepared for RNA extraction as in Materials. Single samples are shown without replication.

REFERENCES

Abramovitch, R. B. and Martin, G. B. (2004) Strategies used by bacterial pathogens to suppress plant defenses. *Curr Opin Plant Biol,* **7,** 356-364.

Bertani, I., Sevo, M., Kojic, M. and Venturi, V. (2003) Role of GacA, LasI, RhlI, Ppk, PsrA, Vfr and ClpXP in the regulation of the stationary-phase sigma factor rpoS/RpoS in Pseudomonas. *Arch Microbiol,* **180,** 264-271.

Boch, J., Joardar, V., Gao, L., Robertson, T. L., Lim, M. and Kunkel, B. N. (2002) Identification of Pseudomonas syringae pv. tomato genes induced during infection of Arabidopsis thaliana. *Mol Microbiol,* **44,** 73-88.

Bordi, C., Lamy, M. C., Ventre, I., Termine, E., Hachani, A., Fillet, S.*, et al.* (2010) Regulatory RNAs and the HptB/RetS signalling pathways fine-tune Pseudomonas aeruginosa pathogenesis. *Mol Microbiol*.

Brencic, A. and Lory, S. (2009) Determination of the regulon and identification of novel mRNA targets of Pseudomonas aeruginosa RsmA. *Mol Microbiol,* **72,** 612-632.

Brencic, A., McFarland, K. A., McManus, H. R., Castang, S., Mogno, I., Dove, S. L.*, et al.* (2009) The GacS/GacA signal transduction system of Pseudomonas aeruginosa acts exclusively through its control over the transcription of the RsmY and RsmZ regulatory small RNAs. *Mol Microbiol,* **73,** 434-445.

Brencic, A. and Winans, S. C. (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiol Mol Biol Rev,* **69,** 155-194.

Bretz, J., Losada, L., Lisboa, K. and Hutcheson, S. W. (2002) Lon protease functions as a negative regulator of type III protein secretion in Pseudomonas syringae. *Mol Microbiol,* **45,** 397-409.

Buell, C. R., Joardar, V., Lindeberg, M., Selengut, J., Paulsen, I. T., Gwinn, M. L.*, et al.* (2003) The complete genome sequence of the Arabidopsis and tomato pathogen Pseudomonas syringae pv. tomato DC3000. *Proc Natl Acad Sci U S A,* **100,** 10181-10186.

Caetano-Anolles, G. (1993) Amplifying DNA with arbitrary oligonucleotide primers. *PCR Methods Appl,* **3,** 85-94.

Chatterjee, A., Cui, Y., Yang, H., Collmer, A., Alfano, J. R. and Chatterjee, A. K. (2003) GacA, the response regulator of a two-component system, acts as a master regulator in Pseudomonas syringae pv. tomato DC3000 by controlling regulatory RNA, transcriptional activators, and alternate sigma factors. *Mol Plant Microbe Interact,* **16,** 1106-1117.

Cuppels, D. A. and Ainsworth, T. (1995) Molecular and Physiological Characterization of Pseudomonas syringae pv. tomato and Pseudomonas syringae pv. maculicola Strains That Produce the Phytotoxin Coronatine. *Appl Environ Microbiol,* **61,** 3530-3536.

Deng, X., Xiao, Y., Lan, L., Zhou, J. M. and Tang, X. (2009) Pseudomonas syringae pv. phaseolicola Mutants Compromised for type III secretion system gene induction. *Mol Plant Microbe Interact,* **22,** 964-976.

Goodman, A. L., Kulasekara, B., Rietsch, A., Boyd, D., Smith, R. S. and Lory, S. (2004) A signaling network reciprocally regulates genes associated with acute infection and chronic persistence in Pseudomonas aeruginosa. *Dev Cell,* **7,** 745-754.

Goodman, A. L., Merighi, M., Hyodo, M., Ventre, I., Filloux, A. and Lory, S. (2009) Direct interaction between sensor kinase proteins mediates acute and chronic disease phenotypes in a bacterial pathogen. *Genes Dev,* **23,** 249-259.

Grimm, C., Aufsatz, W. and Panopoulos, N. J. (1995) The hrpRS locus of Pseudomonas syringae pv. phaseolicola constitutes a complex regulatory unit. *Mol Microbiol,* **15,** 155-165.

Hendrickson, E. L., Guevera, P. and Ausubel, F. M. (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,** 3508-3516.

Hernandez-Morales, A., De la Torre-Zavala, S., Ibarra-Laclette, E., Hernandez-Flores, J. L., Jofre-Garfias, A. E., Martinez-Antonio, A.*, et al.* (2009) Transcriptional profile of Pseudomonas syringae pv. phaseolicola NPS3121 in response to tissue extracts from a susceptible Phaseolus vulgaris L. cultivar. *BMC Microbiol,* **9,** 257.

Hsu, J. L., Chen, H. C., Peng, H. L. and Chang, H. Y. (2008) Characterization of the histidine-containing phosphotransfer protein B-mediated multistep phosphorelay system in Pseudomonas aeruginosa PAO1. *J Biol Chem,* **283,** 9933-9944.

Hutcheson, S. W., Bretz, J., Sussan, T., Jin, S. and Pak, K. (2001) Enhancer-binding proteins HrpR and HrpS interact to regulate hrp-encoded type III protein secretion in Pseudomonas syringae strains. *J Bacteriol,* **183,** 5589-5598.

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

Jing, X., Jaw, J., Robinson, H. H. and Schubot, F. D. (2010) Crystal structure and oligomeric state of the RetS signaling kinase sensory domain. *Proteins,* **78,** 1631-1640.

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

Laskowski, M. A. and Kazmierczak, B. I. (2006) Mutational analysis of RetS, an unusual sensor kinase-response regulator hybrid required for Pseudomonas aeruginosa virulence. *Infect Immun,* **74,** 4462-4473.

Laskowski, M. A., Osborn, E. and Kazmierczak, B. I. (2004) A novel sensor kinase-response regulator hybrid regulates type III secretion and is required for virulence in Pseudomonas aeruginosa. *Mol Microbiol,* **54,** 1090-1103.

Losada, L. C. and Hutcheson, S. W. (2005) Type III secretion chaperones of Pseudomonas syringae protect effectors from Lon-associated degradation. *Mol Microbiol,* **55,** 941-953.

Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H.*, et al.* (2009) CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res,* **37,** D205-210.

Marchler-Bauer, A. and Bryant, S. H. (2004) CD-Search: protein domain annotations on the fly. *Nucleic Acids Res,* **32,** W327-331.

Mougous, J. D., Cuff, M. E., Raunser, S., Shen, A., Zhou, M., Gifford, C. A.*, et al.* (2006) A virulence locus of Pseudomonas aeruginosa encodes a protein secretion apparatus. *Science,* **312,** 1526-1530.

Murray, T. S. and Kazmierczak, B. I. (2008) Pseudomonas aeruginosa exhibits sliding motility in the absence of type IV pili and flagella. *J Bacteriol,* **190,** 2700-2708.

Peters, J. E. and Craig, N. L. (2000) Tn7 transposes proximal to DNA double-strand breaks and into regions where chromosomal DNA replication terminates. *Mol Cell,* **6,** 573-582.

Preiter, K., Brooks, D. M., Penaloza-Vazquez, A., Sreedharan, A., Bender, C. L. and Kunkel, B. N. (2005) Novel virulence gene of Pseudomonas syringae pv. tomato strain DC3000. *J Bacteriol,* **187,** 7805-7814.

Rahme, L. G., Mindrinos, M. N. and Panopoulos, N. J. (1992) Plant and environmental sensory signals control the expression of hrp genes in Pseudomonas syringae pv. phaseolicola. *J Bacteriol,* **174,** 3499-3507.

Rashid, M. H. and Kornberg, A. (2000) Inorganic polyphosphate is needed for swimming, swarming, and twitching motilities of Pseudomonas aeruginosa. *Proc Natl Acad Sci U S A,* **97,** 4885-4890.

Records, A. R. and Gross, D. C. (2010) Sensor kinases RetS and LadS regulate Pseudomonas syringae type VI secretion and virulence factors. *J Bacteriol*.

Stonehouse, E., Kovacikova, G., Taylor, R. K. and Skorupski, K. (2008) Integration host factor positively regulates virulence gene expression in Vibrio cholerae. *J Bacteriol,* **190,** 4736-4748.

The Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. *Nature,* **408,** 796-815.

van Dijk, K., Fouts, D. E., Rehm, A. H., Hill, A. R., Collmer, A. and Alfano, J. R. (1999) The Avr (effector) proteins HrmA (HopPsyA) and AvrPto are secreted in culture from Pseudomonas syringae pathovars via the Hrp (type III) protein secretion system in a temperature- and pH-sensitive manner. *J Bacteriol,* **181,** 4790-4797.

Ventre, I., Goodman, A. L., Vallet-Gely, I., Vasseur, P., Soscia, C., Molin, S*., et al.* (2006) Multiple sensors control reciprocal expression of Pseudomonas aeruginosa regulatory RNA and virulence genes. *Proc Natl Acad Sci U S A,* **103,** 171-176.

Whalen, M. C., Innes, R. W., Bent, A. F. and Staskawicz, B. J. (1991) Identification of Pseudomonas syringae pathogens of Arabidopsis and a bacterial locus determining avirulence on both Arabidopsis and soybean. *Plant Cell,* **3,** 49-59.

Windgassen, M., Urban, A. and Jaeger, K. E. (2000) Rapid gene inactivation in Pseudomonas aeruginosa. *FEMS Microbiol Lett,* **193,** 201-205.

Wolfgang, M. C., Lee, V. T., Gilmore, M. E. and Lory, S. (2003) Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell,* **4,** 253-263.

Xiao, Y., Heu, S., Yi, J., Lu, Y. and Hutcheson, S. W. (1994) Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of Pseudomonas syringae pv. syringae Pss61 hrp and hrmA genes. *J Bacteriol,* **176,** 1025-1036.

Xiao, Y., Lan, L., Yin, C., Deng, X., Baker, D., Zhou, J. M.*, et al.* (2007) Two-component sensor RhpS promotes induction of Pseudomonas syringae type III secretion system by repressing negative regulator RhpR. *Mol Plant Microbe Interact,* **20,** 223-234.

Zolfaghar, I., Angus, A. A., Kang, P. J., To, A., Evans, D. J. and Fleiszig, S. M. (2005) Mutation of retS, encoding a putative hybrid two-component regulatory protein in Pseudomonas aeruginosa, attenuates multiple virulence mechanisms. *Microbes Infect,* **7,** 1305-1316.

Zolfaghar, I., Evans, D. J., Ronaghi, R. and Fleiszig, S. M. (2006) Type III secretion-dependent modulation of innate immunity as one of multiple factors regulated by Pseudomonas aeruginosa RetS. *Infect Immun,* **74,** 3880-3889.