

**CELLULAR METABOLISM AND ITS EFFECT ON THE TYPE III
SECRETION SYSTEM OF *Dickeya dadantii* 3937**

by

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ABSTRACT

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Nutrition, in both eukaryotes and prokaryotes, is vital to the life and well-being of the species. In organisms such as *Escherichia coli*, metabolism and its regulation have been well established, whereas in *Dickeya dadantii* 3937, the metabolic pathways and their effects on other processes have not been elucidated. Little is known is how carbon metabolism is able to regulate virulence and pathogenicity in this organism. In this work, we have investigated how the metabolic network contributes to positively and negatively regulating the pathogenicity of *D. dadantii* 3937.

Chapter 1 provides an overview of the history and virulence processes in the organism. In Chapter 2, *D. dadantii* 3937 was tested in different carbon sources for its ability to induce the type III secretion system (T3SS). *D. dadantii* 3937 was able to express the T3SS in carbon sources that are upstream of the dihydroxyacetone phosphate/glyceraldehyde-3-phosphate conversion point in the Embden-Meyerhof-Parnas pathway of glycolysis. A mutation was made in the *pgi* gene, which encodes phosphoglucoisomerase, critical for the conversion of glucose-6-phosphate to fructose-6-phosphate. The *pgi* mutant had reduced expression of *hrpL* (the master

regulator of T3SS expression), *hrpA* (the type III secretion pilus), and *rpoN* (the nitrogen-related sigma factor) genes, but increased expression of *gcpA* (diguanylate cyclase), in comparison to the wild-type strain. Fructose-6-phosphate supplementation restored T3SS gene expression to near wild-type levels. Complementation of the *pgi* mutant with the *pgi* gene restored swimming, swarming, *in vivo* virulence, and expression of T3SS genes. These results suggest that the metabolic gene *pgi*, and its intermediate fructose-6-phosphate, may indirectly play a role in the expression of virulence factors in *D. dadantii* 3937.

In Chapter 3, a mutant of *D. dadantii* 3937 was produced by a *miniHimar* Tn5 RB1 transposon insertion. This mutant was unable to grow on minimal media with fructose. The insertion mapped to a 1,650bp gene product, which sequenced to the *fruA* gene, which corresponds to the fructose permease, which is the sugar transport component of the fructose phosphotransferase system (PTS). This mutant had increased *hrpS*, *hrpL*, and *hrpA* gene transcription. A *fruB* mutant showed slightly decreased T3SS activity, but *fruB* overexpression in the wild-type, and complementation in the *fruB* mutant showed increased *hrpS* expression. The FruB protein also interacted with HrpX at the protein level using a yeast 2 hybrid assay, showing increased β -galactosidase activity in comparison to empty vector control, HrpX, and FruB alone. This suggests that the fructose PTS plays a part in regulating the T3SS of *D. dadantii* 3937.

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List of abbreviations

Amp	Ampicillin sodium
c-di-GMP	Cyclic diguanosine monophosphate
Cm	Chloramphenicol
DGC	Diguanylate cyclase
Ecp	EAL-containing protein
Gcp	GGDEF-containing protein
GFP	Green fluorescent protein
Hrp	Hypersensitive response and pathogenicity
Km	Kanamycin sulfate
LB	Luria-Bertani media
MM	Minimal salts media
OD _x	Optical density at specific wavelength
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PGI	Phosphoglucoisomerase
Pel	Pectate lyase gene products
qRT-PCR/qPCR	quantitative Reverse Transcriptase-Polymerase chain reaction
RpoS	RNA polymerase subunit, stationary phase sigma factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis
Sp	Spectinomycin
T2SS	Type II secretion system
T3SS	Type III secretion system
UspA	Universal stress protein A
CRP	cAMP receptor protein

CHAPTER 1

Introduction

1.1 *Dickeya dadantii* 3937

1.1.1 Background and Significance of *Dickeya dadantii* 3937

Erwinia chrysanthemi 3937 is a Gram-negative phytopathogen. It was first observed to cause disease on greenhouse stocks of Chrysanthemums in New York, and closely neighboring states, in the 1950s. Burkholder and his associates determined that the isolated organism was a new species of *Erwinia*, and thus proposed the name *Erwinia chrysanthemi* (Burkholder, 1953). Over the course of time, better isolation techniques, molecular genotyping/phenotyping and modeling studies have shown that *E. chrysanthemi* 3937 was actually divergent from the *Erwinia* sp., and thus was renamed *Dickeya dadantii*.

D. dadantii is a globally dispersed pathogen with a wide host range. It has been found in almost all regions of the United States, as well as in Central and South America, East and West Asia. It has been known to affect bananas, different varieties of carnations and other flowering plants, potato, carrot, and the model host plant *Saintpaulia ionantha* (African violet), and a host of others (EPPO). It grows in humid, temperate climates, usually between 25°C-30°C. It has been cultured from water-logged environments, such as marshes, rivers and streams (Cother & Gilbert, 1990). During the infection process, once the bacterium has gained a stronghold within the host, it causes severe soft-rot and wilting. Soft-rot manifests itself as severe maceration, or decaying of the plant tissues, while wilting is the blockage or reduction of water transport or water pressure, respectively, thus leading to the loss of rigidity in the plant. Plant death is imminent if the wilt is permanent. *D. dadantii* is also a soil-dwelling microorganism, which can make its elimination extremely

difficult once established, and can infect newly seeded plants. It is extremely hardy, being found to exist for weeks in cattle fecal material (Lohuis, 1990). Because of the infectious nature of the organism, it can cost the farmer thousands of dollars in preventive measures to reduce or possibly eliminate the organism from crops.

The complete genome sequence of *D. dadantii* strain 3937 has been sequenced (Glasner et al., 2011). Phenotypically, *D. dadantii* 3937 is rod-shaped, with an average length and diameter of approximately 1.8 and 0.6 microns, respectively. For motility, it uses peritrichous flagella, which usually number on average 8 to 11 per cell (EPPO). Genetically, it is closely related to *Yersinia* sp. and *Escherichia coli*. This relatedness to *E. coli* makes *D. dadantii* 3937 extremely easy to work with and makes *D. dadantii* 3937 an excellent model for plant pathogen studies. Due to the similarities, most, if not all molecular techniques used for *E. coli* can be used to study this phytopathogen.

1.1.2 Virulence mechanisms of *D. dadantii* 3937

1.1.2.1 Type II secretion system

In order for *D. dadantii* 3937 to cause severe maceration and wilting of host tissue, it uses a plethora of plant cell-wall degrading enzymes (PCWDE). These enzymes are released via an ATP-dependent secretion system notated the Type II secretion system, or T2SS. The T2SS is configured via the Out system, which consists of membrane-bound, as well as soluble proteins which secrete pectinolytic enzymes into the extracellular space. The Out locus is comprised of 15 genes located within 5 transcriptional units. The transport of the Out proteins, as well as the pectinolytic enzymes, are shuttled to the periplasmic space via the general

secretory system (Sandkvist, 2001). The pectinolytic enzymes are secreted into the extracellular space, where interact with host plant tissues, and cause the breakdown of the long-chain carbohydrate pectin, which undergoes structural changes ultimately into smaller, more manageable sugar sources. These are transported into the bacterial cell, and used in pathways leading to central metabolism (Figure 1). It is the action of these PCWDE which ultimately cause the symptoms seen in the plant, such as soft-rot and plant wilt.

The regulatory pathway of pectate lyase production is positively and negatively influenced by many cellular factors (Hugouvieux-Cotte-Pattat et al., 1996, Nasser et al., 2005). Their synthesis is positively regulated by global systems such as the quorum-sensing pathway, which is encoded by *expI* and *expR* (Nasser et al., 1998). The carbon regulated cAMP receptor protein CRP also assists in positive regulation of pectate lyase activity (Hugouvieux-Cotte-Pattat et al., 1996, Reverchon & Robert-Baudouy, 1987). Negative regulation is afforded by PecT and PecS, which are thought to be repressors specific for pectate lyase secretion, but have recently been found to co-regulate a specific protein HrpN, which is in the T3SS regulon (Nasser et al., 2005). Negative regulation is also provided by KdgR, which is a pectate lyase repressor that, once it binds to pectin-based intermediates, derepresses transcription of the *pel* operon (Hugouvieux-Cotte-Pattat et al., 1996). Pel enzyme secretion is also regulated by the iron status of the cell, in which Fur, or the ferric uptake receptor, normally provides a negative effect on the transcription of the *pel* operon (Franza et al., 2002) (Figure 2).

1.1.2.2 Type III secretion system

Plants recognize specific antigens exhibited by pathogens that try to colonize them. These are known as pathogen-associated molecular patterns, or PAMPs. MAMPs, or microbe-associated molecular patterns, have been substituted in the literature, but basically describe the same mechanism for plants recognizing potential pathogen entry to activate the first stage of immunity, called PTI, or pattern-triggered immunity (Dubery et al., 2012) (Figure 3a). In order for a phytopathogen such as *D. dadantii* 3937 to successfully infect the host, it must evade the basal and systemic immune responses of the plant. To perform this feat, it uses a specific translocation system which injects specific effectors into the host cell, in order to somehow neutralize the effector-triggered immune system, or ETI (Figure 3b). The plant then responds to the attack by synthesizing specific proteins to neutralize the injected bacterial effectors (Figure 3c).

The type III secretion system, or T3SS, used by *D. dadantii* 3937 contains approximately 32 ORFs, some of which have no putative functionality (Glasner et al., 2011, Yang et al., 2002, Yang et al., 2010). Evolutionarily, the *D. dadantii* 3937 T3SS basal assembly is similar to the motor and membrane-anchoring proteins in the flagellar system (Tampakaki et al., 2004) (Figure 4a, d). Analogous to most other secretion systems, the T3SS uses ATP-hydrolysis to propel harpins and effectors through the cytoplasmic, periplasmic space, and outer membranes into the host cell cytoplasm (Alfano & Collmer, 1997, Buttner, 2002, Buttner, 2012, Buttner & Bonas, 2006, Buttner & He, 2009, Coombes, 2009, He, 1998, Kuwae & Abe, 2005). Once inside the host, these various harpins and effectors modulate host cell physiology to ultimately inhibit cellular responses to pathogen infection. There are two main

modules for regulation of the T3SS; the HrpXY pathway and the GacSA-*rsmB* pathway.

1.1.2.2.1 The HrpXY pathway of T3SS regulation

To sense its external environment, *D. dadantii* 3937 uses multiple mechanisms. It couples signal input from various sensors to effectively create a global response to pathogen interaction. For the T3SS, one of the initial sensors responsible for activation is the HrpX and HrpY two component signal transduction system (TCS), which belongs to the CheY-superfamily of phosphoaccepting proteins (Figure 5). HrpX is a 56 kDa protein, annotated as a membrane-bound, orthodox sensor histidine kinase, which under extracellular stimulation, autophosphorylates. Similar to the EnvZ protein of *E. coli*, sequence annotation has revealed that HrpX contains a putative single PAS domain, along with multiple PAS folds, an ATP-like binding site, and a dimerization and phosphoaccepting site (Glasner, 2003). Once dimerization has occurred, the putative PAS domain in HrpX is responsible for sensing unknown signals, which in all likelihood is small ligand-mediated. Once the signal has been perceived and conformational change has occurred, HrpX then hydrolyzes one molecule of ATP on a histidine residue located within the phosphoaccepting/catalytic site. This phosphate, once liberated, is transferred to the downstream cognate response regulator HrpY. HrpY is a 23 kDa soluble protein, which is responsible for accepting the phosphate from HrpX. HrpY belongs to the LuxR-family of transcriptional regulators, therefore having regulatory and effector domains. The phosphoaccepting site in the regulatory domain is predicted to be

located on aspartate 57, and a point mutation in this amino acid functionally prevent phosphorylation and activation of HrpY (Merighi et al., 2003). There is a weak sigma 70 consensus sequence upstream of the *hrpX* transcriptional start site. Also, there is no rho- dependent or independent terminator sequence located between *hrpX* and *hrpY*, indicating that this is a single transcriptional unit; therefore we can conclude that the transcription of these genes is constitutive. When HrpY is active, it then binds to a consensus sequence located in the promoter region of *hrpS* (Merighi et al., 2003).

Two more intermediate components located within the T3SS regulatory cascade needed for activation are RpoN and HrpS. RpoN, or sigma 54 ($\sigma 54$), is responsible for the regulation of nitrogen utilization genes (Reitzer, 2003) (Figure 5). HrpS is a 36 kDa enhancer DNA-binding protein included in the NtrC-family that is responsible for interacting with RpoN, and thus allows full transcription of its regulon genes (Bush & Dixon, 2012, Wei et al., 2000). The effector domain of HrpS, responsible for binding the enhancer-like element (ELE) located within the promoter of some genes, is of the Fis-type, which is also similar to the consensus sequence recognized by NtrC. HrpS also has an AAA+ ATPase domain, but it has not been shown experimentally to bind or enzymatically hydrolyze ATP. Once HrpS couples with RpoN, this allows efficient open complex formation and transcription of the primary regulator of the T3SS in *D. dadantii* 3937, *hrpL* (Li, 2010, Tang et al., 2006, Wei et al., 2000, Yamazaki et al., 2012, Yang et al., 2008, Yang et al., 2010).

The master regulator of the T3SS in *D. dadantii* 3937 is HrpL. HrpL is a 21 kDa protein, annotated as an alternative sigma factor located within the ECF-family

(Chatterjee et al., 2002, Wei & Beer, 1995). In order to promote transcription of its regulon, it binds to the core RNA polymerase, similar to the sigma 70 mechanism of RNAP interaction, and diffuses along the DNA to a consensus sequence known as the “*hrp* box.” Once active, HrpL can then advance to promoting the transcription of its regulon genes, such as the T3SS pilus *hrpA*, and various effector and harpin proteins, such as DspE and HrpN.

1.1.2.2.2 The GacS/A-*rsmB* pathway of T3SS regulation

HrpL is tightly regulated to prevent transcription of its regulon genes outside of HrpXY stimulation. This additional mode of regulation is provided by the GacS-GacA TCS. GacS is a 102 kDa membrane-spanning sensor kinase, similar to the BarA protein in *E. coli*, which under appropriate environmental stimulation autophosphorylates and relays the phosphate to its cognate response regulator (Figure 6). GacS is unorthodox; normal histidine kinases relay the phosphate to the transmitter domain, which relay the phosphate to the response regulator, while tripartite histidine kinases, such as GacS, after ATP hydrolysis, transmit the phosphate to a histidine/aspartate phosphorelay domain located within the protein, and ultimately to the response regulator. GacA, the cognate response regulator for GacS, is a 24 kDa soluble protein, which has a receiver domain capable of accepting a phosphate molecule. This acceptance causes a conformational change within the protein to allow the effector domain to recognize specific consensus sequences located within the DNA strand to initiate transcription of regulated genes. Both GacS and GacA are included in the CheY-superfamily of phosphotransfer proteins,

much like most other TCSs. The GacS/GacA TCS performs its positive role on *hrpL* translation by increasing cellular concentrations of a small regulatory RNA called *rsmB*. *rsmB*, which is 341bp in size, is structurally and functionally similar to *csrB* of *E. coli*. *rsmB* is noncoding, therefore it performs its function due to the secondary structure formed in solution. RsmA is a small 6.83kDa protein which is important in many cellular processes, such as motility, alternative carbon source usage, virulence, etc (Romeo, 1998, Romeo et al., 2013) (Figure 6). RsmA is a negative regulator of *hrpL* as well as negatively autoregulating its own translation, by promoting time-dependent degradation by binding the 5' untranslated region. *rsmB* antagonizes the function of RsmA on *hrpL* mRNA, by binding up to 18 units of RsmA to one molecule of *rsmB* at specific sites located within the stem-loop regions of *rsmB* (Liu et al., 1997). This makes *rsmB* an efficient, positive regulator of the T3SS in *D. dadantii* 3937.

1.2 Putative metabolic contributors to virulence in *D. dadantii* 3937

1.2.1 The Phosphoenolpyruvate-Phosphotransferase System

In a specific environment, the bacteria must selectively use nutrient supplies that will allow optimal cellular and metabolic efficiency. To perform this feat, bacteria have evolved mechanisms which will efficiently use sources which are commonplace to its niche. The phosphoenolpyruvate (PEP):Phosphotransferase system (PTS) consists of two main modules; a core module which is common to most sugars which are transported via the PTS system, and a sugar-specific module which recognizes membrane-spanning permeases for each individual sugar (Figure 7). Historically, the PTS was found in *E. coli* in 1964 by as a system which is able to

generate hexose phosphates via specific sugars (Kundig, 1964). After that, homologous proteins were found in other Gram-negative and low G+C Gram-positive bacteria. The core module contains Enzyme I (EI) and the histidine protein (HPr). This module is considered “generic,” in the fact that they are general contributors of phosphate flux into the general PTS pathway. EI, encoded by the *ptsI* gene, is a 63 kDa protein that is first in the phosphotransfer cascade, binding phosphoenolpyruvate and enzymatically removing a phosphate. The EI-phosphate is linked to a histidine residue, which is optimally placed once the protein dimerizes, and thus binds a Mg^{2+} cofactor, which is required for phosphotransfer (Postma et al., 1993, Deutscher et al., 2006). Continuing downstream, the phosphate is then transferred to HPr, or the histidine protein. HPr, encoded by the *ptsH* gene, is a 9 kDa singular protein which binds a phosphate on His-15 at the N-1 position on the imidazole ring of histidine. The protein interaction site on HPr is non-specific. It is able to interact with both EI, and also Enzyme II of various sugar-specific modules. This is absolutely necessary, since it will increase the affinity of phosphotransfer to various EIIs, without considerable changes in its architecture.

The specific modules of the PTS, or Enzyme II complexes, interact with HPr from the core module, and relay the phosphate to the incoming sugar molecule from the extracellular environment. The components EII_x and EII_y, where *x* is specific to a particular carbon substrate and *y* can be up to three associated protein subunits, are the sugar specific entities, i.e. EIIA^{Glc} and EII (BC)^{Glc}, is specific for glucose. According to the literature, they are broken down into specific (super) families, based on the transported sugar, i.e. the glucose/fructose/lactose superfamily, the

ascorbate/galacitol superfamily, the mannose family, and the dihydroxyacetone family (Deutscher et al., 2006) . Not all organisms have all of the PTS families, but the mechanism by which they transfer the phosphate is almost identical.

The fact that all steps of the phosphorylation cascade are reversible, this creates a stepwise point in putative cellular regulation. This concept is seen in the cAMP-CRP regulatory cascade. Since most *Enterobacteriaceae* preferably utilize glucose over other carbon sources, Enzyme IIA^{Glc} is seen as the regulatory switch over carbon metabolism. When the phosphate flux is high, due to increased glucose consumption, this will lead to a dephosphorylated state of EIIA^{Glc}. This interacts with adenylate cyclase, and in turn inhibits the production of cAMP (Evelyne Krin, 2002) (Figure 7). Studies have shown that both dephospho- and phosphorylated forms of EIIA^{Glc} can interact with adenylate cyclase, albeit the prior with less affinity (Notley-McRobb et al., 1997). Dephosphorylated EIIA^{Glc} can also interact with sugar-specific permeases to prevent the conformational change necessary for transporting sugars across the membrane (Magasanik, 1970). Bacteria also have evolved mechanisms to restrict the use of carbon which is not considered “optimal.” In most *Enterobacteriaceae*, this is termed “carbon catabolite repression,” or CCR (Figure 7). In *D. dadantii* 3937 and also *E. coli*, CCR is active when glucose is present, and the cell will preferentially use it over all other sugars which may be available in the immediate environment. The activation of CCR is preempted by a phosphorelay system which transfers a phosphate group from an intermediate of the Emden-Meyerhof-Parnas pathway of glycolysis (Figure 7a), phosphoenolpyruvate, ultimately to a membrane protein responsible for

phosphorylating the incoming glucose molecule into its esterified form. Inducer exclusion also plays a role in regulation of selective carbon utilization, by blocking the flow of carbon through other permease genes, such as LacY (lactose permease).

In some bacteria, the PTS can play a role in processes such as motility. Various components of the core PTS module, such as HPr, have been shown to modulate motility due to interference in the CheY-mediated regulation of flagellar rotation (Neumann et al., 2012). Virulence processes, such as biofilm formation, can also be modulated via fluxes in PTS-mediated phosphate transfer. In *Vibrio sp.* a mutation in $EIIA^{Glc}$ was found to decrease the ability of *Vibrio* to form a biofilm in certain environmental conditions, which is mandatory in *Vibro sp.* to establish itself within the host (Houot et al., 2010, Minato et al., 2013, Visick et al., 2007).

1.2.2 Carbon-based metabolic control of virulence processes

The process of cellular metabolism is a complex progression of intertwined pathways, which convene together for a common goal; to optimize usage of available compounds to meet the needs of the cell. The two main pathways of all living organism, are the Embden-Meyerhof-Parnas (EMP) and tricarboxylic acid (TCA) cycles. These are the core pathways, with hundreds of branching pathways either leading to, or deriving from the central core (Figure)With this mass assemblage of interconnections, the ability of the cell to regulate pathways based on the availability of specific precursors is necessary to maintain a homeostatic environment, without excessive flux and/or accumulation.

Even though the cell tries to maintain a state of equilibrium, there are some instances where the intracellular environment, which is always in flux, can cause

temporal and spatial imbalances of some processes. In pathogenic organisms, one of those processes is virulence. The process of virulence induction is taxing on the cell. The organization of some virulence operons contain hundreds of genes, which, when induced, can impart an extremely hard burden on the cell. In *Salmonella*, induction of the *Salmonella* pathogenicity islands (SPI) I and II, caused a major reduction in the growth of the microorganism (Sturm et al., 2011). This is a direct consequence of the load placed on the organism to prepare itself to invade the host.

In the last few years, investigators have been examining how the cell uses the abundance or accumulation of some carbon compounds to actually regulate virulence processes directly or indirectly, to try and preserve some form of homeostasis. If the cell has an abundance of compounds to use as potential energy sources, then there would be no reason to activate induction of virulence processes. In *Samonella*, the organism modulates its metabolism to meet the needs of virulence induction (Sturm et al., 2011). *Pseudomonas entomophila*, an organism which infects *Drosophila melanogaster*, needs to have a secondary metabolite called dipteracin in order to be lethal to the fruit fly (Vallet-Gely et al., 2010).

Some of the regulators used in global modulation of carbon metabolism are also starting to get attention as of late. Cra (FruR) is a negative regulator of the fructose operon in many Gram-negative bacteria, including *D. dadantii* 3937, *E. coli* and others. Recently, Cra has been shown in enterohemorrhagic *E. coli* (EHEC) to bind the promoter region of the master regulator of the T3SS, *ler*, or LEE-encoded regulator (Njoroge et al., 2013). This is a very prominent finding, and one of the first to show that carbon regulators are actually involved in direct regulation of

virulence. More research needs to be implemented in this area, which, in the future, may possibly be used as a therapeutic target for antimicrobial therapy.

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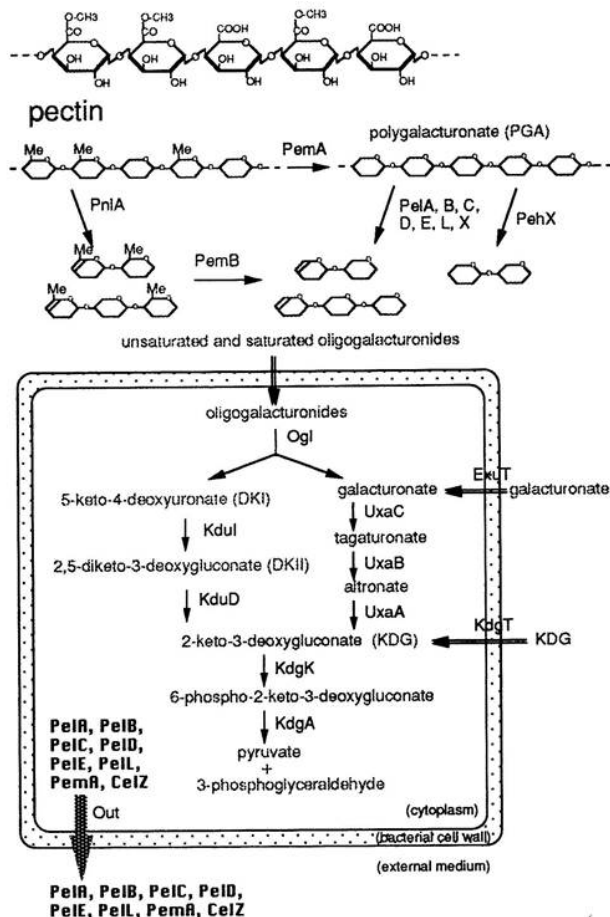
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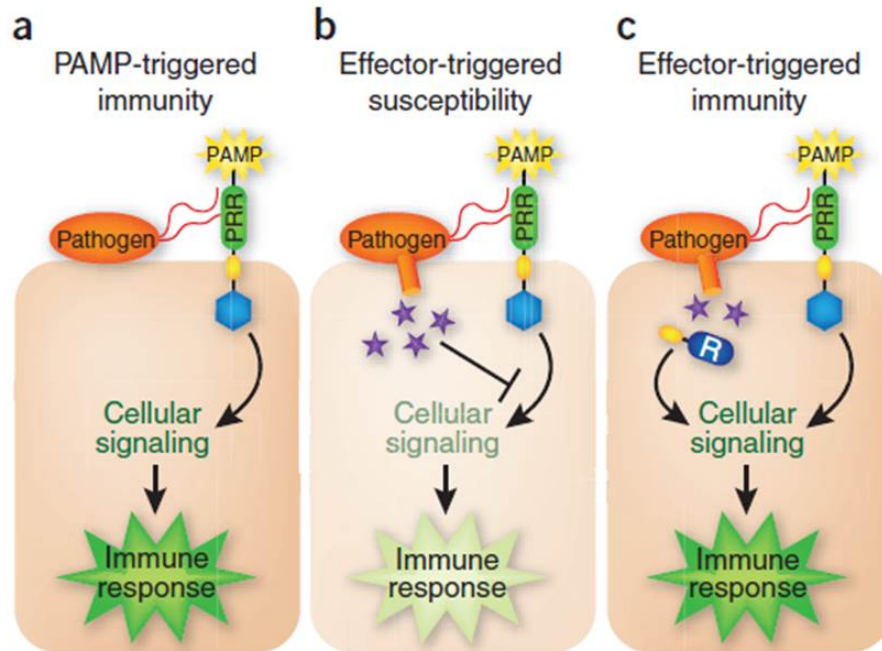
Pectin Degradation Pathway

Nicole Hugouvieux-Cotte-Pattat, Guy Condemine, William Nasser, and Sylvie Reverchon

Annu. Rev. Microbiol. 1996. 50:213–57

Figure 1: Pectin Degradation Pathway

The upper part of the figure shows the detailed structure of pectin. Pectinases outside the cell are used for pectin degradation (but note that PeiX and PemB are a periplasmic and a membrane protein, respectively). The Out proteins (*large arrow*) are involved in the secretion of most of the pectinases and of the cellulase CelZ. *Double arrows* indicate sugar transport systems. The catabolism of transported oligogalacturonides takes place within the cytoplasm.

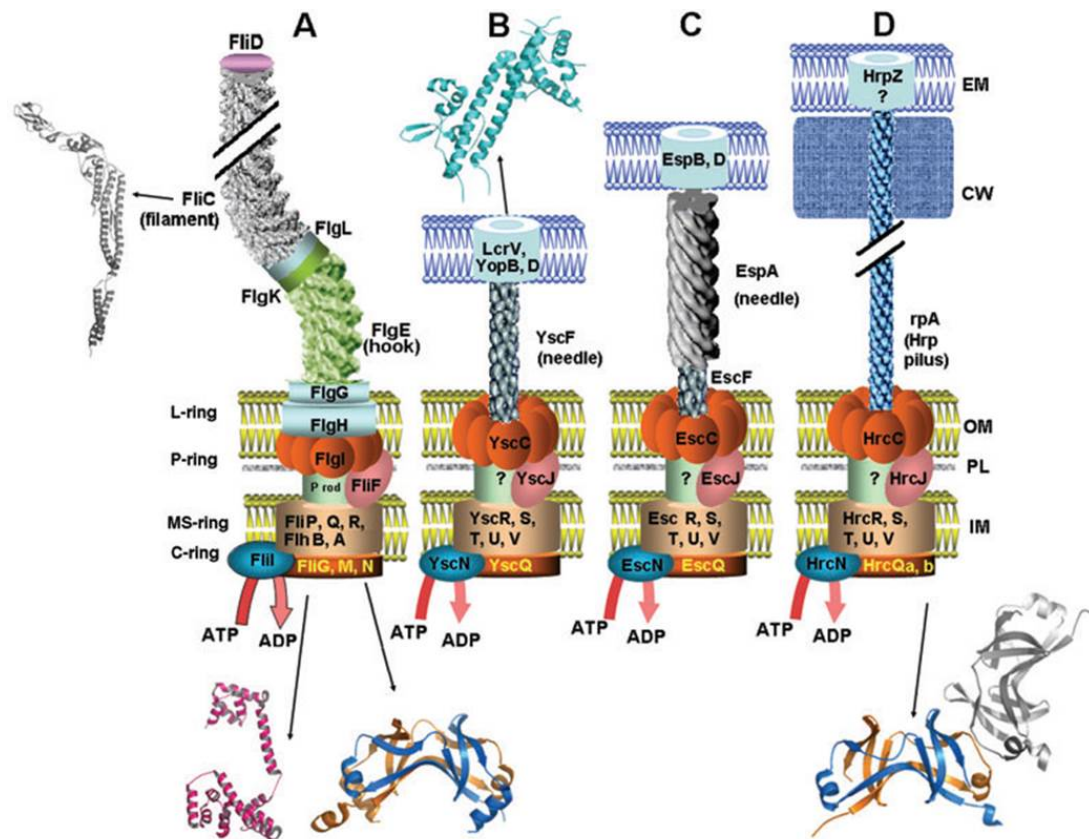


PTI- and ETI-triggered immunity

Corné M J Pieterse, Antonio Leon-Reyes, Sjoerd Van der Ent & Saskia C M Van Wees
Nature Chemical Biology 5, 308 - 316 (2009)

Figure 3: PTI- and ETI-triggered immunity

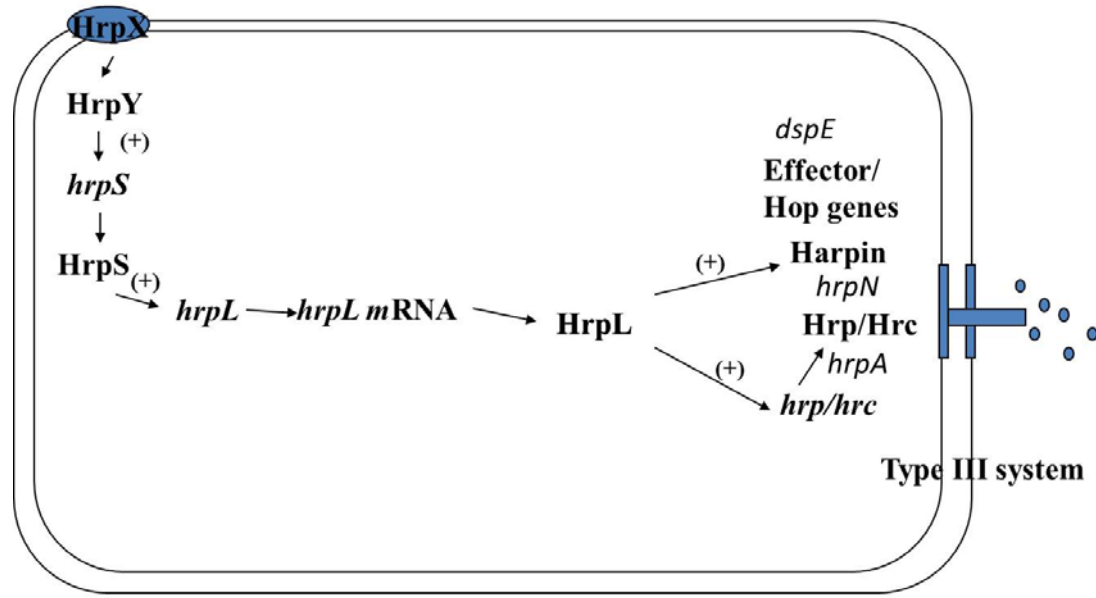
Plant immunity diagram. (a) When pathogens attack the plant, the initial trigger, pathogen-associated molecular patterns (PAMPs), activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream phosphorylation signaling cascade that leads to PAMP-triggered immunity (PTI). (b) Virulent pathogens have acquired effectors and harpins (purple stars) that suppress PTI, resulting in effector-triggered susceptibility (ETS). (c) R proteins by the plant, which are acquired and recognize these attacker-specific effectors, results in a secondary immune response called effector-triggered immunity (ETI).



A.P. Tampakaki, V. E. Fadouloglou, A. D. Gazi, N. J. Panopoulos and M. Kokkinidis
 Cellular Microbiology (2004) 6(9), 805–816

Figure 4: Conserved relationship of the flagellar and the type III secretion apparatus

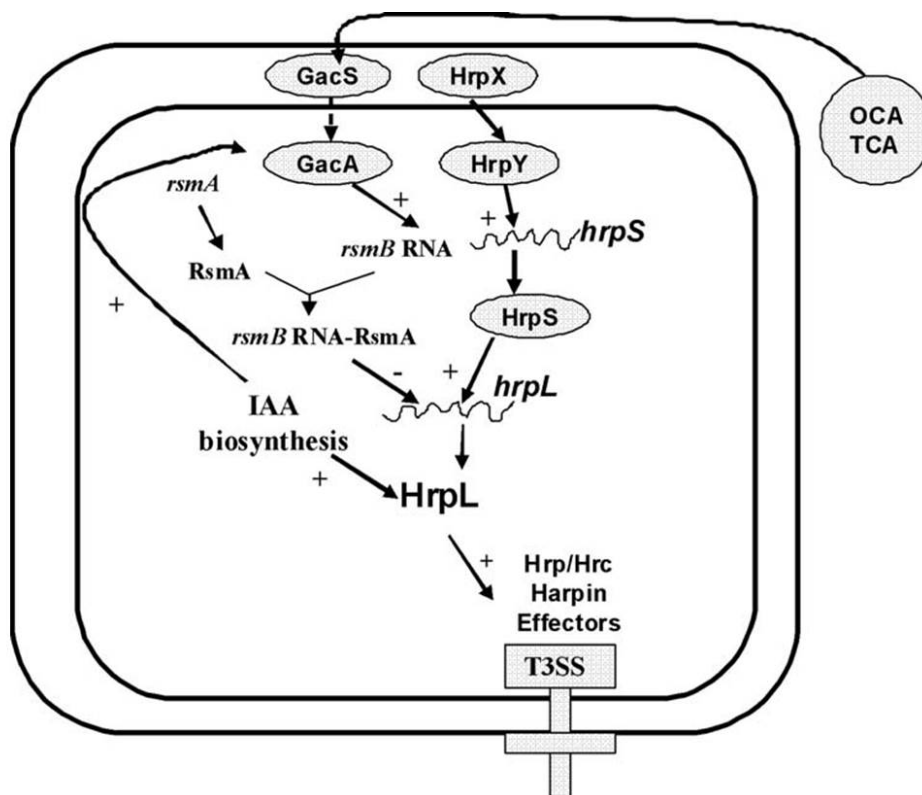
Comparison of the T3SS and flagellar system, A, and T3SS in *Yersinia* (B), *Escherichia coli*(C) and *Pseudomonas syringae* (similar to *D. dadantii* 3937)(D). Only conserved proteins in the T3SSs, and their flagellar homologues, have been drawn and are labeled by position and coloring. Some of the flagellar proteins have no homologs in the T3SS. The question mark in T3SS indicates that a bridging structure in the inner membrane has not been identified. The major constituent of the needle is YscF in *Yersinia*, EspA in *E. coli*, HrpA in *P. syringae* and *D. dadantii* 3937. Pore-forming proteins are drawn with the same color in the eukaryotic cell membrane (EM): LcrV, YopB, YopD in *Yersinia*, and EspB, D in *E. coli* . In plant pathogens, putative translocator proteins are HrpZ (*P. syringae*) and HrpF (*X. campestris*). OM, outer membrane; PL, peptidoglycan layer; IM, inner membrane of the bacterium; EM, eukaryotic membrane; CW, cell wall of the plant cells. The various components are not drawn to scale.



Li, Y., *ClpXP Protease Regulates the Type III Secretion System of Dickeya dadantii 3937 and Is Essential for the Bacterial Virulence*. Mol Plant Microbe Interact, 2010. 23(7): p. 871-78.

Figure 5: HrpX/Y regulatory pathway in *D. dadantii* 3937

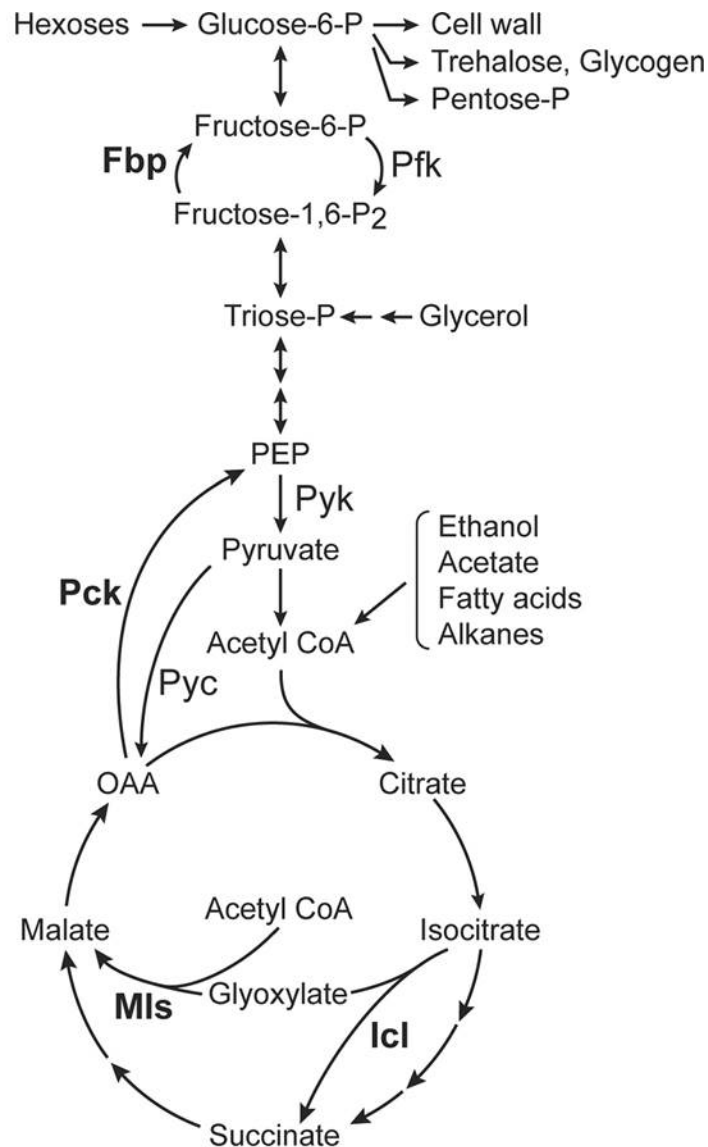
Working model of the *hrp* regulatory cascade in *D. dadantii* 3937. The HrpX sensor perceives undefined metabolic and stress signals and activates HrpY via phosphorylation (indicated by ~P). A parallel phosphorylation mechanism may also activate HrpY *in planta*. HrpY then activates *hrpS* and its own operon; HrpS and sigma54 activate *hrpL*; and HrpL enables RNA polymerase core enzyme to activate promoters containing *hrp* boxes (e.g., the harpins *dspE* and *hrpN*, as well as *hrp* secretion gene *hrpA*).



Yang S, Peng Q, San Francisco M, Wang Y, Zeng Q, et al. (2008)
 Type III Secretion System Genes of *Dickeya dadantii* 3937 Are Induced by Plant Phenolic Acids.
 PLoS ONE 3(8):

Figure 6: GacS/GacA-*rsmB* regulation in *D. dadantii* 3937

Unknown signals activate GacS, the sensor kinase and relay a phosphate to GacA, its cognate response regulator. GacA binds to the promoter region of *rsmB*, and promotes its transcription. *rsmB* then antagonizes RsmA's inhibitory function on *hrpL* mRNA.



Jardón R et al. *Eukaryotic Cell* 2008;7:1742-1749

Fig. 7a. Embden-Meyerhof-Parnas and tricarboxylic pathways.

Gluconeogenic enzymes are indicated in bold letters. Their glycolytic counterparts are also indicated. Pyruvate carboxylase (Pyc), an anaplerotic enzyme, might be needed both in gluconeogenesis and in glycolysis, depending on the carbon source. Fbp, fructose-1, 6-bisphosphatase; Pfk, phosphofructokinase; Pyk, pyruvate kinase; Icl, isocitrate lyase; Mls, malate synthase; OAA, oxaloacetate.

Chapter 2

Metabolic control of the type III secretion system of

***Dickeya dadantii* 3937**

Abstract

Dickeya dadantii 3937, the causative agent of soft rot in host plants, was tested in different carbon sources for its ability to induce the type III secretion system (T3SS). *D. dadantii* 3937 was able to express the T3SS in carbon sources that are upstream of the dihydroxyacetone phosphate/glyceraldehyde-3-phosphate conversion point within the Embden-Meyerhof-Parnas pathway of glycolysis. In contrast, the bacterium was unable to express the T3SS at wild-type levels when carbon sources that enter the pathway either at the conversion point or downstream of it. In order to elucidate the mechanism, a mutation was made in the *pgi* gene, which encodes a phosphoglucosomerase, which promotes the reversible conversion of glucose-6-phosphate to fructose-6-phosphate (Figure 7a). The *pgi* mutant was unable to grow on glucose as the sole carbon source, but when supplemented with fructose the mutant grew almost as well as the wild-type strain. The mutant also had reduced expression of *hrpL* and *hrpA* genes in comparison to the wild-type strain. The *in planta* virulence in the African violet plant, swimming, swarming, and biofilm formation were also reduced in comparison to the wild-type. When fructose-6-phosphate was supplied with fructose at a concentration of 20mM, the T3SS gene expression of the *pgi* mutant was restored to near wild-type levels. Intergenic complementation of the *pgi* gene with its native promoter restored swimming, swarming, *in planta* virulence, and expression of the T3SS genes of the *pgi* mutant. The expression of the diguanylate cyclase *gcpA* was increased 2-fold in the *pgi* mutant vs. the wild-type strain. These results suggest that the metabolic gene *pgi*

and its product fructose-6-phosphate may play a role in the expression of virulence factors in *D. dadantii* 3937.

Introduction

Dickeya dadantii 3937 is a gram negative, necrotrophic phytopathogen of the family Enterobacteriaceae, with a wide host range. It is noted for its plethora of cell wall degrading enzymes, such as cellulases, pectinases, and proteases secreted are by the type 2 secretion system (T2SS) (Hugouvieux-Cotte-Pattat et al., 1996, Nasser et al., 2005). It produces massive cell wall maceration, leading to tissue destruction and ultimately nutritional release. The ability of *D. dadantii* 3937 to successfully colonize the host is dependent on the complex relationship between various systems. It must process specific signals, which after the appropriate cellular conditions are met, activate specific processes to ultimately cause disease.

The type III secretion system (T3SS) is a highly specific transport system which translocates various effector proteins directly into the cytoplasm of the host cell (Buttner, 2002, Buttner & He, 2009, He, 1998, Vashchenko & Hvozdiak, 2007). Once translocated, the effector proteins modulate the host cell defense system, allowing for efficient colonization by the bacteria. The type III secretion system of *D. dadantii* 3937 consists of approximately 34 open reading frames located in two divergent operons, which contain *hrp* (*h*ypersensitive *r*esponse and *p*athogenicity) genes, which are responsible for secretion, structure, regulation, and virulence (Tang et al., 2006, Yang et al., 2010). Expression of the effector genes and Hrp system are controlled by *hrpL*, an alternative sigma factor which belongs to the ECF

family of sigma factors, and HrpL recognizes a specific consensus sequence located upstream of genes regulated, called the *hrp* box (Tang et al., 2006). Two well-studied pathways are known to activate *hrpL*; the HrpX/Y-HrpS pathway, and the GacS/A-*rsmB* pathway (Figure 8).

HrpX is a sensor histidine kinase which senses some unknown signal located extracellularly, and autophosphorylates. HrpY is the cognate response regulator for HrpX, which accepts the phosphate from HrpX and promotes the transcription of *hrpS*, which belongs to the sigma 54 class of enhancer-binding proteins. Along with HrpS, RpoN, which codes for sigma 54 (σ^{54}), combine with RNA polymerase and bind to the promoter region of *hrpL* and promotes its transcription (Yap et al., 2005, Tang et al., 2006).

GacS and GacA also comprise a two-component signal transduction system. It is a homolog of the BarA-UvrY TCS in *Escherichia coli*. GacS is an unorthodox histidine kinase, which autophosphorylates, and relay a phosphate to GacA, its cognate response regulator (Yang et al., 2008b, Yap et al., 2008). GacA promotes the production of *rsmB*, a regulatory small RNA which sequesters RsmA, an RNA-binding protein which binds *hrpL* mRNA at the 5' untranslated region and promotes its degradation (Yang et al., 2008b, Zeng et al., 2010). The sequestering of RsmA by *rsmB* allows accumulation of *hrpL* mRNA to initiate transcription of its regulon (Figure 8) (Liu et al., 1997). Exact activation of the type III secretion system is unknown, but promising research is being performed. Recently studies have shown that various organic phenolic compounds, which are normally found in plants, are able to elicit a type III secretion system response, while some are able to

suppress T3SS activation in *D. dadantii* 3937 and other phytopathogens (Yamazaki et al., 2012, Li et al., 2009, Yang et al., 2008a).

c-di-GMP is a small compound generated by the condensation of two molecules of guanosine triphosphate. It was first discovered in *Gluconoacetobactor xylinus* (Ross et al., 1986). The molecule is not found in eukaryotes, and thus seems restricted to bacteria. The synthesis and degradation of the c-di-GMP is performed by the actions of diguanylate cyclases (DGC) and phosphodiesterases (PDE), respectively. The diguanylate cyclases contain a specific motif responsible for the synthesis of c-di-GMP, and this is called the GGDEF motif. Mutations in any of the residues render the protein inactive (Chan et al., 2004). In the cell, some of the diguanylate cyclases are membrane-bound, while some are soluble entities (Aldridge et al., 2003, Paul et al., 2004). The degradation of c-di-GMP results in formation of pGpG, which is proposed to be inactive in the cell, and rapidly degraded by PDEs specific for this molecule (Hengge, 2009a, Tamayo et al., 2007). In order for diguanylate cyclases to be active, it must dimerize. The active site of the DGC is located at the dimerization interface, and DGCs also contain a regulatory site, called the I-site, which binds c-di-GMP to prevent further condensation and activity (Hengge, 2009a). Fluctuations in levels of the cyclic diguanosine monophosphate can cause perturbations in regulatory functions within the cell such as biofilm formation, pathogenicity, and motility (Hengge, 2009a, Kalia et al., 2013, Paul et al., 2010, Yi et al., 2010).

Nutritional status, such as carbon availability, also plays a major role in the induction of virulence processes (Brencic & Winans, 2005, Deng et al., 2010, Li et al.,

2009), and investigators are now realizing the importance of carbon-based regulation on cellular processes. Since the metabolic state of the cell can ultimately determine how bacteria will apportion the available resources, it is only appropriate that certain intermediates serve as intracellular signals. It has been shown that during infection, that primary sugars such as glucose and fructose are quickly consumed during disease processes in various soft rot Enterobacteriaceae (SRE) (Effantin et al., 2011), but whether intermediates produced during their consumption are used for signaling is not known. CRP, the catabolite activator protein, is necessary for activation of the T2SS in *D. dadantii* 3937 (Hugouvieux-Cotte-Pattat et al., 1996), but whether it is involved in the T3SS has not been well studied. In *D. dadantii* 3937, activation of pectate lyase production, which is secreted via the Type II secretion system is dependent on metabolism of pectin, a large chain carbohydrate required for induction. Intracellularly, small carbon intermediates, such as 2-keto, 3-deoxygluconate, or KDG, causes derepression of a key repressor protein, KdgR, to allow transcription of almost all pectin-based cell wall degrading enzymes in the organism (Hugouvieux-Cotte-Pattat et al., 1996, Diolez et al., 1986, Reverchon & Robert-Baudouy, 1987).

To date, unlike the T2SS, there are no known metabolic carbon intermediates that actively regulate the HrpX/HrpY pathway of the type III secretion system in *D. dadantii* 3937. Our results suggest that *pgi*, and its product fructose-6-phosphate, plays a key role in nutritional status within the cell, which modulates the activation of the T3SS and associated virulence phenotypes in *D. dadantii* 3937.

Materials and Methods

Bacterial strains, plasmids, and culture media

The bacterial strains and plasmids used are listed in Table 12. *Escherichia coli* and *D. dadantii* 3937 was routinely cultured in Luria-Bertani (LB) broth (10g of tryptone, 10g of sodium chloride, 5g of yeast extract, each per liter) at 28°C. For type III expression studies, *D. dadantii* 3937 was subcultured from an overnight growth in LB, and diluted 1:100 in *hrp*-inducing minimal media (MM) (Yang et al., 2004) at 28°C. Media were supplemented with chloramphenicol (20µg/mL), ampicillin (100µg/mL), and kanamycin (50µg/mL) as needed in both LB and *hrp*-inducing minimal media.

Mutant construction

To create the mutants needed for experimentation, a *pgi* deletion mutant was constructed by marker exchange mutagenesis as described (Yang et al., 2002), with slight modifications. Briefly, two 600bp fragments flanking each target gene were obtained. A chloramphenicol or kanamycin resistance cassette, amplified from pKD3 or pKD4 (Table 12), was ligated with these two fragments using recombinant PCR, and then cloned into pGEMT-Easy (Promega, San Luis Obispo, CA). The recombinant fragment was digested at the NotI site from pGEMT-Easy, and the resultant fragment was gel-purified and ligated into suicide plasmid pWM91, propagated in EC100λpir, and transformed into S17-1λpir. This construct was then transferred into *D. dadantii* 3937 by conjugation using *Escherichia coli* S17-1λpir. To select for strains with chromosomal deletions, transconjugants with

chloramphenicol or kanamycin and ampicillin resistance were plated on mannitol-(L) glutamic acid (MG) agar containing ampicillin and chloramphenicol or kanamycin. *D. dadantii* 3937 colonies having chloramphenicol or kanamycin and ampicillin resistance were cultured in LB broth with no selection overnight, and plated on MG media with 5% sucrose and chloramphenicol or kanamycin. Colonies were then replica plated on separate plates containing chloramphenicol and ampicillin. Colonies which were chloramphenicol or kanamycin resistant and ampicillin sensitive were isolated and confirmed by PCR using outside primers and sequenced. Complementation and overexpression was performed by cloning the various genes and their native promoters into integration vector pTCLS-Cm (Table 12).

FACS assay

The bacterial cells carrying promoter-*gfp* transcriptional fusion plasmids (Table 12) were cultured in LB broth overnight, normalized by OD₆₀₀, and then subcultured 1:100 in *hrp*-inducing minimal media broth at 28°C. Cells were harvested at the time points 12 and 24 hours after growth, and adjusted to approximately 10⁶ CFU/ml with 1X phosphate-buffered saline (PBS, 8.0g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, and 0.24g of KH₂PO₄ per liter, pH adjusted to 7.2- 7.4) before FACS analysis. A four-color BD FACSCalibur (BD Biosciences, San Jose, CA) equipped with 488- and 633-nm lasers was used to analyze the fluorescence intensity of each bacterial cell in a total collection of 10000 cells per measurement. Measurements were performed in triplicate. Bacteria were electronically gated based on forward

and side light-scattering properties. All GFP fluorescence measurements were taken using the 488-nm laser and FL1 channel on the gated population. The results were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

qPCR analysis

qPCR was employed to measure mRNA levels of *hrpL* and *rpoN* gene transcription. Bacteria were cultured overnight in LB broth. A 1:100 dilution of bacterial culture was inoculated in MM for 12 hours. Total RNA was extracted using an RNAeasy Mini-Kit (Qiagen, Valencia, CA) and cleaned up of any contaminating genomic DNA with a Turbo DNase-free kit (Ambion, Austin, TX). The resultant RNA was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA). *hrpL*, *rpoN*, and *rplU* (reference gene) (Zeng et al., 2010) message levels were quantified using the EXPRESS SyBr GreenER Universal qPCR Supermix (Life Technologies, Grand Island, NY).

Northern Analysis

Bacterial strains were grown overnight in LB media, and subcultured 1:100 in 30mL of MM media for 12 hours with aeration. Total RNA was extracted using an RNAeasy mini-kit (Qiagen, Valencia, CA), and cleaned up of any contaminating genomic DNA with a Turbo DNase-free kit (Ambion, Austin, TX). RNA samples were detected and analyzed using *rsmA* and *rsmB* probes labeled with biotin (Ambion BrightStar Psorlen-Biotin labeling and BioDetect kits, Austin, TX).

Western Analysis

For Western blotting, cells were grown overnight in LB media, and were sub-cultured 1:100 in MM for 12 and 24 hours. The cells were pelleted and suspended in 1X PBS, and adjusted to an OD₆₀₀ of 1.0. The resultant suspensions were diluted 1:1 with 2X SDS-PAGE sample buffer. The proteins were separated by SDS-PAGE, and blotted to a PVDF membrane using a Trans-Blot semi-dry apparatus (BioRad, Hercules, CA). The resultant blot was then subjected to immunodetection. The PVDF blotted membrane was washed three times with 1X PBS, and allowed to incubate in blocking solution (2% skim milk) overnight. The blocked membrane was washed two times with 1X PBS/Triton-X100 solution, and a third wash included the primary anti-RpoS polyclonal antibody for 1.5 hours. The solution was discarded, the membrane was washed twice with 1X PBS/Triton-X100, and a third wash included the secondary goat/anti-rabbit conjugated antibody with alkaline phosphatase for 1.5 hours. The solution was discarded, and washed twice with alkaline phosphatase reaction buffer, and a third wash included the chromogenic detection substrate and incubated for 20 minutes. The resultant band was quantified using the ImageJ (NIH) program to obtain relative intensity measurements.

Biofilm formation

In order to examine biofilm formation of the respective strains, cells were subjected to the protocol by O' Toole (O'Toole et al., 1999). Bacteria were grown overnight in LB broth at 28°C and adjusted to an OD₆₀₀ of 1.0. A 1:100 dilution was performed in

either fresh LB, or MM supplemented with the appropriate carbon source in a sterile, non-tissue culture treated 96-well microtiter plate. The cells were allowed to grow at room temperature ($\sim 25^{\circ}\text{C}$) for 48 hours. Adhering cells were subjected to staining with 1% crystal violet and solubilized with 95% ethanol. Absorbance was taken at an OD_{600} in triplicate.

Virulence assay

D. dadantii 3937 and associated strains were grown in LB media overnight. Cells were collected by centrifugation, and adjusted to an OD_{600} of 1.0 in 50mM sodium phosphate buffer, pH 7.0. Cells were then adjusted to a concentration of $\sim 10^7$ CFU/mL. A small hole was made in a single leaf of *Saintpaulia ionathia* (African violet), and a volume of 50 μL was pressure-infiltrated into the apoplastic spaces of the plant using a 1 mL syringe. The plants were allowed to incubate in 100% humidity for 72 hours. A total of 5 leaves from 5 individual plants per strain were used for assessment of virulence.

Pectate lyase activity

Bacteria were grown overnight in LB media. A 1:100 dilution was subcultured into MM supplemented with 0.5% polygalacturonic acid (PGA) overnight to induce pectate lyase activity. 10 μL of overnight induced sample was inoculated on premade Pel assay plates (0.5% PGA, 1% Yeast extract, 50 μM CaCl_2 , 50mM Tris-Cl (pH 8.5), 0.8% Agar, 50 $\mu\text{g}/\text{mL}$ kanamycin). Using a No.2 cork borer, a small hole was made in the center of the plate, and sealed with agarose supplemented with

kanamycin. The plates were allowed to incubate overnight at 28°C. The plates were developed using 5N H₂SO₄ for 2 minutes with swirling, washed with water and photographed.

Swimming and Swarming Assessment

Bacteria were grown overnight in LB broth with appropriate antibiotics at 28°C. Bacteria were washed in 1X PBS, and resuspended to an OD₆₀₀ of 1.0. For the swimming assay, 0.2% agar was used, while for the swarming assay, 0.4% agar was used. A 10 µL spot was placed in the center of the mannitol-glutamic acid plates (MG), and the plates were incubated for 24 hours at 28°C. The images were captured with a digital camera (Olympus) and analyzed using Adobe Photoshop software (<http://www.adobe.com>).

Results

T3SS expression in the *pgi* mutant

A test of 5 carbon sources on the expression of the T3SS in *D. dadantii* 3937 was performed (Table 1). It was observed that some carbon sources inhibited the expression of the T3SS, while some induced the expression. Glucose and fructose positively affected expression, while glycerol, casamino acids, and pyruvate negatively affected the expression levels of *hrpS* and *hrpA* (Table 1). *D. dadantii* 3937 grew at very similar rates in all carbon sources used in this study, which eliminated the possibility of decreased expression due to differences in growth (data not shown, DNS).

Since glucose and pyruvate inversely affect the expression of T3SS genes, we hypothesize that the intermediates in the Emdem-Meyerhof-Parnas pathway may have an effect on T3SS expression. In order to determine the mechanism by which different carbon sources influence the expression of type III secretion genes, a mutation of the *pgi* gene within the Emdem-Meyerhof-Parnas pathway was made. The *pgi* gene, which encodes phosphoglucosomerase, catalyzes the reversible conversion of glucose-6-phosphate to fructose-6-phosphate. The *pgi* mutant was unable to grow as well as the wild-type strain with glucose as the sole carbon source. When grown on fructose, the *pgi* mutant grew at a rate comparable to the wild-type strain (DNS). Because of this, fructose was chosen to be the sole carbon source for the remainder of the study.

In order to observe the transcriptional activity of the T3SS under *pgi*-deficient conditions, a promoter-probe reporter was constructed, in which promoter regions of specific type III genes were cloned in front of promoterless GFP gene. The promoter activities of *hrpS* (an enhancer protein), *hrpL* (the master regulator of T3SS expression), and *hrpA* (the needle-like pilus) were all reduced in the *pgi* mutant in comparison to the wild-type at 12, and 24 hours of growth (Table 2). Notably, in the case of *hrpS*, transient expression changes were seen in the *pgi* mutant strain. In order to verify if the *pgi* mutation was responsible for the reduction seen in expression of the T3SS, the *pgi* open reading frame and its native promoter was cloned into the integration vector pTCLS-Cm. FACS analysis was performed to observe if T3SS expression could be restored in the complemented strain. Our study showed that the promoter activities of *hrpS*, *hrpL*, and *hrpA* were

all restored to almost wild-type levels at 12 and 24 hours of bacterial growth (Table 2). Gene expression of *hrpXY*, the response regulator, was unchanged between wild-type and the mutant (Table 3).

The *pgi* mutation doesn't affect the T3SS through the RsmA/*rsmB* pathway

In *D. dadantii* 3937, the T3SS can be further regulated by the GacS-GacA-*rsmB* RNA regulatory pathway, that can reduce the time-dependent degradation of *hrpL* mRNA via RsmA at the posttranscriptional level (Li et al., 2009, Yang et al., 2008a). In order to detect whether the reduction in *hrpL* mRNA was due to a decrease in the amount of *rsmB* production, Northern analysis was performed. Interestingly, compared to the wild-type strain, there were similar levels of *rsmB* RNA produced in the *pgi* mutant, while there was a significant decrease in *rsmA* message levels (Figure 9).

Since the RsmA protein promotes the degradation of *hrpL* mRNA, we expect little contribution of RsmA on *hrpL* mRNA degradation in the *pgi* mutant. We measured the *hrpL* mRNA level in wild-type and *pgi* mutant. As expected, there was an approximate 2.5-fold reduction in *hrpL* mRNA in the *pgi* mutant in comparison to the wild-type (Figure 10) which is similar to the 3-fold reduction of promoter activity of *hrpL* in the *pgi* mutant (Table 2).

rpoN, or sigma⁵⁴, is needed for the full induction of *hrpL* expression at transcriptional level (Tang et al., 2006). To monitor if *rpoN* expression was different between the wild-type, Δ *pgi*, and Δ *pgi*-complemented strains, qPCR was performed. In accordance with the decrease seen in *hrpL* expression, *rpoN* relative expression

was lower in the Δpgi strain versus the wild-type. The Δpgi -complemented strain had a slightly higher, but non-significant increase in *rpoN* message levels (Figure 11).

Fructose-6-Phosphate is necessary for full T3SS induction in *Dickeya dadantii* 3937

The functional activity of phosphoglucosomerase is to catalyze the reversible conversion of glucose-6-phosphate to fructose-6-phosphate. When fructose is used as the sole carbon source, it passes through FruA, the fructose permease and is concomitantly phosphorylated to fructose 1-phosphate. Fructose-1-phosphate is subsequently phosphorylated to fructose-1, 6-bisphosphate via FruK, and then enters the Emden-Meyerhof-Parnas pathway of central carbon metabolism (Postma et al., 1993). For the *pgi* mutant, when grown on fructose as the sole carbon source, the only notable potential supply of the metabolite fructose-6-phosphate is produced via gluconeogenesis, since *D. dadantii* 3937 has a non-functional Entner-Doudoroff pathway. We therefore complemented the fructose media with the compound disodium fructose-6-phosphate (Sigma, St. Louis, MO), at a combined final concentration of 20mM. Our results showed that *hrpS*, *hrpL*, and *hrpA* transcription was restored to near wild-type levels (Table 2), providing evidence that the product of the *pgi* gene, fructose-6-phosphate, is a potential signaling molecule, or possibly an effector of the type III secretion system in *D. dadantii* 3937.

***uspA* expression is altered in the *pgi* mutant, but doesn't affect T3SS expression**

The universal stress protein is responsible for protection against various environmental stresses, such as metabolite deficiencies, and exposure to heat and antibiotics (Freestone et al., 1997, Gawande & Griffiths, 2005, Nystrom & Neidhardt, 1996, Nystrom & Neidhardt, 1994, Zhang & Griffiths, 2003). Previous studies have shown that UspA was metabolically controlled by the glycolytic intermediate fructose-6-phosphate (Persson et al., 2007). Because of this, *uspA* expression was investigated in the wild-type, Δpgi , and Δpgi -complemented strains. In the *pgi* mutant strain, the expression of *uspA* was increased in contrast to the wild-type and Δpgi -complemented strains (Table 4).

We further decided to examine if we could detect a phenotypic change in the T3SS expression in a *D. dadantii* 3937 *uspA* mutant. *uspA* deletion mutant construction in *D. dadantii* 3937 was attempted, but unsuccessful after multiple efforts. The gene was therefore deemed essential. In order to clarify if there was an impact on the type III secretion system of *D. dadantii* 3937 due to an overproduction of this protein, the *uspA* ORF and its native promoter was cloned into plasmid pTCLS-Cm, integrated into the genome, and expressed in the wild-type strain. Expression of *hrpS*, *hrpL*, and *hrpA*, was observed at 12 and 24 hours. With a single-copy overexpression of *uspA*, there was no statistical difference on the expression of *hrpS*, *hrpL*, and *hrpA*, allowing us to conclude that the *pgi* mutation and its production of fructose-6-phosphate works through another pathway independent of *uspA* (Table 5).

To eliminate any further contribution of *uspA* to the potential negative regulation of the T3SS, we cloned the *nptII* promoter upstream of the *uspA* ORF. This was also integrated within the intergenic region of *D. dadantii* 3937. Even after strong, constitutive expression of *uspA*, there was no effect on the expression for *hrpS*, *hrpL*, and *hrpA* (Table 5).

Absence of *pgi* affects the expression of RpoS in *D. dadantii* 3937

In a *pgi* mutant of *Escherichia coli*, there was an increase in the production of the general stress sigma factor, RpoS (Farewell et al., 1998, Gawande & Griffiths, 2005, Persson et al., 2007). Therefore, we assessed the transcription of the *rpoS* promoter in the wild-type and *pgi* mutant strains. Compared to the *pgi* mutant, there was a noticeable increase from wild-type levels in the transcriptional activity of *rpoS* at 12 and 24 hours (Table 6).

Because RpoS is tightly regulated at the transcriptional, as well as the post-translational level, we also quantified its production via Western Blot analysis (Hengge, 2009b). Following the same trend as the FACS results, the *pgi* mutant strain contained increased amounts of detectable RpoS protein when compared to the wild-type and Δ *pgi*-complemented strains at 12 and 24 hours (Figure 12).

RpoS induction is dependent on a multitude of factors, and the intermediate UDP-glucose has been shown to be an intracellular signaling molecule (Böhringer, 1995). In order to discern if this intermediate was necessary for *rpoS* regulation, we supplemented the MM media with a sublethal concentration (0.002%) of glucose, and measured *rpoS* transcription via FACS analysis. At 12 and 24 hours, expression

of *rpoS* was not restored to wild-type or complement levels after sublethal glucose supplementation (Table 6).

Diguanylate cyclase expression is increased in the *pgi* mutant

c-di-GMP, or cyclic diguanosine monophosphate, is formed via the condensation of two molecules of guanosine triphosphate, GTP, and subsequently degraded into pGpG, and ultimately into GMP via phosphodiesterases. The small signaling molecule c-di-GMP has been shown to play a pleotropic role in bacterial phenotypes, such as biofilm formation, virulence, and motility (Armitage, 1992, Monds et al., 2007, Paul et al., 2010, Yi et al., 2010). Because of its pleotropic effects, we decided to observe the expression of three genes, *gcpA*, *gcpE*, and *ecpC*, which encode two diguanylate cyclases and a phosphodiesterase, respectively. In the wild-type, there was high-level expression of *ecpC* at 12 hours (Table 7), which would dictate that the c-di-GMP levels in the cell are probably low throughout the lifespan of the bacteria. At 24 hours, the expression level of *ecpC* in the wild-type and *pgi* mutant strains were similar. For *gcpE*, there was no significant difference between the three strains at 6, 12, and 24 hours. The expression of *gcpA* was initially increased in the wild-type at 6 hours, but this increase quickly diminished at 12 and 24 hours based on FACS analysis, with the *pgi* mutant increasing the expression of *gcpA* greater than 2-fold at 24 hours (Table 7).

In the *pgi/gcpA* double mutant, the expression of *hrpS* remained the same as the wild-type and Δ *pgi* strains, but the expression of *hrpL* was restored to wild-type

levels (Table 8). Unexpectedly, the transcriptional activity of *hrpA* was not restored to wild type levels (Table 8).

The expression of the catabolite activator protein CRP is increased in the *pgi* mutant

CRP, or the cyclic AMP receptor protein, is a protein responsible for regulating global carbon usage in the bacterial cell (Shimada et al., 2011). In order for it to perform its function, it must bind a molecule of cAMP. 3'-5' cyclic adenosine monophosphate, or cAMP, is a small signaling molecule formed by the cyclization of a molecule of adenosine triphosphate by the enzyme adenylate cyclase (Yang & Epstein, 1983). CRP has also been shown to play a role in virulence regulation (Heroven et al., 2012). Since fructose was used as the sole carbon source, CRP is expected to be active, due to the increase in phosphorylated EIIA^{Glc} to promote adenylate cyclase activation (Gorke & Stulke, 2008). To observe if there was a difference in *crp* expression between the wild-type, Δpgi , $\Delta pgi::pgi$ strains, a GFP-reporter with the *crp* promoter region was constructed. At 12 hours, the expression of CRP was increased in the Δpgi and $\Delta pgi::pgi$ strains vs. wild-type 7-fold and 5-fold, respectively (Table 9). At 24 hours, the expression of *crp* increased to ca. 9-fold in the *pgi* mutant strain, while the expression decreased in the $\Delta pgi::pgi$ strain to approximately 3-fold (Table 9). An increase was also seen in the *pgi/gcpA* mutant strain, with the increases in *crp* expression mirroring the increase seen in the *pgi* mutant, with increases of 6.6-fold and 8.7-fold at 12 and 24 hours, respectively (Table 9).

Pel enzyme secretion is unaffected in the *pgi* mutant

Since the metabolic balance has possibly been altered in the *pgi* mutant, this may result in the inability of the strain to effectually regulate cellular processes. Pectate lyase regulation is dependent on intermediates produced via the degradation of pectin. The pectate lyases (Pel) secreted by the type II secretion system are used by *D. dadantii* 3937 to produce maceration of host plant tissues. To examine Pel-specific activity, the cells were grown in minimal salts media (MM) supplemented with 0.5% v/v polygalacturonic acid (PGA). The supernatant, as well as the cell fraction was used to examine Pel activity. Upon inoculation of each sample on the agar plate containing PGA, the *pgi* mutant did not show a reduction in Pel-specific activity in comparison to the wild-type and complemented strains (Figure 13).

Swimming and swarming is compromised in the *pgi* mutant

In most plant pathogenic bacteria, motility is necessary for successful colonization (Antunez-Lamas et al., 2009, Cesbron et al., 2006, Ottemann & Miller, 1997). Motility is dynamic and regulated by a multitude of factors, and each plays an important role in how the cell responds to signal input. Consequently then, the cell can appropriately modulate flagellar function to either inhibit or induce movement, by tumbling or running (Armitage, 1992). We examined the swimming and swarming ability of the *pgi* mutant in comparison to the wild-type and *pgi*-complemented strains. In the *pgi* mutant, motility on both the swimming and

swarming plates was completely diminished, while in the *pgi*-complemented strain, motility was restored to wild-type levels (Figure 14).

Biofilm formation is compromised in the *pgi* mutant

In most bacterial strains, the formation of biofilm, or any other extracellular matrix, is essential for virulence and surviving in harsh environments (Barak et al., 2007, Jahn et al., 2011, O'Toole et al., 1999). By using a biofilm, this aids in the bacteria's ability to colonize the host, thus enhancing their survival. Cells were cultured, diluted, and inoculated into fresh MM containing either glucose or fructose, and also LB media. Crystal violet staining was performed, and biofilm formation was assessed between the wild-type and *pgi* mutant strains. At an OD₆₀₀, biofilm formation was reduced in the *pgi* mutant compared to the wild-type strain (Figure 15).

***In planta* virulence is reduced in the *pgi* mutant**

It has been shown that a *Pseudomonas syringae* mutant is deficient in the ability to cause localized cell death in the plant (Deng et al., 2010). *D. dadantii* 3937 is a phytopathogen; therefore intact virulence mechanisms are critical to its survival (Brencic & Winans, 2005, Buttner & He, 2009, Charkowski et al., 1997, Li, 2010, Okinaka et al., 2002, Tang et al., 2006, Yamazaki et al., 2011, Yang et al., 2002, Yang et al., 2008b, Yang et al., 2010, Yang et al., 2004). In order to observe if a mutation in *pgi* would decrease the ability of the bacteria to infect the host plant, wild-type, Δ *pgi*, and Δ *pgi*-complemented strains were inoculated into *Saintpaulia ionathia* (African

violet) at a concentration of 10^7 CFU/mL. After 72 hours incubation, the Δpgi strain was deficient in its ability to cause disease in the host plant, while the virulence of the Δpgi -complemented strain was restored to near wild-type maceration levels (Figure 16).

Discussion

The ability of *D. dadantii* 3937 to cause maceration on the host plant is dependent on multiple factors. The T3SS allows subversion of the host cell's immune system via translocation of virulence factors, and in the interim, the bacteria can synthesize the appropriate enzymes for use in pectinolysis and secrete them via the T2SS. It secretes these enzymes into the external milieu to cause concurrent maceration and tissue damage. The cell's usage of the T2SS and T3SS, albeit extremely necessary, are only a small part of the complete battery of processes needed to effectively reside in the host and multiply.

The *pgi* mutation affects the T3SS at *hrpL* transcriptional level

In *D. dadantii* 3937, we have demonstrated that the expression of T3SS virulence genes *hrpS*, *hrpL*, and *hrpA* are reduced when specific nutrient sources are introduced into the media (Table 1). Carbon sources which are upstream of the glyceraldehyde-3-phosphate/dihydroxyacetone phosphate interchange point, such as glucose and fructose, allow normal induction of the T3SS. Sources downstream, such as glycerol, casamino acids, and pyruvate, inhibit induction. When the *pgi* gene, which encodes phosphoglucoisomerase is mutated in *D. dadantii* 3937, this mutation causes a similar phenotype that is seen when carbon sources at or below

the interchange point are used, even though fructose was used as the sole carbon source. It should be noted that, the carbon sources used in Table 1 also caused a stable reduction in *hrpS*, which suggests that there may be a more global effect on the cell than what is seen in the *pgi* mutant (Table 2), since *hrpS* expression changes were transient in that strain. Changes in expression of *hrpL* and *hrpA* were very consistent in the *pgi* mutant. Because of this observation, we concentrated our efforts on pathways which would allow us to postulate a regulatory mechanism for the effects on *hrpL* expression in the *pgi* mutant. To confirm that the effect seen in the *pgi* mutant was downstream of the HrpX/HrpY signaling cascade, the promoter activity of the *hrpXY* operon, which are genes responsible the extracellular activation of the T3SS, was unchanged from the wild-type (Table 3). This therefore provides evidence that an effect of the *pgi* mutation occurs at the *hrpL* level.

The reduction in T3SS expression is downstream of glucose-6-phosphate in the *pgi* mutant

In the *pgi* mutant, the enzyme phosphoglucosomerase is not available, and if grown on glucose, will produce lethal amounts of glucose-6-phosphate. Because of this, fructose was used as the sole carbon source. The molecule glucose-6-phosphate is used as a precursor for the formation of ADP- and UDP-glucose, which are important for the formation of outer membrane synthesis, pentose phosphate pathway precursors, as well as glycogen formation. A *pgi* mutant in *E. coli* was defective in UDP-glucose formation (Böhringer, 1995), which is thought to be an intracellular signal for RpoS production. Li observed that in *D. dadantii* 3937, virulence was increased in an *rpoS* mutant (Li, 2010). Therefore, we looked at the

transcriptional expression, as well as the protein level of *rpoS*. In the *pgi* mutant, there was increased transcriptional expression of *rpoS*, as well as increased RpoS protein levels (Table 6 and Figure 12). With intergenic complementation of the *pgi* gene, the expression of *rpoS* was reduced near to wild-type levels (Figure 12). Therefore, to see if the presence of glucose-6-phosphate would be able to restore the virulence of *D. dadantii* 3937 by reducing RpoS levels and possibly restoring redox balance, we used sublethal doses of glucose to allow the production of glucose-6-phosphate in the cell. When 0.002% glucose was used in addition to fructose supplementation, the expression of *rpoS*, *hrpL* and *hrpA* was still not returned to wild-type levels (Table 6 and Table 10). This observation led us to believe that the metabolic regulation of the T3SS through RpoS in *D. dadantii* 3937 is downstream of glucose-6-phosphate in the *pgi* mutant.

RsmA/*rsmB*-RNA is not the effector of differential T3SS expression in the *pgi* mutant

The T3SS in *D. dadantii* 3937 is also co-regulated by the RsmA/*rsmB* pathway. In this pathway, the production of *rsmB* via the GacS-GacA two-component signal transduction system sequesters RsmA, a CsrA homolog, and prevents RsmA from binding *hrpL* mRNA, and promote its time-dependent degradation (Yang et al., 2008b, Liu et al., 1997, Romeo, 1998). In the *pgi* mutant, the production of *rsmA* mRNA is reduced in comparison to the wild-type strain, while the production of *rsmB* mRNA is similar between the two strains (Figure 9). In addition, RsmA is positively regulated by RpoS (Yakhnin et al., 2011). With increases in transcriptional, and also protein levels of RpoS in the *pgi* mutant (Table

6 and Figure 12), this should, in essence, increase cellular levels of RsmA. This is not observed in the Northern blot (Figure 9), but shows that *rsmA* mRNA levels are decreased in the *pgi* mutant. The reduced *rsmA* mRNA level may suggest that some other mechanism takes precedence over the *rpoS*-mediated regulatory pathway in the *pgi* mutant (Li, 2010). We postulated that the reduction in *hrpL* mRNA is not dependent on a functional RsmA/*rsmB* RNA pathway in the *pgi* mutant, and since there was no significant difference of *hrpS* transcription, the *pgi* mutation's effect on the T3SS must be at the *hrpL* level.

Pectate lyase production is unaffected in the *pgi* mutant

Chatterjee has shown that pectate lyase production in *D. dadantii* 3937 is negatively regulated, in part, by RsmA (Chatterjee et al., 1995). In the *pgi* mutant, the mRNA of *rsmA* is decreased from the wild-type (Figure 9). Interestingly, the pectate lyase supernatant and cell fraction levels via plate assay were not significantly different from the wild-type (Figure 13), although the *in planta* virulence of the *pgi* mutant was decreased from the wild-type and Δ *pgi*-complemented levels (Figure 16). This doesn't seem to correlate with the pectate lyase activity, since maceration is caused by the secretion of pectate lyases into the surrounding milieu. The regulation of pectate lyase production is multifactoral. Possibly, the reduction in *rsmA* is not sufficient to cause a concurrent decrease in pectate lyase production in the *pgi* mutant. In addition, the outer membrane of the *pgi* mutant may be compromised, since the precursor glucose-6-phosphate is not available. This may inadvertently cause periplasmic and outer membrane instability, which may impact the ability of the *pgi* mutant to infect its host. This

theoretically may manifest itself via decreased maceration of the host, or increased susceptibility to host defenses.

UspA doesn't contribute to T3SS regulation in *D. dadantii* 3937

When the fructose media was supplemented with fructose-6-phosphate, the expression of *hrpL* and *hrpA* was restored to wild-type levels (Table 2). UspA, the universal stress protein, is a DNA transcriptional regulator which has been characterized to play a role in the stress response in *Escherichia coli* (Freestone et al., 1997, Gawande & Griffiths, 2005, Nystrom & Neidhardt, 1994). It also plays a role in the virulence of *Salmonella typhimurium* (Liu et al., 2007), but its effect in *D. dadantii* 3937 is unknown. Previous research has shown that in a *pgi* mutant in *E. coli*, the transcription of *uspA* is increased in comparison to the wild-type strain, which was mirrored in our *pgi* mutant (Table 4). The function of UspA is controlled by the metabolic intermediate fructose-6-phosphate (Persson et al., 2007). Since *E. coli* and *D. dadantii* 3937 are fairly close genetically, we postulated that UspA might be causing the reduction of *hrpL* at the transcriptional level, due to a deficiency in fructose-6-phosphate in the *pgi* mutant. In *D. dadantii* 3937, we were not able to generate a mutation in this gene; therefore using single-copy native and *nptII* promoter overexpression of *uspA*, the *pgi* mutant was still unable to generate an effect on the expression of *hrpL* and *hrpA* transcription different from the wild-type strain (Table 5). This allowed us to conclude that *pgi*, and the deficient production of fructose-6-phosphate, was indirectly controlling T3SS expression through some other mechanism, but not through UspA.

RpoN production is affected by the *pgi* mutation

RpoN (sigma54), which is encoded by the *rpoN* gene, is responsible for a host of regulatory processes, including nitrogen assimilation (Reitzer, 2003). In the *pgi* mutant, there is a reduction of *hrpL* mRNA in comparison to the wild-type strain (Figure 10). Due to the reduction of *hrpL* mRNA in the mutant, we looked at other regulators which are responsible for *hrpL* transcription. In *D. dadantii* 3937, *hrpL* expression is dependent on two known proteins, HrpS and RpoN (Tang et al., 2006, Yang et al., 2008b, Yang et al., 2010, Yap et al., 2008, Yi et al., 2010). Since the transcription of *hrpS* in both the wild-type and mutant was transient and not significantly different and we were observing a reduction in *hrpL* transcription in the *pgi* mutant (Table 2), we looked at cellular expression of *rpoN*. In the *pgi* mutant, there was a reduction in *rpoN* mRNA levels in comparison to the wild-type strain (Figure 11). The reduction was eliminated by functionally complementing the mutant with an intergenic copy of *pgi* (Figure 11). This suggests that the absence of *pgi* is affecting the expression of *hrpL* through *rpoN*.

Diguanylate cyclase expression is affected in the *pgi* mutant

Cyclic diguanosine monophosphate, or c-di-GMP, is a small signaling molecule which has been getting a lot of attention as of late. It has been shown to play a pleiotropic role in many bacterial phenotypes, such as motility, virulence, biofilm formation, cell division, and cell morphology (Ham, 2013, Hengge, 2009a, Monds et al., 2007, Pesavento & Hengge, 2009, Yi et al., 2010). Yi et al. showed that in the phosphodiesterase mutant strains *ecpB* and *ecpC* of *D. dadantii* 3937, the *rpoN* levels were decreased in those strains (Yi et al., 2010). Because the *rpoN* mRNA

levels were lower in the *pgi* mutant than the wild-type (Figure 11), it is possible that metabolic flux within the cell is causing modulation in the activity of DGCs and PDEs and may play a role in the reduction of *rpoN*. We assessed the transcription of diguanylate cyclases *gcpA* and *gcpE*, and phosphodiesterase *ecpC* in the wild-type, Δ *pgi*, and Δ *pgi::pgi* strains. In the *pgi* mutant, there was a 2-fold increase in transcriptional expression of the diguanylate cyclase *gcpA* from 12 to 24 hours, but the expression of the diguanylate cyclase *gcpE*, and the phosphodiesterase *ecpC* was fairly constant throughout the time points tested (Table 7). This shift in *gcpA* expression may be sufficient to increase the cellular levels of c-di-GMP, and impact the phenotypes seen in the *pgi* mutant, such as the reduction in *hrpL* and *hrpA* expression levels, possibly through modulation of *rpoN*. In order to see if a *gcpA* mutation could restore the expression of *hrpL* and *hrpA*, a double mutant was generated in *pgi* and *gcpA*. Unexpectedly, *hrpL* expression could be restored, but *hrpA* expression wasn't (Table 8). The expression of *hrpA* in differing *hrpL* backgrounds has been previously reported (Zeng et al., 2012). It is possible that in the *pgi* mutant, there are additional factors required for full induction of *hrpA*, since in the *pgi/gcpA* double mutant *hrpL* was restored to near wild-type levels. This concept is not well understood, and a hypothesis is given in the next section.

CRP expression is affected in the *pgi* mutant

The CRP protein is extremely important in cellular metabolism. The ability of the cell to utilize carbon sources other than glucose is dependent on a functional cAMP:CRP complex, which allows for high specificity recognition of consensus sequences, optimal promoter clearance and association with RNA Polymerase, and

ultimately production of enzymes needed for utilization of those alternative carbon sources, such as fructose and lactose (Kuhlman et al., 2007, Ryu et al., 1995). In this work, *crp* expression was increased ca. 6-fold and 9-fold in the *pgi* and *pgi/gcpA* mutant strains, while in the *pgi::pgi* strain, the increase was limited to 5-fold and 3-fold, at 12 and 24 hours, respectively, showing partial complementation in the Δ *pgi::pgi* strain (Table 9). The expression of *crp* is dependent on two factors, 1) the presence of the transcriptional regulator Fis, and 2) the presence of cAMP:CRP itself (Gonzalez-Gil et al., 1998). In the *pgi* mutant, previous research has shown that the cellular concentration of ppGpp, the cellular stress alarmone, is increased due to the metabolic deficiency of fructose-6-phosphate (Persson et al., 2007). ppGpp is a negative regulator of Fis, preventing transcription from the promoter by inhibiting RNAP binding via DksA (Mallik et al., 2006). This regulatory paradigm in the *pgi* and *pgi/gcpA* mutant strains might explain the increase in *crp* expression. Interestingly, in the *pgi/gcpA* double mutant the expression of *hrpL*, the T3SS master regulator was restored to near wild-type levels, but *hrpA* expression wasn't recovered (Table 8). In the promoter region of *hrpA*, there is a putative CRP-binding site located at the -70 position relative to the translational start site (Figure 17). This site is not 100% to consensus, but it is possible that, during high levels of CRP in the cytoplasm, the binding of CRP to this site might actually be repressing *hrpA* transcription, since in the *pgi* and *pgi/gcpA* mutant strains, *hrpA* transcription was not restored to wild-type levels under all conditions tested with the exception of *pgi* gene complementation and fructose-6-phosphate supplementation (Table 2).

Motility is decreased in the *pgi* mutant

Motility is necessary for full virulence in most plant pathogens, and also in *D. dadantii* 3937 (Antunez-Lamas et al., 2009). The increase in *gcpA* transcription, which would effectively increase c-di-GMP levels, would manifest itself in modulations in motility as well. Increases in c-di-GMP levels have been shown in some bacteria to cause decreased motility (Ham, 2013, Hengge, 2009a, Monds et al., 2007, Yi et al., 2010). Also, certain c-di-GMP effector proteins, such as YcgR, have been known to interact with specific proteins of the flagellar apparatus to modulate their function (Paul et al., 2010). The *pgi* mutant was non-motile, and motility was restored when the mutant was functionally complemented (Figure 14). Also, preliminary TEM micrographs showed that the *pgi* mutant had intact flagella on the cell surface (DNS). The increases in intracellular levels of c-di-GMP might possibly be affecting flagellar function through specific effectors modulated by c-di-GMP (Paul et al., 2010). This would support the hypothesis that motility is partially controlled by increased activity of diguanylate cyclases in the *pgi* mutant, due to increased levels of c-di-GMP.

Biofilm is reduced in the *pgi* mutant

Biofilms are extremely important in the lifestyle of both human and plant pathogens. Biofilm composition varies with the organism, and its composition usually relies on the environment in which the organism inhabits, along with nutrient sources available to it. In some organisms, such as *Mycobacterium tuberculosis*, the organization of the biofilm is predominantly free fatty acids, more specifically mycolic acids (Ojha et al., 2008). Because biofilm formation is often

necessary for the establishment of bacteria within their host, it is important to assess its production. When rich media was used, both the wild-type and *pgi* mutant strains formed a biofilm at a very low level. Under conditions that are favorable for the induction of biofilm, such as in minimal salts media, the wild-type strain produced a biofilm at a very high level, while the *pgi* mutant was unable to do so (Figure 15). Also, the *pgi* mutant may be incapable of producing intermediates responsible for the efficient production of the biofilm, even in the presence of increased c-di-GMP levels. Interestingly, in fructose media the wild-type strain formed an even greater amount of biofilm, reflected in the crystal violet staining results.

In summary, a mutation in *pgi*, encoding phosphoglucosomerase, was found to exhibit decreased levels of expression for T3SS genes *hrpL* and *hrpA*, exhibited increased diguanylate cyclase *gcpA* transcription leading to increased c-di-GMP levels, had decreased motility, and also reduced *in planta* pathogenicity. Intergenic complementation of the *pgi* gene restored T3SS gene expression to wild-type levels, as well as swimming and swarming. Supplementation of the media with fructose-6-phosphate was found to restore the expression of *hrpL* and *hrpA* to near wild-type levels in the *pgi* mutant. UspA, the universal stress protein, was found to modulate pathogenicity in *Salmonella*, and also to be metabolically controlled by fructose-6-phosphate. But in the present study, overexpression of *uspA* did not affect T3SS gene expression in *D. dadantii* 3937. The increased production of CRP in the *pgi* and *pgi/gcpA* mutant strains might possibly be reducing *hrpA* transcription, by binding to a putative site located within the *hrpA* promoter region.

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Table 1: Promoter activity of *hrpS* and *hrpA* genes in *D. dadantii* 3937 (3937) in glucose (glu), fructose (fru), glycerol (gly), casamino acid (CAA), and pyruvate (pyr) media

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b
	24hr
3937 (p <i>hrpS</i>)-glu	126.20±1.82
3937 (p <i>hrpS</i>)-fru	112.41±14.09
3937 (p <i>hrpS</i>)-gly	35.04±1.60*
3937 (p <i>hrpS</i>)-CAA	16.37±0.44*
3937 (p <i>hrpS</i>)-pyr	50.99±3.01*
3937 (pEV) ^c	2.36 ± 0.12
3937 (p <i>hrpA</i>)-glu	129.00±0.80
3937 (p <i>hrpA</i>)-fru	134.90±11.55
3937 (p <i>hrpA</i>)-gly	5.74±0.13*
3937 (p <i>hrpA</i>)-CAA	6.63±0.18*
3937 (p <i>hrpA</i>)-pyr	20.32±1.23*

^aWild-type *D.dadantii* 3937 was used for this and future experiments.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test), referenced to glucose media. Additional carbon sources were added in addition to glucose (combined, 20mM)

^cpEV, empty vector control. Values was similar throughout this and following experiments.

Table 2: Promoter activity of *hrpS*, *hrpL*, and *hrpA* genes in *D. dadantii* 3937 (3937) vs. Δpgi and $\Delta pgi::pgi$ strains with or without Fructose-6-phosphate

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (<i>phrpS</i>)	58.46±2.51	96.48±1.20
Δpgi (<i>phrpS</i>)	73.54±3.58	86.56±11.19
$\Delta pgi::pgi$ (<i>phrpS</i>)	60.55±0.51	106.24±1.05
3937 (<i>phrpS</i>)-F6P	99.48±0.93	118.96±12.76
Δpgi (<i>phrpS</i>)-F6P	108.99±16.16	125.94±18.97
3937 (<i>phrpL</i>)	14.39±0.59	15.33±0.21
Δpgi (<i>phrpL</i>)	5.38±0.15*	8.15±1.77*
$\Delta pgi::pgi$ (<i>phrpL</i>)	14.78±0.77	15.82±1.05
3937 (<i>phrpL</i>)-F6P	22.47±4.90	31.06±6.33
Δpgi (<i>phrpL</i>)-F6P	18.45±0.59	31.67±3.81
3937 (<i>phrpA</i>)	113.48±15.56	159.71±3.16
Δpgi (<i>phrpA</i>)	9.59±0.40*	22.57±1.29*
$\Delta pgi::pgi$ (<i>phrpA</i>)	97.17±4.20	113.85±2.81
3937 (<i>phrpA</i>)-F6P	100.55±13.88*	184.28±14.67
Δpgi (<i>phrpA</i>)-F6P	53.14±9.77	162.06±15.42

^a Δpgi mutant is a markerless deletion mutant of the *pgi* gene in the 3937 background, without antibiotic selection. $\Delta pgi::pgi$ is the *pgi* mutant strain with a chromosomally integrated copy of the *pgi* gene with its native promoter, located between the *lacY-prt* locus in 3937. Fructose-6-phosphate was supplemented to the fructose media for a total concentration of 20mM

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 3: Promoter activity of *hrpXY* genes in *D. dadantii* 3937 (3937) vs. Δ *pgi* strain

Strain^a/Promoter	Mean Fluorescent Intensity (MFI)^b	
	12hr	24hr
3937 (phrpXY)	32.40±0.35	41.39±1.35
Δ<i>pgi</i> (phrpXY)	32.85±1.27	39.45±0.62

^a Δ *pgi* mutant has been described previously.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 4: Promoter activity of the *uspA* gene in *D. dadantii* 3937 (3937) vs. Δpgi and $\Delta pgi::pgi$ strains

Strain^a/Promoter	Mean Fluorescence Intensity (MFI)^b	
	12hr	24hr
3937 (<i>puspA</i>)	130.73±3.09	141.09±22.48
Δpgi (<i>puspA</i>)	264.77±7.99*	379.11±6.84*
$\Delta pgi::pgi$ (<i>puspA</i>)	142.57±5.29	161.24±4.02

^a Δpgi and $\Delta pgi::pgi$ have been described previously.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 5: Promoter activity of *hrpS*, *hrpL*, and *hrpA* genes in *D. dadantii* 3937 (3937) vs. 3937::*uspA* and 3937::*nptII-uspA*

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpS)	92.72±16.87	95.71±4.28
3937::<i>uspA</i> (phrpS)	91.54±6.59	124.02±10.33*
3937::<i>nptII-uspA</i> (phrpS)	73.81±1.52	98.30±2.80
3937 (phrpL)	15.28±5.09	18.98±1.22
3937::<i>uspA</i> (phrpL)	15.18±0.91	24.47±2.32
3937::<i>nptII-uspA</i> (phrpL)	11.26±1.40	20.10±2.67
3937 (phrpA)	91.37±7.79	138.94±14.12
3937::<i>uspA</i> (phrpA)	89.23±3.15	111.80±6.15
3937::<i>nptII-uspA</i> (phrpA)	70.05±7.96	189.19±16.62*

^a3937::*uspA* and 3937::*nptII-uspA* are the wild-type strain with a chromosomally integrated copy of the *uspA* gene with its native or the constitutively expressing *nptII* promoter, located between the *lacY-prt* locus in 3937 respectively.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 6: Promoter activity of the *rpoS* gene in *D. dadantii* 3937 (3937) and the Δpgi strain, with and without 0.002% glucose supplementation.

Strain^a/Promoter	Mean Fluorescence Intensity (MFI)^b	
	12hr	24hr
3937 (<i>prpoS</i>)	11.93 0.23	10.59 0.08
Δpgi (<i>prpoS</i>)	15.38 0.60*	20.90 0.82*
3937 (<i>prpoS</i>)-0.002% glu	15.18 1.26	11.39 1.34
Δpgi (<i>prpoS</i>)-0.002% glu	20.13 1.46*	28.24 3.30*

^a Δpgi has been described previously. Fructose was used as the sole carbon source, and glucose added at the concentration where indicated.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and *pgi* mutant strains ($P < 0.05$, Student's T-test)

Table 7: Promoter activity of *gcpA*, *gcpE*, and *ecpC* genes in *D. dadantii* 3937 (3937) vs. *Δpgi* strains

Strain^a/Promoter	Mean Fluorescence Intensity (MFI)^b		
	6hr	12hr	24hr
3937 (<i>pgcpA</i>)	461.62±16.81	461.05±1.93	387.79±5.14
<i>Δpgi</i> (<i>pgcpA</i>)	356.95±29.13	627.00±32.97*	858.03±57.55*
3937 (<i>pgcpE</i>)	40.91±1.26	53.77±0.74	50.65±1.03
<i>Δpgi</i> (<i>pgcpE</i>)	33.35±0.36	48.36±1.11	56.70±2.82
3937 (<i>pecpC</i>)	424.06±11.12	732.41±3.18	515.18±5.72
<i>Δpgi</i> (<i>pecpC</i>)	372.88±8.32	527.58±8.02*	525.84±7.00

^a*Δpgi* has been described previously.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and *pgi* mutant strains (P<0.05, Student's T-test)

Table 8: Promoter activity of *hrpS*, *hrpL*, and *hrpA* genes in *D. dadantii* 3937 (3937) vs. Δpgi and $\Delta pgi/\Delta gcpA$ mutant strains

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (<i>phrpS</i>)	67.86±0.18	84.90±1.09
Δpgi (<i>phrpS</i>)	73.54±3.58	86.56±11.19
$\Delta pgi/\Delta gcpA$ (<i>phrpS</i>)	68.03±5.41	105.60±1.38
3937 (<i>phrpL</i>)	19.05±3.38	23.78±2.56
Δpgi (<i>phrpL</i>)	2.18±0.02*	3.04±0.13*
$\Delta pgi/\Delta gcpA$ (<i>phrpL</i>)	11.70±0.57	22.54±2.37
3937 (<i>phrpA</i>)	121.48±5.69	102.27±1.52
Δpgi (<i>phrpA</i>)	8.56±0.14*	23.43±0.92*
$\Delta pgi/\Delta gcpA$ (<i>phrpA</i>)	9.03±0.30*	28.23±1.54*

^a Δpgi has been described previously. $\Delta pgi/\Delta gcpA$ was constructed by creating a deletion in the *pgi* gene in the *gcpA* mutant strain background

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and *pgi* mutant strains (P<0.05, Student's T-test)

Table 9: Promoter activity of the *crp* gene in *D.dadantii* 3937 vs. Δpgi , $\Delta pgi::pgi$, and $\Delta pgi/\Delta gcpA$ mutant strains

Strain^a/Promoter	Mean Fluorescent Intensity^b	
	12hr	24hr
3937 (pcrp)	88.94±1.99	100.82±0.71
Δpgi (pcrp)	653.15±29.45*	911.28±10.93*
$\Delta pgi::pgi$(pcrp)	468.54±1.73*	352.25±4.71*
$\Delta pgi/\Delta gcpA$ (pcrp)	589.15±6.13*	876.49±43.27*

^a Δpgi , $\Delta pgi::pgi$, and $\Delta pgi/\Delta gcpA$ have been described previously. Fructose was used as the sole carbon source.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and *pgi* mutant strains (P<0.05, Student's T-test)

Table 10: Promoter activity of *hrpL* and *hrpA* genes in *D. dadantii* 3937 (3937) vs. Δ *pgi* mutant strain with 0.002% glucose supplementation

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpL)	18.36±4.81	24.07±4.69
Δ<i>pgi</i> (phrpL)	11.00±2.30*	11.17±1.00*
3937 (phrpA)	159.88±9.36	255.94±13.60
Δ<i>pgi</i> (phrpA)	51.97±7.39*	55.98±6.20*

^a Δ *pgi* has been described previously. Fructose was used as the sole carbon source, and glucose added at the concentration where indicated.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and *pgi* mutant strains (P<0.05, Student's T-test)

Table 11: Strains and plasmids used in the study

Strains	Relevant characteristics	Reference or Source
<i>Escherichia coli</i>		
S17-1λpir	Conjugation strain, F ⁺ , Sp ^R	(Bouhenni et al., 2005)
EC100λpir	Cloning strain	Laboratory strain
<i>Dickeya dadantii</i>		
3937	Wild type strain, <i>Saintpaulia</i> isolate	N. Hugouvieux-Pattat
3937::<i>uspA</i>	3937 with chromosomal insertion of <i>lacY-uspA-cm-prt</i>	This study
Δpgi	Deletion of the phosphoglucosomerase gene, <i>pgi</i>	This study
Δpgi::<i>pgi</i>	Δ pgi with chromosomal insertion of <i>lacY-pgi-cm-prt</i>	This study
Δpgi/ΔgcpA	Double mutation in <i>pgi</i> and <i>gcpA</i> genes	This study
Plasmids		
pMiniHimar RB1	Transposon vector, Km ^R	(Bouhenni et al., 2005)
pKD3	Antibiotic resistance cassette containing plasmid with FRT excision sites, Cm ^R	(Datsenko & Wanner, 2000)
pKD4	Antibiotic resistance cassette containing plasmid with FRT excision sites, Km ^R	(Datsenko & Wanner, 2000)
pRK415	Complementation/marker exchange vector, Tc ^R	(Keen et al., 1988)
pGEMT-Easy	Cloning vector, Amp ^R	Promega
pWM91	Suicide vector; <i>oriR6K mobRP4 lacZa</i> (of pBluescript II) <i>sacB</i> ; Suc ^c Amp ^R	(Metcalf et al., 1996)
pTCLS-Cm	6.4-kb <i>lacY-cm-prt</i> region cloned in pGEM-T Easy, Cm ^R	(Yap et al., 2005)
pFLP2	Flippase <i>FRT</i> excision vector, Amp ^R	(Hoang et al., 1998)
pPROBE-AT	Long-life promoter probe GFP expressing vector, Amp ^R	(Miller et al., 2000)
phrpS	pPROBE-AT derivative with PCR fragment containing <i>hrpS</i> promoter, Amp ^R	(Yang et al., 2008a)
phrpL	pPROBE-AT derivative with	(Yang et al., 2008a)

phrpA	PCR fragment containing <i>hrpL</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	(Yang et al., 2008a)
puspA	<i>hrpA</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	This study
prpoS	<i>uspA</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	This study
pgcpA	<i>rpoS</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	(Yi et al., 2010)
pgcpE	<i>gcpA</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	(Yi et al., 2010)
pecpC	<i>gcpE</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing	(Yi et al., 2010)
pcrp	<i>ecpC</i> promoter, Amp ^R pPROBE-AT derivative with PCR fragment containing <i>crp</i> promoter, Amp ^R	This study

a Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Sp^R, spectinomycin resistance.

Table 12: Primers used in the study

Primers	Sequence 5' to 3'	Source
pgiH1Frd	AGCGTTTCGTCAACGATTCGCTGAGCGGC	This study
pgiH1RC	GAAGCAGCTCCAGCCTACACATGTTTATCTCCTGCT	This study
pgiH2Frd	GGATAGGAACTAAGGAGGATATTCATATGTTGCCGCTATAACCT	This study
pgiH2RC	ACAGCATCTGATTGATGATGGCACAGTCAAT	This study
uspAH1Frd	CAGCATTGCAATATCAGCGTCTTGCGGTCGCCCTGACG	This study
uspAH1RC	GAAGCAGCTCCAGCCTACACAAACGCTCCTCTCTTGCTG	This study
uspAH2Frd	GGATAGGAACTAAGGAGGATATTCATATGGCAGACGCGTTGCCACC	This study
uspAH2RC	AGCGTAGGCAACTTGCGTCCGGCCCAGATTTC	This study
gcpAH1Frd	AAAGGATCCGGTCTGCACACGGCGAT	This study
gcpAH1RC	GAAGCAGCTCCAGCCTACACACATTAGCGATCA	This study
gcpAH2Frd	GGAATAGGAACTAAGGAGGATATTCATATGGTACGGATTTCGC	This study
gcpAH2RC	AAAGGTACCCACTTGCGGGGCTGAAAG	This study
pgifrdintXhoI	TCCTCGAGCAGCCCGACCATGAAC	This study
pgiRCintXhoI	TCCTCGAGTCATGCTGCGCC	This study
uspAnatprmfrdXhoI	ATCGCTCGAGAGTTGCCCGGGTGTTCCTTC	This study
uspAnatprmRCXhoI	TCAGCTCGAGTTACTCCTCTTCTTCACGCA	This study
nptIIfrduspAXhoI	TCAGCTCGAGTGTAGGCTGGAGCTG	This study
nptIIRCuspAfrdcomp	CTTTATGAGTCTATCCATGCCGATTGTCTGTTGTGCCC	This study
rpoSprmfrdXbaI	ATGCTCTAGATGTGGGTAAGATGTTGC	This study
rpoSprmRCSacI	AGTCGAGCTCATTACAGCATCTTCATG	This study
rsmAnorthFrd	ATGCTTATTTTGACTCGTCGAGTTGG	This study

rsmAnorthRC	TCAATAGGAGGTAGGCTGAGACTTCTC	This study
rsmBnorthFrd	GGTGGTCGTCTATAAACCGCG	This study
rsmBnorthRC	TAGTTCGTTTGCAGCAGTCC	This study
rplUFrd	TCGACGGCGGCAAAATCAAG	Zeng
rplURC	AAGCGCTGATGCCGGTGATT	Zeng
hrpLFrd	GCAAGCGGTATCGAACCGT	This study
hrpLRC	ACTTCCAACGCATCGTCGCTG	This study
rpoNFrd	ACTGGCGCTGGAAAGCAACC	Zeng
rpoNRC	GGCAGCTCGTCGGGCATATC	Zeng

Italicized font indicates restriction enzyme site. Frd, forward; RC, reverse complement

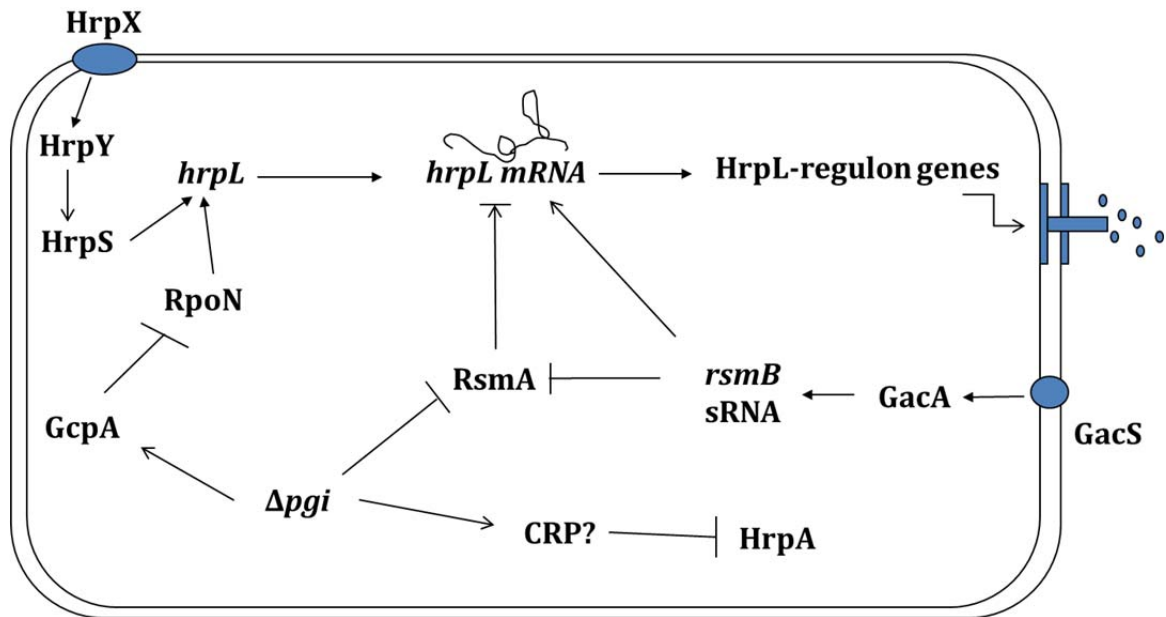


Fig. 8: Regulatory pathway of the *pgi* gene on the T3SS of *D. dadantii* 3937.

The T3SS is primarily regulated via the HrpX/Y and the GacS/A pathways. In the *pgi* mutant, there are increases in the diguanylate cyclase GcpA, in which increased c-di-GMP levels decrease the production of RpoN. Increases in CRP were observed, which may possibly decrease *hrpA* transcription via binding to putative consensus site in the promoter. The inhibition of RsmA production could possibly be due to increased RpoS production in the *pgi* mutant, preventing *rsmA* transcription. HrpX/HrpY constitutes a two component signal transduction system.

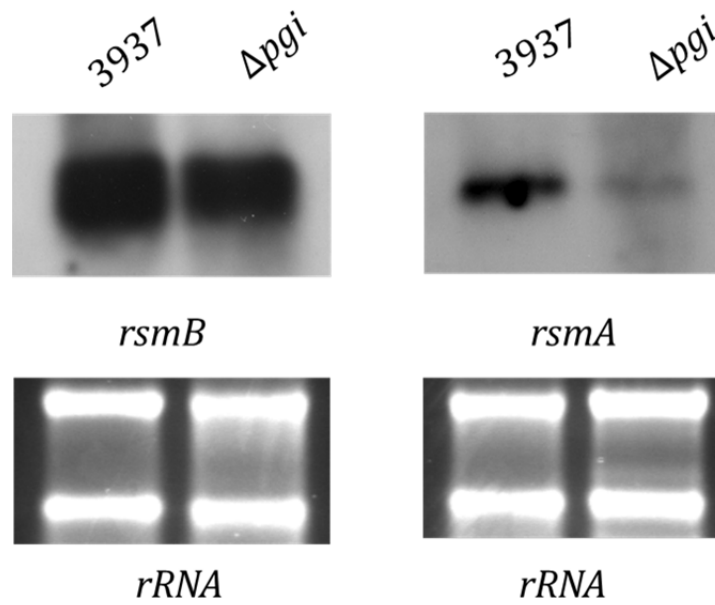


Fig 9: RNA levels of *rsmA* and *rsmB* in *D. dadantii* 3937 vs. *pgi* mutant. Northern blot analysis of *rsmA* and *rsmB* in *D. dadantii* 3937 (lane 1) and *pgi* mutant (lane 2). Similar results were obtained in two independent experiments.

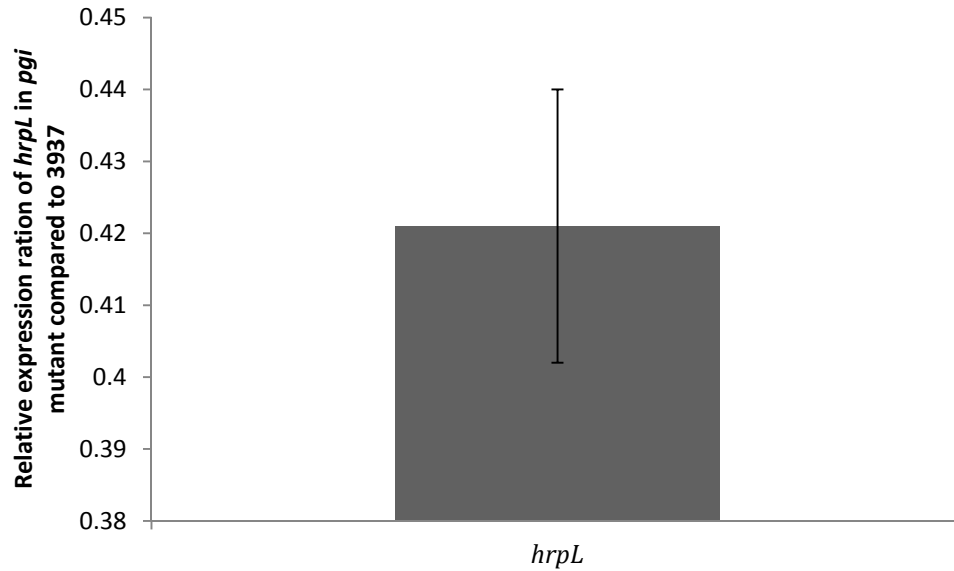


Fig. 10: *hrpL* mRNA expression ratios of *D.dadantii* 3937 vs. Δ *pgi* mutant strain. Bacterial strains were grown in minimal media for 12 hours. Total cellular RNA was isolated, reverse transcribed, and the message levels were measured by qRT-PCR. Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutants ($P < 0.01$, Student's T-test)

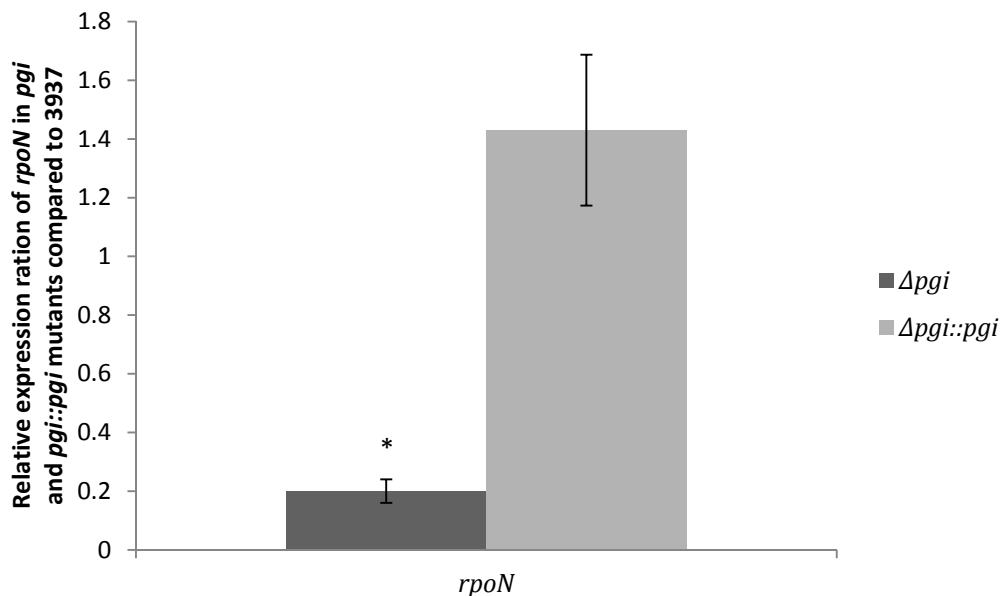


Fig. 11: *rpoN* mRNA expression ratios of *D.dadantii* 3937 vs. Δpgi and $\Delta pgi::pgi$ mutant strains. Bacterial strains were grown in minimal media for 12 hours. Total cellular RNA was isolated, reverse transcribed, and the message levels were measured by qRT-PCR. Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains ($P < 0.01$, Student's T-test)

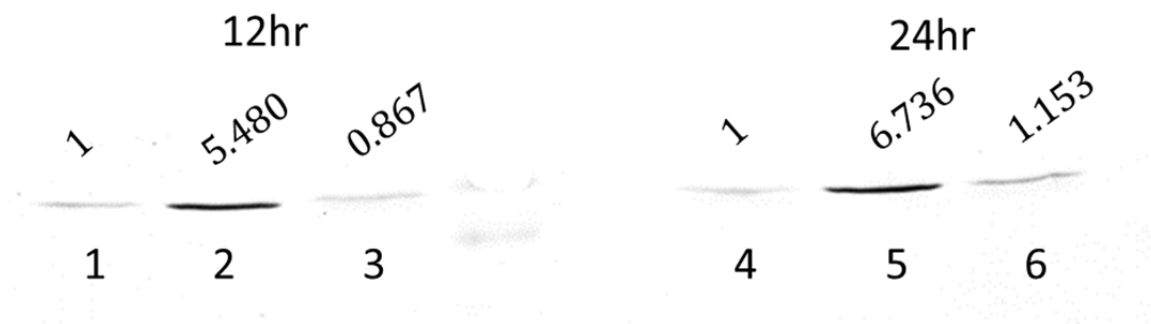


Fig. 12: Western blot of RpoS protein levels in *D. dadantii* 3937. 3937 (lane 1, 4), *pgi* mutant (Lane 2, 5), and *pgi*-complemented strain (Lane 3, 6). Cells were grown overnight in LB broth. A 1:100 dilution was performed in MM media, and cultured for 12 and 24 hours. Total protein was extracted and subjected to SDS-PAGE, and blotted using an anti-RpoS antibody.

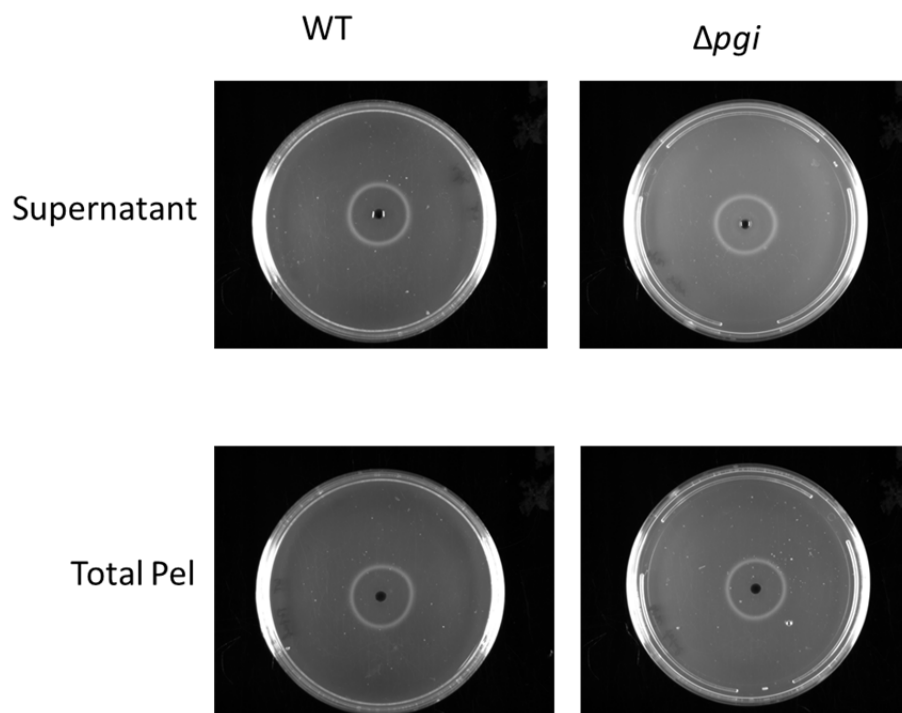


Fig. 13: Pectate lyase activity of the total cell extract between *D. dadantii* 3937 (3937) and *pgi* mutant strain. 10 μ L was deposited into a premade, agarose-sealed hole. The plates were allowed to incubate for 24 hours at 28°C, developed with 5N H₂SO₄ and photographed. Kanamycin was added to prevent bacterial growth. Similar results were obtained between duplicate experiments.

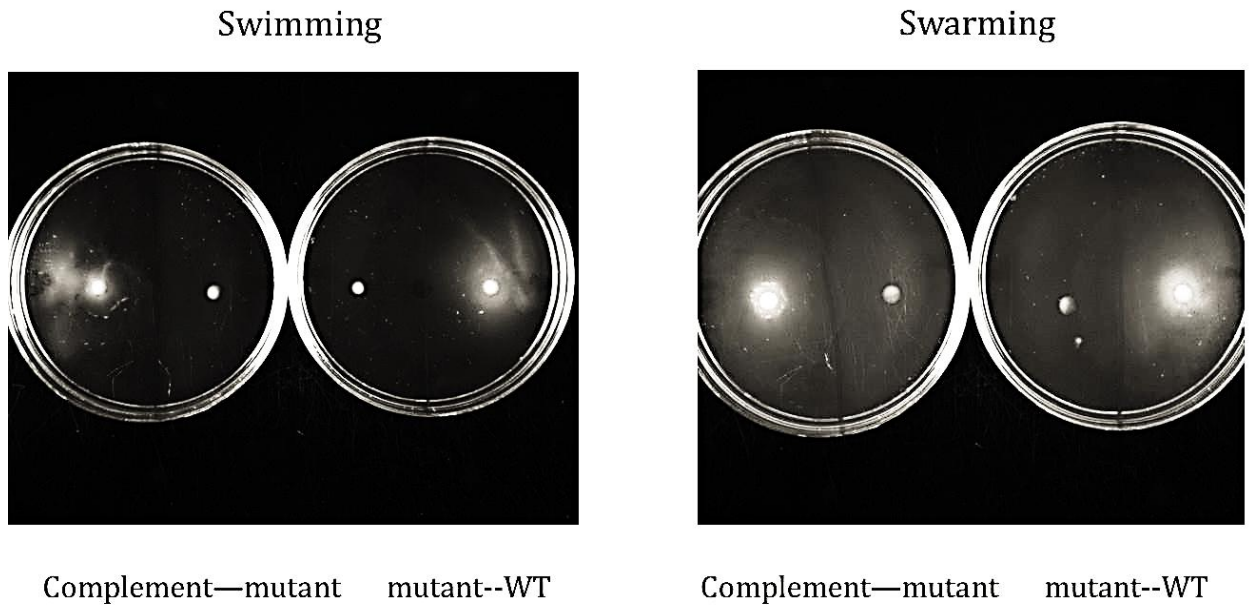


Fig. 14: Swimming and swarming of *D. dadantii* 3937 (WT), Δpgi (mutant), and $\Delta pgi::pgi$ (complement) strains. Swimming was performed on 0.2% MG agar plates. Swarming was performed on 0.4% MG agar plates. 10 μ L was deposited on the center of the plates, and allowed to incubate for 24 hours. Similar results were obtained between duplicate experiments.

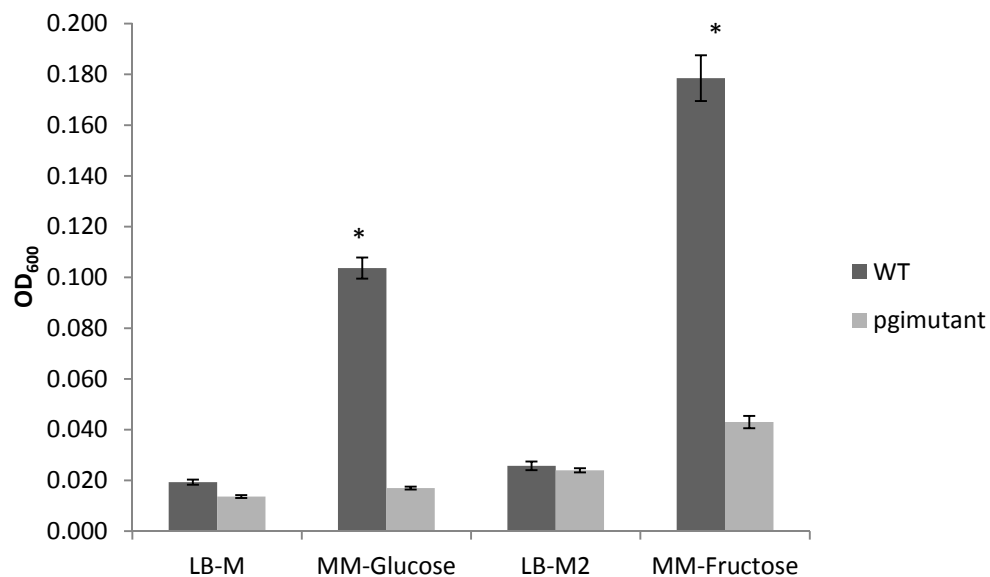


Fig. 15: Biofilm formation in *D. dadantii* 3937 vs. the *pgi* mutant. Cells were cultured overnight in LB broth. Cells were washed in 50mM sodium phosphate buffer, and a 1:100 dilution was made in either LB or MM media. The protocol to assess biofilm production was performed as described in the Materials and Methods.

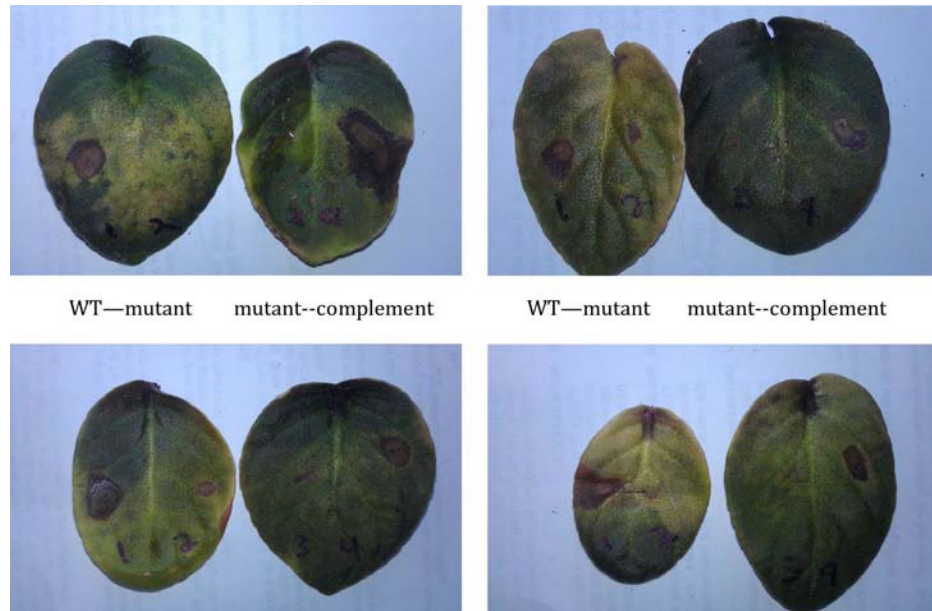


Fig. 16: In planta virulence of *D. dadantii* 3937 (WT), Δpgi (mutant), and $\Delta pgi::pgi$ (complement) strains. Strains were grown overnight in LB media, washed in 50mM sodium phosphate, pH 7.0, and adjusted to an $OD_{600}=0.7$. 50 μ L of cells were pressure infiltrated into the apoplastic space, and allowed to incubate for 72 hours at 100% humidity @ 28°C.

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ATACGGTAAAAATTCAGCTGCCGACGCTGCGTTCGCGTACGGAAATCATTCTGCCGTTATT
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AGTTCATAGCGAAATCACTTTATCAATTAATTGTTAACTGGAGATATTTATCATG

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Fig. 17: *hrpA* promoter region. The translational start codon, ATG, is shown in bold print. The Shine-Dalgarno sequence is shown in italics, while the putative CRP binding site is underlined

Chapter 3

Phosphotransferase-mediated regulation of the type III secretion system of *Dickeya dadantii* 3937

Abstract

Dickeya dadantii 3937 was mutagenized by a *miniHimar* Tn5 RB1 transposon insertion, that displayed increased *hrpS* gene expression, the type III secretion system (T3SS) enhancer-binding protein. The transposon insertion sequenced to the *fruA* gene, which was annotated as the fructose permease component of the fructose phosphotransferase system, or PTS. A clean non-polar *fruA* mutant was constructed in the bacteria, and the mutant had increased *hrpS* and *hrpL*, as well as increased *hrpA* gene at transcriptional level. FruB, a diphosphoryl transfer protein located within the fructose PTS, relays a phosphate to FruA. The *fruB* mutant showed decreased T3SS transcriptional activity, but *fruB* overexpression in the wild-type showed increased *hrpS*, as well as *hrpL* and *hrpA* expression. In addition, HrpX, sensor kinase of the HrpX/Y two component system, was found to be indispensable for T3SS function in *fruA* and *fruB* mutants. A yeast two-hybrid assay showed that the FruB protein specifically interacted with the HrpX protein. These data strongly suggest that the fructose PTS plays a role in regulating the T3SS of *D. dadantii* 3937 possibly through modulation of the activity of HrpX. Swimming and swarming was also found to be altered in the *fruB* mutant. Here we propose a model that explains how the fructose PTS controls the expression of genes for the T3SS.

Introduction

Dickeya dadantii 3937 is a gram negative, facultative rod shaped bacterium which causes diseases on a wide range of host plants (Burkholder, 1953). It uses proteolytic enzymes, which are secreted by the Type II Secretion System (T2SS), to extract nutrients from host tissues (Hugouvieux-Cotte-Pattat et al., 1996). In order to evade host responses, *D. dadantii* 3937 uses a specialized protein translocation system called the Type III Secretion System (T3SS) (Buttner & He, 2009). The *hrp* system (*h*ypersensitive *r*esponse and *p*athogenicity) is located within a divergent operon, and its exact triggering signal is unknown (Tang et al., 2006). The T3SS is activated by a two component signal transduction system, HrpX and HrpY, which encode the sensor histidine kinase, and its cognate response regulator, respectively (Fig. 18). HrpY then promotes transcription of *hrpS*, which encodes a sigma 54 enhancer-like binding protein. When HrpS couples with RpoN, it promotes transcription of *hrpL*. Considered the master regulator of Type III secretion, *hrpL*, which encodes an alternative sigma factor, binds to a specialized consensus sequence (called the “*hrp* box”) located within the corresponding promoter regions of the HrpL-regulon genes (Tang et al., 2006).

The *Dickeya* T3SS can also be positively regulated by the GacS-GacA two component signal transduction system (Fig. 18) (Yap et al., 2008, Yang et al., 2008b). Unknown signals promote autophosphorylation of the sensor histidine kinase GacS, which in turn relays the phosphate to GacA, its cognate response regulator. GacA promotes the transcription of *rsmB*, a regulatory small RNA which sequesters RsmA, an RNA-binding protein. RsmA binds to the 5' untranslated region of *hrpL*, and

promotes its time-dependent degradation. *rsmB* therefore promotes the stability of *hrpL* mRNA by binding up to 18 units of RsmA per *rsmB* molecule (Liu et al., 1997).

The introduction of sugars into the intracellular space for utilization in metabolism can occur via a number of ways, but the most notable is through the phosphoenolpyruvate-dependent phosphotransferase system, or PEP-PTS (Tchieu et al., 2001). In this system, a phosphate donated from an intermediate of the Emden-Meyerhof-Parnas pathway of glycolysis, phosphoenolpyruvate (PEP), is relayed through a cascade of phosphotransfer proteins onto the incoming sugar molecule. The incoming sugar molecule diffuses through a permease specific for the molecule, is subsequently phosphorylated, and then is utilized for metabolism or other cellular processes (Postma et al., 1993). Some of the components of the phosphotransferase system are shared amongst the different PTSs, such as EI and the HPr protein, which encode the enzyme I and histidine protein, respectively (Saier, 1976). PTSs in some bacteria, such as *Escherichia coli*, have multiple phosphotransfer systems for each specific sugar to enter the cell (Postma et al., 1993).

It has been previously shown that specific components of the phosphotransferase system are important regulatory components (Chavarria et al., 2012, Stulke & Hillen, 1998). Processes such as catabolite repression and inducer exclusion, virulence, biofilm formation, chemotaxis, etc., all rely on the phosphorylation status of one or more of the phosphorelay components (Chavarria et al., 2012, Feldheim et al., 1990). In *Vibrio sp.*, levels of Enzyme IIA^{Glc} affect the virulence of the organism. Within the same organism, the PEP-dependent

phosphotransferase system also modulates biofilm formation through multiple, independent pathways (Visick et al., 2007). In most organisms, motility is also affected by phosphorylation status of specific proteins. In *E. coli* and other organisms, increasing levels of Enzyme IIA^{Glc} are known to interact with CheY (Neumann et al., 2012), which encodes for the sensor kinase within the methyl-accepting chemotaxis system (MCP) (Ordal, 1985).

In related organisms, such as *Pantoea stewartii*, signals which have promoted activation are pH, temperature, osmolarity, and nitrogen levels (Merighi et al., 2003). Yang et al. have shown that specific plant compounds, such as *p*-coumaric acid and other phenolic compounds are able to modulate the T3SS of organisms such as *Pseudomonas aeruginosa* and *D. dadantii* 3937 (Yang et al., 2008a, Yang et al., 2007, Yamazaki et al., 2012). These are conditions which either activate or inhibit the T3SS, however, it is not known whether prior environmental conditions promote autophosphorylation of HrpX, or if some intracellular signaling mechanism is at work to elicit the desired response.

The interplay between carbon metabolism and virulence processes has not been well described (Poncet et al., 2009). In this work, we have found that various components of the fructose phosphotransferase system are able to modulate the transcription of specific type III secretion system genes of *D. dadantii* 3937. We have demonstrated that mutations in genes within the fructose operon, *fruA*, the fructose permease, *fruK*, 1-phosphofructokinase, and *fruB*, the diphosphoryl transport protein, affect the transcription of *hrpS* in *D. dadantii* 3937, possibly

through modulation of the activity of HrpX. This is one of the first reports which illustrates that the fructose PTS plays an active role in the T3SS of *D. dadantii* 3937.

Materials and Methods

Bacterial strains, plasmids, and culture media

The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* and *D. dadantii* 3937 was routinely cultured in Luria-Bertani (LB) broth (10g of tryptone, 10g of sodium chloride, 5g of yeast extract, each per liter), or mannitol-glutamic acid (MG) media (Yang et al., 2008a, Yang et al., 2008b, Zeng et al., 2010, Zou et al., 2012) at 28°C. For type III expression studies, *D. dadantii* 3937 was subcultured from an overnight growth in LB, and diluted 1:100 in *hrp*-inducing minimal salts media (MM) (Yamazaki et al., 2011, Yang et al., 2008a, Yang et al., 2008b, Zeng et al., 2010, Zou et al., 2012) at 28°C. Media was supplemented with chloramphenicol (20µg/mL), ampicillin (100µg/mL), kanamycin (50 µg/mL), and gentamicin (15 µg/mL) as needed in LB, MM, and MG media.

Mutant construction

To create the mutants needed for experimentation, mutants were constructed by marker exchange mutagenesis (Yang et al., 2002), with slight modifications. Briefly, two 600bp fragments flanking each target gene were obtained. Kanamycin and chloramphenicol resistance cassettes, amplified from either pKD4 or pKD3 (Table 22), respectively, were ligated with the flanking region fragments of the mutants using recombinant PCR, and then cloned into pGEMT-Easy

(Promega, Madison, WI). The recombinant fragment was digested at the NotI site from pGEMT-Easy, and the resultant fragment was gel-purified and ligated into suicidal plasmid pWM91, propagated in EC100 λ pir, and transformed into S17-1 λ pir. This construct was then transferred into *D. dadantii* 3937 by conjugation. To select strains with chromosomal deletions, transconjugants with chloramphenicol/kanamycin and ampicillin resistance were plated on MG agar containing ampicillin and chloramphenicol/kanamycin. *D. dadantii* 3937 colonies having either kanamycin or chloramphenicol and ampicillin resistance were cultured in Luria-Bertani broth with no selection overnight, and plated on MG media with 5% sucrose and kanamycin or chloramphenicol. Colonies were then replica plated on separate plates containing kanamycin/chloramphenicol and ampicillin. Colonies which were kanamycin/chloramphenicol resistant and ampicillin sensitive were isolated and confirmed by PCR using outside primers. *fruA* complementation was performed by cloning the *fruA* ORF into pML123, while *fruB* complementation and overexpression was performed by cloning the *fruB* gene and its native promoter into chromosomal integration vector pTCLS-Cm.

FACS assay

The bacterial cells carrying promoter-*gfp* transcriptional fusion plasmids were cultured in LB broth overnight, normalized by OD₆₀₀, and then subcultured 1:100 in *hrp*-inducing minimal media broth at 28°C. Cells were harvested at the time points 6, 12 and 24 hours, and adjusted to approximately 10⁶ CFU/ml with 1X phosphate-buffered saline (PBS, 8.0g of NaCl, 0.2g of KCl, 1.44g of Na₂HPO₄, and

0.24g of KH_2PO_4 per liter, pH adjusted to 7.2- 7.4) before FACS analysis. A four-color BD FACSCalibur (BD Biosciences, San Jose, CA) equipped with 488- and 633-nm lasers was used to analyze the fluorescence intensity of each bacterial cell in a total collection of around 10000 cells per measurement. Measurements were performed in triplicate. Bacteria were electronically gated based on forward and side light-scattering properties. All GFP fluorescence measurements were taken using the 488-nm laser and FL1 channel on the gated population. The results were analyzed using CellQuest software (BD Biosciences, San Jose, CA).

Northern Analysis

Bacterial strains were grown overnight in LB media, and subcultured 1:100 in 30mL of MM media for 12 hours with aeration. Total RNA was extracted using an RNAeasy mini-kit (Qiagen, Valencia, CA), and cleaned up of any contaminating genomic DNA with a Turbo DNase-free kit (Ambion, Austin, TX). RNA samples were detected and analyzed using *rsmA* and *rsmB* probes labeled with biotin (Ambion BrightStar Psorlen-Biotin labeling kit and BrightStar BioDetect, Austin, TX).

Yeast two hybrid analysis

Plasmids pEG202 and pACT2 were chosen to express the LexA transcriptional domain and Gal4 activation domain, respectively (Legrain et al., 1994). To construct pEG202-FruB and pACT2-HrpX, *hrpX* and *fruB* open reading frames were amplified from *D. dadantii* 3937 genomic DNA, and cloned into pGEMT-Easy. The plasmids were sequenced and digested with BamHI. The *fruB* and *hrpX*

open reading frames were ligated into pEG202 and pACT2, respectively, at the BamHI site, and propagated in *E. coli* EC100 λ pir. The plasmids were isolated and transformed into *S. cerevisiae* CTY10-5d and Y2 strains. Transformants were grown to mid-log phase with plasmid selection in SC medium lacking histidine and leucine containing 2% glucose. B-galactosidase activity was measured in permeabilized cells and expressed in Miller units as previously described (Strogolova et al., 2012).

Pectate lyase activity

Bacteria were grown overnight in LB media. A 1:100 dilution was subcultured into MM media supplemented with 0.5% polygalacturonic acid (PGA) overnight to induce pectate lyase (Pel) activity. 10 μ L of overnight induced sample was inoculated on premade Pel assay plates (1% PGA, 1% Yeast extract, 50 μ M CaCl₂, 50mM Tris-Cl (pH 8.5), 0.8% Agar). Kanamycin was added to prevent bacterial growth. The plates were allowed to incubate overnight at 28°C. The plates were developed using 5N H₂SO₄ for 2 minutes with swirling, washed with water and photographed.

Swimming and swarming assessment

Bacteria were grown overnight in LB broth with appropriate antibiotics at 28°C. Bacteria were washed in 1X PBS, and resuspended to an OD₆₀₀ of 1.0. For the swimming assay, 0.2% agar was used, while for the swarming assay, 0.4% agar was used. A 10 μ L spot was placed in the center of the mannitol-glutamic acid plates (MG), and the plates were incubated for 24 hours at 28°C. The images were

captured with a digital camera (Olympus) and analyzed using Adobe Photoshop software.

Results

FruA, the fructose permease, plays a negative role in T3SS expression

A transposon mutant with an increase in *hrpS* expression was observed compared to the wild-type strain (data not shown). Sequencing results showed that the transposon integrated into gene ASAPID: 0020028, which is annotated as *fruA* (Fig. 19). Because of the increase, this prompted us to further study the mechanism's effect on the T3SS. *fruA* is located in a three-gene operon which contains *fruB*, encoding the diphosphoryl transfer protein, *fruK*, which encodes 1-phosphofructokinase, and *fruA*, the fructose permease.

Validation of the *hrpS* increase in the transposon mutant was performed by constructing a deletion mutant in *D. dadantii* 3937 of *fruA*. In concert with the transposon phenotype, there was an increase in *hrpS* transcriptional activity (Table 13). Since HrpS upregulates the transcription of *hrpL*, and *hrpA* is a HrpL-regulon gene, promoter activities of *hrpL* and *hrpA* were also examined. Compared with the wild-type, increases in GFP fluorescence of *hrpL*, as well as *hrpA*, were seen in the *fruA* mutant (Table 13). To test if the mutation could be functionally complemented, the *fruA* mutant was transformed with a plasmid, pMLfruA, in which the open reading frame of *fruA* was cloned and the expression of *hrpS*, *hrpL*, and *hrpA* was examined. T3SS gene expression was restored to near wild-type levels (Table 13).

Because the type III secretion system is also regulated via the RsmA/*rsmB* system, the transcriptional activity of *rsmA* and *rsmB* were examined by FACS. The mRNAs of *rsmA* and *rsmB* were also measured by Northern blot. Similar promoter activities and mRNA levels of *rsmA* and *rsmB* were observed between *D. dadantii* 3937 and *fruA* mutant strains (Table 14 and Fig. 20). In summary, *hrpS*, *hrpL* and *hrpA* expression levels were increased in *fruA* mutant in comparison to the wild-type strain *D. dadantii* 3937. Our results suggest that FruA affects T3SS expression through the HrpS-HrpL, but not the RsmA-*rsmB* pathways. In this study, the *fruA* mutant was unable to grow on fructose as the sole carbon source (data not shown); therefore glucose was used at a final concentration of 20mM for T3SS expression assays described below.

FruB, the diphosphoryl transfer protein, plays a positive role in T3SS expression

In the fructose PTS, PEP donates a phosphate group to EI, which in turn relays the phosphate to FruB. FruB then relays the phosphate to FruA, which subsequently attaches the phosphate to the incoming fructose molecule to create fructose 1-phosphate. FruK then attaches another phosphate to create fructose 1, 6-bisphosphate, which enters the Emden-Meyerhof-Parnas pathway of glycolysis. In order to further clarify the role of fructose PTS components on T3SS expression, a mutation was created in *fruB*. Interestingly, the promoter activities of *hrpS*, *hrpL*, and *hrpA* were reduced in the *fruB* mutant versus the wild-type (Table 15).

Complementation of the *fruB* mutant with a *fruB* gene coupled with its native

promoter into the chromosome of the mutant strain at an intergenic region increased the transcription of *hrpS*, *hrpL*, and *hrpA* similar to the wild-type level (Table 15).

Since the T3SS is also regulated through the RsmA/*rsmB* pathway, we examined if there were any effects on this pathway in the *fruB* mutant. The transcriptional and RNA levels of *rsmA* and *rsmB* were examined by FACS and Northern blot analysis. Our result showed that similar promoter activities and RNA levels of *rsmA* and *rsmB* were observed between *fruB* mutant and wild-type (Table 14 and Fig. 20). In addition, the transcription of *hrpX* and *hrpY*, the sensor histidine kinase and the response regulator, respectively, which are required for T3SS activation, was not affected in both the *fruA* and *fruB* mutants in comparison to the wild-type strain, which discloses that the increased transcription of *hrpS* is not due to overexpression of the *hrpXY* operon (Table 13 and Table 15).

In order to ascertain if *fruA* or *fruB* altered its own expression by modulating the transcription of the operon, fructose operon transcription was checked in the wild type, *fruA*, and *fruB* mutants. There was no statistical difference in the transcription of the fructose operon (Table 16). In summary, the results above suggested that the components of the fructose PTS, FruA and FruB, affect T3SS expression through the HrpS-HrpL pathway, but do not interfere with HrpX/Y, or the fructose operon at the transcriptional level.

In an attempt to clarify the roles of other genes in the fructose operon and their impact on the T3SS, a deletion mutation was also made in *fruK*, or 1-phosphofructokinase. A mutation in this gene also showed an increase in *hrpS*, *hrpL*,

and *hrpA* transcription (Table 17), similar to the *fruA* mutant phenotype (Table 13). This result demonstrates that components in fructose PTS play a role in altering expression of the T3SS.

The glucose-specific PTS protein EIIA^{Glu} does not affect the T3SS of *D. dadantii* **3937**

In most bacteria, glucose is the preferred energy substrate, and the cells undergo catabolite repression, as well as inducer exclusion, to use glucose before any other carbon source (Kornberg et al., 1980, Deutscher, 2008). Glucose is a PTS-transported sugar; therefore, it uses the exclusive glucose PTS to subsequently phosphorylate the incoming glucose molecule for downstream metabolism. Enzyme IIA^{Glc}, the product of PTS *crr* gene, is a specific phosphotransfer protein responsible for coupling the general PTS proteins EI and HPr, which are common to other PTSs, to the glucose permease PtsG (Gabor et al., 2011, Postma et al., 1993, Tchieu et al., 2001). This in turn will allow the relayed phosphate to be translocated to the incoming glucose molecule. Phosphorylated, as well as non-phosphorylated EIIA^{Glc} has been shown in previous literature to interact with and activate adenylate cyclase to produce cAMP, which can ultimately pair with the CRP protein to transcriptionally regulate genes within its regulon (Notley-McRobb et al., 1997).

Based on the results acquired, FruB plays a role in the transcription of T3SS genes. Both EIIA^{Glc} and FruB have phosphotransfer abilities, and EIIA^{Glc} has a wide range of regulatory functions, and in *Vibrio sp.*, plays a role in virulence of the organism, by altering its ability to colonize the mouse intestine (Houot et al., 2010).

Because of this, we looked at the impact of EIIA^{Glc} on T3SS expression in *D. dadantii* 3937. A mutation was made in the *crr* gene, and the expression of *hrpS*, *hrpL*, and *hrpA* was assessed. Because the growth of the *crr* mutant was slower than the wild-type strain on glucose, fructose was used as the carbon source. The transcriptional expression of *hrpS*, *hrpL*, and *hrpA* was not significantly different than the wild-type strain (Table 18). The T3SS expression of the *crr* mutant in a different carbon source, mannitol, was tested as well, and the results in comparison to the wild-type strain were non-significant (Table 18). These results suggest that Enzyme IIA^{Glc} doesn't play an active role in the transcription of T3SS genes *hrpS*, *hrpL*, and *hrpA* in *D. dadantii* 3937, and that the contribution of fructose PTS to the expression of the T3SS is specific.

HrpX is indispensable for T3SS function in the *fruA* mutant

The type III secretion system is dependent on environmental cues to facilitate its function of causing disease in host plants. HrpS, which causes induction of the master regulator HrpL in *D. dadantii* 3937, is dependent on the two-component signal transduction system, HrpX and HrpY. In the *fruA* mutant, an increase in *hrpS* transcription was observed in comparison to the wild-type strain 3937 (Table 13). In order to explicate the mechanism of the *hrpS* transcriptional increase in the *fruA* mutant, we created mutations located within the HrpX/Y signaling pathway. As expected, in the *hrpX* mutant, the transcription of *hrpS*, *hrpL*, and *hrpA* was reduced in comparison to the wild-type strain (Table 19). The same trend was also seen in the *hrpY* mutant, with a reduction in *hrpS* and *hrpA* gene

expression (Table 19). In order to see if the *hrpS* induction caused by the *fruA* mutation was possibly due to an effect on *hrpX*, a strain containing a mutation in *hrpX* and *fruA* was constructed. No observable differences were observed in the transcription of *hrpS* and *hrpL* in the *hrpX* and the *hrpX/fruA* mutant strains, respectively (Table 19). This finding suggests that the increase in *hrpS* transcription is reliant on a functional *hrpX* gene, and the contribution of increased *hrpS* expression in the *fruA* mutant may be relayed through HrpX.

The phosphotransfer function of FruB impacts the T3SS of *D. dadantii* 3937

Under fructose utilization, FruB specifically interacts and transfers a phosphate to its cognate acceptor, FruA. According to previous studies, phosphorylated FruB also has the capability to modulate the activity of other proteins which play a major role in global regulatory functions, such as the nitrogen phosphotransferase system (Pfluger & de Lorenzo, 2008). In this study, the *fruB* mutation decreased, while the *fruA* and *fruK* mutations increased T3SS expression in *D. dadantii* 3937 (Tables 13, 15, and 17). An intergenic complementation of the *fruB* gene restored expression to wild-type levels in the *fruB* mutant, and enhanced T3 expression when the same copy of the gene was incorporated into the wild-type genome within an intergenic region (Table 15). These results suggest that FruB may possibly be directly or indirectly affecting T3S expression.

In order to elucidate the mechanism by which FruB is influencing T3SS expression, we placed a copy of *fruB* with its native promoter in the intergenic region of the *hrpX* mutant. In the mutant strain, *hrpS* T3SS gene expression was

similar as the *hrpX* mutant (Table 20). This finding suggests that the phosphotransfer function of FruB is also dependent on a functional HrpX protein similar to the phenotype seen in the *hrpX/fruA* double mutant (Table 19).

FruB interacts with HrpX in the yeast two-hybrid system assay

HrpY directly interacts with HrpX in order to become phosphorylated and perform its DNA-binding function on the promoter of *hrpS*. FruB also interacts with its cognate receiver, FruA, to phosphorylate the incoming fructose molecule. Transcriptional expression of the enhancer-like binding protein *hrpS* was not increased in the *hrpX/fruA* double mutant, or the *hrpX* mutant with *fruB* complementation strains (Table 19 and Table 20). However, in the wild-type and *fruB* mutant, both with *fruB* complementation, the expression of *hrpS* was increased approximately 1.5-fold (Table 15). FruB is a phosphotransfer protein; therefore it is possible that FruB interacts with HrpX and further increases the transcription of *hrpS* by increasing subsequent phosphorylation of HrpY. In order to investigate this hypothesis, we tested the protein-protein interaction between HrpX and FruB using the yeast 2-hybrid method (Y2H). Fusions of *hrpX* and *fruB* to plasmids pACT2 and pEG202, respectively, were constructed and transformed into strains CTY10-5d and Y2. After crossing, the strains were subjected to quantitative β -galactosidase measurements. The interaction between both empty vectors pACT2 and pEG202 yielded very little β -galactosidase expression (Fig. 21). The vector containing pACT2::*HrpX* also yielded very little expression. The pEG202::*FruB* vector yielded some low level background β -galactosidase expression, but this is a common

occurrence with the Y2H transcriptional domain fusions (Creasey et al., 2003). Interestingly, the strain containing both pEG202::FruB and pACT2::HrpX had a 3-fold increase in the production of β -galactosidase (Fig. 21).

Non-specific phosphodonors affect the T3SS

Many proteins are activated or repressed by several post-translational modifications, such as phosphorylation, glycosylation, ubiquitination, and polyadenylation. In most eubacteria, phosphorylation is the main form of post-translational modifications that cause an effect on downstream targets. Acetyl phosphate is an intermediate formed via downstream metabolism of acetyl-CoA, which ultimately proceeds to the formation of acetate.

Previous research has shown that this compound is an intracellular signaling molecule, which can modulate different cellular phenotypes (Fredericks et al., 2006, Pruss & Wolfe, 1994, Wolfe et al., 2003). Because the phosphorylation status of FruB affected the T3SS, we wanted to observe the effect of acetyl phosphate *in vivo* on the T3SS. Therefore, we constructed a *pta* and an *ackA* mutant, which is unable to produce acetyl phosphate and acetate, respectively, from acetyl-CoA. Because of these mutations, the *pta* mutant should be devoid of any notable acetyl phosphate supply, while the *ackA* mutant should have an accumulation of acetyl phosphate within the cell. In the *pta* mutant, the expression of *hrpS*, *hrpL*, and *hrpA* were not statistically different from the wild-type strain. But in the *ackA* mutant, *hrpA*, *hrpL*, and *hrpS* were decreased in comparison to the wild-type strain (Table 21). These results demonstrate that, within the T3SS of *D. dadantii* 3937, a deficiency of acetyl

phosphate has a negligible impact on the T3SS, while an accumulation of the molecule negatively impacts the T3SS *in vivo*.

Swimming and swarming is affected, but pectate lyase activity is unaltered in the *fruA* and *fruB* mutants

Virulence and motility are often correlated. When members of the MCP pathway were mutagenized, the ability of the organism to infect multiple plant species was compromised in *D. dadantii* 3937 (Antunez-Lamas et al., 2009). Researchers have shown that components in the core PTS (EI and HPr) are able to modulate motility in *E. coli* (Neumann et al., 2012). In order to investigate if there was an effect on motility in our mutants, we tested the swimming and swarming abilities of *D. dadantii* 3937, *fruA*, and *fruB* mutant strains. Our results showed that, in comparison to the wild-type strain, swimming and swarming motility in the *fruA* mutant was similar, while the *fruB* mutant showed increased motility (Fig. 22). These results suggest that the fructose PTS, more specifically FruB, is a modulator of bacterial motility in *D. dadantii* 3937.

Once inside the host, plant pathogens must use proteolytic means to digest tissues and cause concomitant nutrient release. In *D. dadantii* 3937, the type II secretion system (T2SS), another major virulence factor besides the T3SS, is used for this purpose. Taking metabolic cues from the immediate environment, *D. dadantii* 3937 uses this information to initiate pectate lyase production, and release a host of cellulases, pectate lyases (Pels), and polygalacturonases through the T2SS

to macerate host tissues, and breakdown long chain carbohydrates into usable, small chain carbon sources which can enter various metabolic pathways.

Since the metabolism of pectin is a major regulator of pectate lyase enzyme production (Hugouvieux-Cotte-Pattat et al., 1996, Peng et al., 2006, Yang et al., 2008b), we assessed the activity of the T2SS in response to the potential metabolic permutations that might be happening due to mutations in the fructose operon. To examine the potential effects, we examined the intracellular production and extracellular secretion of the Pel enzymes, which are responsible for host tissue destruction. In the wild type, $\Delta fruA$, and $\Delta fruB$ strains, there was a zone of enzymatic activity (halo) of similar size around the inoculation site in the assay plate in the total cell extract preparation of all the strains (Fig. 23). This observation demonstrates that there was no effect on pectate lyase activity in the *fruA* and *fruB* mutants.

Discussion

D. dadantii 3937 is a necrotrophic, saprophytic plant pathogen which, under optimal conditions, causes soft rot on the surface and fleshy portions of the host plant (Burkholder, 1953, Yang et al., 2004). Using various virulence factors such as the type II and III secretion systems, as well as motility, it is able to effectively translocate through natural wounds and cause disease (Antunez-Lamas et al., 2009, Bauer et al., 1994, Buttner, 2002, Cesbron et al., 2006, Peng et al., 2006, Yang et al., 2008b). There are several factors, such as PecS, PecT, SlyA, and cyclic diguanylate monophosphate (c-di-GMP), and some of which are still unknown, have been

identified recently to play a role in the regulation or functionality of the T3SS (Hugouvieux-Cotte-Pattat et al., 1996, Nasser et al., 2005, Zou et al., 2012, Yi et al., 2010, Zeng et al., 2010, Yang et al., 2008a). As research progresses, we are coming to know that various intracellular signaling mechanisms are also contributing to a vast interconnected cascade which ultimately fine-tunes the virulence response to the host.

In this work, we have found that components of the fructose phosphotransferase system are actively playing a role in the functionality of the T3SS of *D. dadantii* 3937. The fructose PTS consists of a three-gene operon which is comprised of *fruB*, *fruK*, and *fruA* (Walter Jr & Anderson, 1973, Postma et al., 1993, Saier, 1976, Tchieu et al., 2001). When fructose is used as a carbon source, PEP, or phosphoenolpyruvate, donates a phosphate group to EI, Enzyme I, which in turn relays the phosphate to FruB. FruB then relays the phosphate to FruA, which subsequently attaches the phosphate to the incoming fructose molecule to create fructose 1-phosphate (Ramseier et al., 1995, Walter Jr & Anderson, 1973). FruK then couples another phosphate to create fructose 1, 6-bisphosphate, which can then enter the Emden-Meyerhof-Parnas pathway of glycolysis (Feldheim et al., 1990). FruA, the fructose permease, was observed to play a negative role in the expression of the T3SS, which was observed by generating a deletion mutation in *fruA*, causing the concomitant increase in the expression of *hrpS*, *hrpL*, and *hrpA* genes in this study (Table 13). When the mutation was functionally complemented *in trans*, transcriptional expression of the above reporter constructs was returned to near wild-type levels (Table 13).

The *fruK* gene, which encodes 1-phosphofructokinase, when mutated, also showed the same phenotype as *fruA* (Table 17). FruK, downstream of FruB and FruA in the fructose PTS cascade, promotes the formation of fructose 1,6 bisphosphate when fructose is used in the media. When glucose is used as the sole carbon source, FruA, once phosphorylated by FruB on a cysteine residue, retains its phosphate, and is unable to donate its phosphate to an incoming molecule of fructose to create the phosphoester fructose-1-phosphate (Prior & Kornberg, 1988, Reizer, 1996). One hypothesis to support the *fruK* phenotype observed is that, once phosphorylated, FruA is no longer able to accept phosphotransfer from FruB. Therefore, since FruK is downstream of FruA and relies on the FruA's function of generating fructose-1-phosphate in the cytoplasm, FruK's activity is functionally negated. Residual phosphorylated FruB in the *fruK* mutant is still able to interact with HrpX, similar to the phenotype observed in the *fruA* mutant. Interestingly, when *fruB* was mutated, T3SS expression was reduced in comparison to the wild-type strain (Table 15). Note that since the promoter activities of *rsmA* and *rsmB*, and the mRNA levels of *rsmA* and *rsmB* were similar between *D. dadantii* 3937 and the *fruA* and *fruB* mutants (Table 14 and Fig. 20), it can be concluded that the effect of the fructose PTS on the T3SS doesn't appear to be modulated through the RsmA-*rsmB* pathway. To our immediate knowledge, this is one of the first findings in phytobacteria which illustrates that components of the PTS system actively play a role in the control of the T3SS.

Our results showed that the *hrpS* induction caused by the *fruA* mutation was eliminated in the bacterial strain with both *hrpX* and *fruA* genes deleted (Table 19),

and also with a single copy overexpression of *fruB* in the *hrpX* mutant (Table 20). Similar expression levels of *hrpS* and *hrpL* were observed in the *hrpX/fruA* double mutant and in the *hrpX* mutant overexpressed with *fruB* (Table 19 and Table 20). FruB is a diphosphotransfer protein, which relays the bound phosphate to its cognate receiver FruA (Postma et al., 1993, Saier, 1976). This result suggests that *fruA* and *fruB* mutations affect the T3SS through the HrpX-HrpY two component signal transduction pathway. This finding also suggests that the contribution of increased *hrpS* expression in the *fruA* mutant and the wild-type strain with *fruB* overexpression is relayed through HrpX, since the increase in *hrpS* expression was negated in the *hrpX/fruA* and *hrpX/fruB* overexpression strains. Transcriptional levels of the fructose operon are low, which can be seen under glucose supplementation (Table 16); therefore, it can be hypothesized that the net movement of phosphate in the PEP-PTS is biased to the core proteins EI, HPr, and also EIIGlc. Enzyme I is pleotropic, and for that reason, low cellular levels of FruB can still accept phosphates from Enzyme I, and thus relay this phosphate to FruA or other accepting targets (Pfluger & de Lorenzo, 2008). It is not currently known if the phosphorylated or non-phosphorylated form of FruB is causing the effect seen on the T3SS of *D. dadantii* 3937.

According to our findings, single-copy overexpression of *fruB* in the wild-type strain increased the transcription of *hrpS* from the wild-type strain (Table 15). This led to the concomitant increase in *hrpL* transcription, which provides strong evidence that *fruB* is interacting at the level of HrpX, either by direct interaction, or via some unknown intermediate factor. The yeast two-hybrid technique is routinely

used to check protein-protein interactions (Creasey et al., 2003, Hays et al., 2000, Rajagopala et al., 2009). To check for direct interaction between *hrpX* and *fruB*, their open reading frames were cloned into plasmids pACT2 and pEG202, respectively. In the yeast two-hybrid assay, the interaction between *fruB* and *hrpX* was 3-fold higher than the background level of *fruB* alone (Fig. 21). This provides evidence that the fructose PTS and signal-sensing component of the T3SS are interacting at the protein level. Due to the increase in *hrpS* transcription in the wild-type and *fruB* mutant with *fruB* complementation, this suggests that the possible interaction of the two proteins plays a positive role in the activation or enhancement of the T3SS in *D. dadantii* 3937. Since HrpX is a two-component sensor kinase, this result also suggests that FruB may affect the T3SS through altering the autophosphorylation activity of HrpX.

The expression of the fructose operon is under carbon catabolite repression (CCR) when glucose is used as the sole carbon source. Therefore, the fructose operon is under strict suppression via Cra, the fructose operon repressor, as well as a low level of cAMP produced due to that repression (Ramseier et al., 1993, Ryu et al., 1995). When fructose is used as the carbon source, the intermediate fructose-1-phosphate, binds to Cra to cause derepression of the operon, while CRP is activated due to a phosphorylated EIIA^{Glc} associating with adenylate cyclase to cause production of the CRP-ligand cyclic AMP (Deutscher, 2008). This may cause an increased level of transcription from the *fru* operon. There was a low level of expression from the *fru* promoter, which remained constant throughout the time points observed in the wild-type, *fruA* and *fruB* mutants under glucose

supplementation (Table 16), which indicates that catabolite repression was intact, thus allowing normal leakage from the promoter. Since similar promoter activities of the *fru* operon were observed among wild type, *fruA* and *fruB* mutants (Table 16), this also negates the possibility that the *fruA* or *fruB* mutations were increasing or decreasing the transcription of the *fru* operon in *D. dadantii* 3937. Also, in the *crr* mutant, using fructose and mannitol as the sole carbon sources, the expression levels of the T3SS genes were similar to the wild-type strain (Table 18). This suggests that the metabolic consequence of varying the pool of cAMP doesn't directly affect the transcription of the T3SS. This finding also suggests that the effect of *fruA* and *fruB* mutations were independent of CCR.

During the infection process, bacteria often move from the site of infection to an area within the host which is more metabolically profitable for the organism. This is done through the swimming and/or swarming processes. Our results showed that, in comparison to the wild-type strain, swimming and swarming motility in the *fruA* mutant was similar, while the *fruB* mutant showed increased motility (Fig. 22). Our results also suggest that the fructose PTS, more specifically FruB, is a modulator of bacterial motility in *D. dadantii* 3937. It is now known that there is crosstalk between the carbon phosphotransferase system and the methyl-accepting chemotaxis system of organisms such as *V. cholera* and *E. coli* (Houot et al., 2010, Visick et al., 2007, Neumann et al., 2012). This crosstalk seems to provide information about the intracellular and extracellular environment by PTS modulation of kinase structure, leading to variations in CheA/CheY interactions. In the *fruB* mutant, an increase in motility (swimming and swarming) was observed in

comparison to the wild-type and the *fruA* mutant (Fig. 22). Our findings suggest that functional levels of phosphorylated FruB are increased in the *fruA* mutant, and completely absent in the *fruB* mutant. This may inadvertently increase the interaction between the EI-HPr-EIIA^{Glc} cascade and the MCP system of the bacterium, since FruB is no longer able to titrate phosphate away from HPr and/or EI, thus causing a modulatory effect on motility. RsmA (CsrA) has been shown to stabilize *flhDC* mRNA (Wei et al., 2001), which is the master regulator of flagellar synthesis. Note that since similar expression levels of *rsmA* and *rsmB* were observed between the wild-type and *fruB* mutant, the contribution of RsmA on the motility of the *fruB* mutant should be negligible. Reduced *hrpL* expression was also observed in *fruB* mutant of *D. dadantii* 3937. In *Erwinia amylovora*, HrpL, which exhibits a similar function as the master regulator of T3SS genes in *D. dadantii* 3937, exhibited an inverse role in motility, since a *hrpL* mutant in comparison to the wild-type strain was more motile, but was unable to infect apple seedlings (Cesbron et al., 2006).

In summary, we have provided evidence that altering phosphate levels in *D. dadantii* 3937, from phosphoproteins such as FruB, or, metabolic sources such as acetyl phosphate, have an impact on T3SS gene expression. In accordance with previous literature, generic phosphodonors, such as acetyl phosphate, are also able to modulate the T3SS (Fredericks et al., 2006, Gueriri et al., 2008, Lima et al., 2012, Wolfe et al., 2003). In addition, by mutating specific components, such as *fruA* or *fruB*, T3SS gene transcription in the bacteria was either enhanced or reduced, as well as modulating swimming or swarming. Furthermore, we showed within the

bacteria that HrpX, the sensor kinase responsible for responding to intra- and extra-cellular conditions, was indispensable for responding to fluxes within the fructose PTS. Finally, *D. dadantii* 3937 has an intact CCR, and preferentially utilizes glucose to any other carbon source. Since a mutation in *crr*, or EIIA^{Glc}, did not produce a difference in *hrpS* and *hrpL* T3SS gene transcription, our experimental data suggest that PTS interactions at HrpX are specific for the fructose PTS.

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Table 13. Promoter activity of *hrpS*, *hrpL*, and *hrpA* genes in wild-type *D. dadantii* 3937 (3937) vs. the *fruA* mutant in minimal media.

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpS)	91.83 ± 1.59	107.66 ± 1.36
Δ<i>fruA</i> (phrpS)	115.57 ± 4.87*	126.86 ± 6.76*
Δ<i>fruA</i>:pML<i>fruA</i> (phrpS)	67.39 ± 7.49	81.98 ± 2.80
3937 (pAT)^c	2.37 ± 0.34	2.66 ± 0.78
3937 (phrpL)	12.93 ± 0.54	22.74 ± 1.88
Δ<i>fruA</i> (phrpL)	22.74 ± 1.88*	32.74 ± 3.01*
Δ<i>fruA</i>:pML<i>fruA</i> (phrpL)	9.50 ± 0.62	18.12 ± 1.45
3937 (phrpA)	104.49 ± 9.76	164.93 ± 4.20
Δ<i>fruA</i> (phrpA)	243.25 ± 4.12*	516.82 ± 6.23*
Δ<i>fruA</i>:pML<i>fruA</i> (phrpA)	97.50 ± 0.77	134.48 ± 6.21
3937 (phrpXY)	12.75 ± 0.46	13.74 ± 1.35
Δ<i>fruA</i> (phrpXY)	12.62 ± 0.49	14.74 ± 0.46

^aΔ*fruA* is a deletion mutant of the *fruA* gene in the *D. dadantii* 3937 background. Δ*fruA*::*fruA* contains a plasmid-encoded copy of the *fruA* gene driven with a constitutive promoter.

^bGFP intensity was determined on gated population of bacterial cells via FACS analysis. Values of mean fluorescence intensity (MFI) are an average GFP intensity of total bacterial populations with standard deviations. Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

^cEmpty vector (pAT) fluorescence values were similar throughout this and following experiments

Table 14. Promoter activity of the *rsmA* and *rsmB* gene products in *D. dadantii* 3937 vs. *fruA* and *fruB* mutants.

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b	
	12hr	24hr
3937 (prsmA)	176.13±6.43	187.65±6.70
<i>ΔfruA</i> (prsmA)	167.85±1.91	172.52±2.28
<i>ΔfruB</i> (prsmA)	173.38±1.92	194.99±5.16
3937 (prsmB)	1747.32±16.65	1323.85±135.44
<i>ΔfruA</i> (prsmB)	1754.65±111.13	1417.86±132.50
<i>ΔfruB</i>(prsmB)	1554.86±24.61	1411.10±41.22

^a*fruA* and *fruB* mutants have been described previously.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 15. Promoter activity of *hrpS*, *hrpL*, and *hrpA* in wild-type *D. dadantii* 3937 vs. *fruB* mutant in minimal media.

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpS)	91.83 ± 1.59	107.66 ± 1.36
Δ<i>fruB</i>(phrpS)	74.94 ± 2.99*	89.51 ± 0.80*
3937::<i>fruB</i> (phrpS)	143.94 ± 2.68*	155.75 ± 2.78*
Δ<i>fruB</i>::<i>fruB</i> (phrpS)	155.79 ± 2.98*	163.76 ± 4.90*
3937 (phrpL)	12.93 ± 0.54	22.74 ± 1.88
Δ<i>fruB</i>(phrpL)	8.06 ± 0.12	9.15 ± 0.22*
3937::<i>fruB</i> (phrpL)	31.00 ± 1.90*	39.58 ± 2.65*
Δ<i>fruB</i>::<i>fruB</i> (phrpL)	36.98 ± 7.42*	47.79 ± 5.79*
3937 (phrpA)	104.49 ± 9.76	164.93 ± 4.20
Δ<i>fruB</i>(phrpA)	68.64 ± 2.85*	94.13 ± 1.67*
3937::<i>fruB</i> (phrpA)	244.10 ± 19.94*	229.76 ± 33.07*
Δ<i>fruB</i>::<i>fruB</i> (phrpA)	259.87 ± 41.82*	327.84 ± 36.25*
3937 (phrpXY)	12.75 ± 0.46	13.74 ± 1.35
Δ<i>fruB</i> (phrpXY)	14.18 ± 0.67	16.07 ± 0.85

^a Δ*fruB* is a markerless deletion mutant of the *fruB* gene in the *D. dadantii* 3937 background without antibiotic selection. Δ*fruB*::*fruB* is a chromosomally-integrated complementation, with a copy of the *fruB* gene with its native promoter located at an intergenic region of the genome, *lacY-prt*, of 3937

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 16. Promoter activity of the *fru* operon in *D. dadantii* 3937 vs. the *fruA* and *fruB* mutants in minimal media.

Strain^a/Promoter	Mean Fluorescence Intensity (MFI)^b	
	12hr	24hr
3937 (pfruBKA)	6.32 ± 0.29	6.95 ± 0.10
Δ<i>fruA</i> (pfruBKA)	6.35 ± 0.15	6.79 ± 0.20
Δ<i>fruB</i> (pfruBKA)	6.75 ± 0.51	6.87 ± 0.06

^a*fruA* and *fruB* mutants have been described previously.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 17. Promoter activity of *hrpS*, *hrpL*, and *hrpA* in wildtype *D. dadantii* 3937 vs. *fruK* mutant in minimal media.

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (<i>phrpS</i>)	67.39±6.12	81.98±2.28
$\Delta fruK$ (<i>phrpS</i>)	89.41±7.60*	145.54±7.60*
3937 (<i>phrpL</i>)	8.78±0.70	10.52±0.32
$\Delta fruK$ (<i>phrpL</i>)	24.68±1.24*	31.52±0.80*
3937 (<i>phrpA</i>)	38.32±4.53	62.35±5.94
$\Delta fruK$ (<i>phrpA</i>)	150.53±25.90*	230.48±18.55*

^a $\Delta fruK$ is a deletion mutant of the *fruK* gene in the *D. dadantii* 3937 background.

^bGFP intensity was determined on gated population of bacterial cells via FACS analysis. Values of mean fluorescence intensity (MFI) are an average GFP intensity of total bacterial populations with standard deviations. Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 18. Promoter activity of *hrpS*, *hrpL*, and *hrpA* genes in *D. dadantii* 3937 vs. Δcrr strains with fructose and mannitol supplementation.

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b	
	12hr	24hr
3937 (<i>phrpS</i>)-fru	95.58±1.33	107.63±3.64
Δcrr (<i>phrpS</i>)-fru	109.66±1.72*	115.50±2.83
3937 (<i>phrpS</i>)-mtl	74.98±2.12	83.24±2.60
Δcrr (<i>phrpS</i>)-mtl	79.19±3.32	79.51±0.73
3937 (<i>phrpL</i>)-fru	22.47±0.74	24.21±1.93
Δcrr (<i>phrpL</i>)-fru	22.54±1.26	20.92±0.32
3937 (<i>phrpL</i>)-mtl	11.48±0.31	12.05±0.06
Δcrr (<i>phrpL</i>)-mtl	12.00±1.02	11.16±0.58
3937 (<i>phrpA</i>)-fru	174.66±5.27	167.04±3.99
Δcrr (<i>phrpA</i>)-fru	107.97±1.17*	122.87±12.50*
3937 (<i>phrpA</i>)-mtl	37.79±3.49	67.00±8.15
Δcrr (<i>phrpA</i>)-mtl	36.48±3.56	40.43±2.23

^a Δcrr mutant is a *crr* gene deletion in the *D. dadantii* 3937 background. fru, fructose; mtl, mannitol carbon sources.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 19. Promoter activity of *hrpS* and *hrpL* in *D. dadantii* 3937 vs. Δ *hrpX* and the Δ *hrpX*/ Δ *fruA* double mutant and promoter activity of *hrpS* and *hrpA* genes in *D. dadantii* 3937 vs. the *hrpY* mutant in minimal media.

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpS)	81.44 ± 2.50	96.67 ± 9.71
Δ<i>hrpX</i> (phrpS)	29.86 ± 2.00*	27.78 ± 2.59*
Δ<i>hrpX</i>/Δ<i>fruA</i> (phrpS)	29.68 ± 1.23*	21.27 ± 2.46*
3937 (phrpL)	23.15 ± 2.38	20.16 ± 0.34
Δ<i>hrpX</i> (phrpL)	6.83 ± 3.30*	9.24 ± 0.53*
Δ<i>hrpX</i>/Δ<i>fruA</i> (phrpL)	7.94 ± 1.81*	7.65 ± 0.12*
3937 (phrpS)	89.61±5.18	98.88±16.28
Δ<i>hrpY</i>(phrpS)	6.36±0.98*	10.47±0.58*
3937 (phrpA)	69.98±12.14	138.92±10.29
Δ<i>hrpY</i>(phrpA)	10.01±0.71*	10.23 ±0.50*

^a *hrpX* is a deletion mutant of the *hrpX* gene in the *D. dadantii* 3937 background. *fruA* was described previously. *hrpY* is a deletion mutant of the *hrpY* gene in the *D. dadantii* 3937 background.

^b Similar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 20. Promoter activity of *hrpS* and *hrpA* gene products in *D. dadantii* 3937 vs. $\Delta hrpX$ and $\Delta hrpX::fruB$.

Strain ^a /Promoter	Mean Fluorescent Intensity (MFI) ^b	
	12hr	24hr
3937 (phrpS)	81.44 ± 2.50	96.67 ± 9.71
$\Delta hrpX$ (phrpS)	29.86 ± 2.00*	27.78 ± 2.59*
$\Delta hrpX::fruB$ (phrpS)	29.79±3.19*	28.81±3.71*
3937 (phrpA)	72.68±2.31	106.82±10.16
$\Delta hrpX$ (phrpA)	6.83±3.30*	9.24±0.53*
$\Delta hrpX::fruB$ (phrpA)	7.30±0.01*	7.98±1.18*

^a*hrpX* mutant and *fruB* complementation strains have been described previously.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 21. Promoter activity of *hrpS*, *hrpL*, and *hrpA* in *D.dadantii* 3937 vs. *ackA* and *pta* mutants in minimal media.

Strain ^a /Promoter	Mean Fluorescence Intensity (MFI) ^b	
	12hr	24hr
3937 (<i>phrpS</i>)	78.94 ± 4.37	127.64 ± 6.89
Δ <i>ackA</i> (<i>phrpS</i>)	39.39 ± 6.64*	96.81 ± 2.90*
Δ <i>pta</i> (<i>phrpS</i>)	63.97 ± 12.04	114.29 ± 3.20
3937 (<i>phrpL</i>)	12.93 ± 0.54	22.74 ± 1.88
Δ <i>ackA</i> (<i>phrpL</i>)	6.29 ± 0.90*	8.30 ± 1.58*
Δ <i>pta</i> (<i>phrpL</i>)	12.28 ± 0.47	15.81 ± 0.77*
3937 (<i>phrpA</i>)	49.60 ± 6.42	112.94 ± 14.20
Δ <i>ackA</i> (<i>phrpA</i>)	10.86 ± 1.66*	51.72 ± 11.55*
Δ <i>pta</i> (<i>phrpA</i>)	53.97 ± 12.04	77.73 ± 25.71

^a Δ *ackA* and Δ *pta* are deletion mutants of the *ackA* and *pta* genes, respectively, in the *D. dadantii* 3937 background.

^bSimilar results were obtained between two experiments, with three replicates per experiment. Results of one of the experiments are shown here. Asterisks denote statistically significant differences between 3937 and associated mutant strains (P<0.05, Student's T-test)

Table 22. Strains and plasmids used in the study.

Strains	Relevant characteristics	Reference or Source
<i>Escherichia coli</i>		
S17-1 λ pir	Conjugation strain, F ⁺ , Sp ^R	[57]
EC100 λ pir	Cloning strain	
<i>Dickeya dadantii</i>		
3937	Wildtype strain, <i>Saintpaulia</i> isolate	N. Hugouvieux-Pattat
3937:: <i>fruB</i>	3937 with chromosomal insertion of <i>lacY-fruB-cm-prt</i>	This study
Δ <i>fruA</i>	Deletion of the fructose permease gene, <i>fruA</i>	This study
Δ <i>fruB</i>	Deletion of the diphosphoryl transfer protein, <i>fruB</i>	This study
Δ <i>fruB</i> :: <i>fruB</i>	Δ <i>fruB</i> with chromosomal insertion of <i>lacY-fruB-cm-prt</i>	This study
Δ <i>ackA</i>	Deletion of the acetate kinase gene, <i>ackA</i> , Km ^R	This study
Δ <i>pta</i>	Deletion of the phosphoacetyltransferase gene, <i>pta</i> , Cm ^R	This study
Δ <i>hrpX/fruA</i>	Double mutation in <i>hrpX</i> and <i>fruA</i>	This study
Δ <i>hrpY/fruA</i>	Double mutation in <i>hrpY</i> and <i>fruA</i>	This study
Δ <i>hrpY</i>	Mutation in the T3SS response regulator, <i>hrpY</i>	This study
Δ <i>hrpX</i>	Mutation in the T3SS sensor kinase, <i>hrpX</i>	This study
Δ <i>hrpX</i> :: <i>fruB</i>	Δ <i>hrpX</i> with chromosomal insertion of <i>lacY-fruB-cm-prt</i>	This study
Δ <i>hrpY</i> :: <i>fruB</i>	Δ <i>hrpY</i> with chromosomal insertion of <i>lacY-fruB-cm-prt</i>	This study
<i>S. cerevisiae</i>		
CTY10-5d	<i>MATa gal4 gal80 URA3::lexAop-lacZ his3 leu2 ade2 trp1-901</i>	[27]
Y2 (MY1401)	<i>MAT_ ura3_ leu2_ his3_</i>	[58]

Plasmids		
pMiniHimar RB1	Transposon vector, Km ^R	[57]
pKD3	Antibiotic resistance cassette containing plasmid with FRT excision sites, Cm ^R	[59]
pKD4	Antibiotic resistance cassette containing plasmid with FRT excision sites, Km ^R	[59]
pGEMT-Easy	Cloning vector, Amp ^R	Promega
pWM91	Suicide vector; <i>oriR6K mobRP4 lacZa</i> (of pBluescript II) <i>sacB</i> ; Suc ^r Amp ^R	Metcalfe, 1996
pTCLS-Cm	6.4-kb <i>lacY-cm-prt</i> region cloned in pGEM-T Easy, Cm ^R	[60]
pFLP2	Flippase <i>FRT</i> excision vector, Amp ^R	Huang, 1998
pML123	Complementation vector, Gm ^R	Metcalfe, 1993
pMLfruA	pML123 with 1.4kB-PCR fragment containing <i>fruA</i> ORF	
pPROBE-AT	Promoter probe GFP expressing vector, Amp ^R	[61]
phrpS	pPROBE-AT derivative with PCR fragment containing <i>hrpS</i> promoter, Amp ^R	[18]
phrpL	pPROBE-AT derivative with PCR fragment containing <i>hrpL</i> promoter, Amp ^R	[18]
phrpA	pPROBE-AT derivative with PCR fragment containing <i>hrpA</i> promoter, Amp ^R	[18]
phrpXY	pPROBE-AT derivative with PCR fragment containing <i>hrpXY</i> promoter, Amp ^R	This study
pfuBKA	pPROBE-AT derivative with PCR fragment containing <i>fruBKA</i> operon promoter, Amp ^R	This study
pEG202	Vector containing LexA-transcriptional domain, HIS3, Amp ^R	[26]
pEG202::<i>fruB</i>	Vector containing LexA-transcriptional domain,	This study

pACTII	HIS3, containing <i>fruB</i> ORF, Amp ^R Vector containing Gal4- activation domain, Leu, Amp ^R	[26]
pACTII::<i>hrpX</i>	Vector containing Gal4- activation domain, Leu, containing <i>hrpX</i> ORF, Amp ^R	This study

a Amp^R, ampicillin resistance; Cm^R, chloramphenicol resistance; Km^R, kanamycin resistance; Gm^R, gentamicin resistance; Sp^R, spectinomycin resistance.

Table 23: Primers used in the study

Primers	Sequence 5' to 3'	Source
fruAH1F	AGCGTTTCGTCAACGATTCGCTGAGCGGC	This study
fruAH1RC	GAAGCAGCTCCAGCCTACACATGTTTATCTCCTGCT	This study
fruAH2F	GGATAGGAACTAAGGAGGATATTCATATGTTGCCGCTATAACCT	This study
fruAH2RC	ACAGCATCTGATTGATGATGGCACAGTCAAT	This study
fruBH1F	CAGCATTGCAATATCAGCGTCTTGCGGTCGCCCTGACG	This study
fruBH1RC	GAAGCAGCTCCAGCCTACACAAACGCTCCTCTCTTGCTG	This study
fruBH2F	GGATAGGAACTAAGGAGGATATTCATATGGCAGACGCGTTGCCACC	This study
fruBH2RC	AGCGTAGGCAACTTGCGTCCGGCCCAGATTTTC	This study
ackAH1F	CCACGCGATTTTCAGGAAACACCGGC	This study
ackAH1RC	GAAGCAGCTCCAGCCTACACAGGAAGTACC	This study
ackAH2F	GGAATAGGAACTAAGGAGGATATTCATATGATAAGTCTCCCCACTCC	This study
ackAH2RC	TTATTCTTGCTGCCGCCAAAGCTGG	This study
ptaH1F	TGACCCCACTGGAAGGTCTGGTGATGGGTAC	This study
ptaH1RC	GAAGCAGCTCCAGCCTACACAGGCTTAACCTCTGTTTG	This study
ptaH2F	GGAATAGGAACTAAGGAGGATATTCATATGTGCGACGGCATTACC	This study
ptaH2RC	ACCTACATTGTTGTACATGATGGCATGGGC	This study
fruOPpromfrdXbal	AGTCTAGACAGCCCGACCATGAACGGGCG	This study
fruOPpromRCSacl	CTGAGCTCCTGCCGGCGGAAGCACCTAAATG	This study
fruBfrdintXhoI	TCCTCGAGCAGCCCGACCATGAAC	This study
fruBRCintXhoI	TCCTCGAGTCATGCTGCGCC	This study
hrpXYpromfrdX	AGTATCTAGAAATTTGATGGCATTGTCC	This

baI		study
hrpXYpromRCS	ATGCGAGCTCCGCATAATTACGTG	This study
aci		study
hrpYD57A-Frd	ATTTACTGCTGCTGGCCATGAGCATGCCGGG	This study
hrpYD57A-RC	CCCGGCATGCTCATGGCCAGCAGCAGTAAAT	This study
hrpYH1F	CAGCACCTGACCTCCATGCG	This study
hrpYH1RC	GAAGCAGCTCCAGCCTACACATCGCGTTTCCTCC	This study
hrpYH2F	GGAACTAAGGAGGATATTCATATGTGATAGTCTGTAG	This study
hrpYH2RC	GGTTTCGCCTTCCAGTACGATATCAAC	This study
hrpYORFfrd	ATGGATAGACTCATAAAG	This study
hrpYORFRC	TCAGGCGATTAAACG	This study
46578FrdXbaI	ATGCTCTAGACCGGAGAATGCCTGG	This study
46578RCSaI	AGTCGAGCTCTCTCTTCACTAGGCA	This study
hrpYORFfrdEtag	ATGGATAGACTCATAAAGCTGATGATCGCTG	This study
hrpYORFRCetag	AGTCGAGTCAACGCGGTTCCAGTGGGTCCGGATACGGCACC GGCGCACCGGCGATTAAACG	This study
hrpXYnatprmF	ATCGCTCGAGTGGCGGAAATTCTGT	This study
XhoI		study
hrpXYnatprmR	CTTTATGAGTCTATCCATTGCCATCTCCTTG	This study
C		study
nptIIfrdXhoI	TCAGCTCGAGTGTAGGCTGGAGCTG	This study
nptIIRChrpYfrd	CTTTATGAGTCTATCCATGCCGATTGTCTGTTGTGCCC	This study
comp		study
rsmAnorthF	ATGCTTATTTTGA CTGTCGAGTTGG	This study
rsmAnorthRC	TCAATAGGAGGTAGGCTGAGACTTCTC	This study
rsmBnorthF	GGTGGTCGTCTATAAACCGCG	This study
rsmBnorthRC	TAGTTCGTTTGCAGCAGTCC	This study

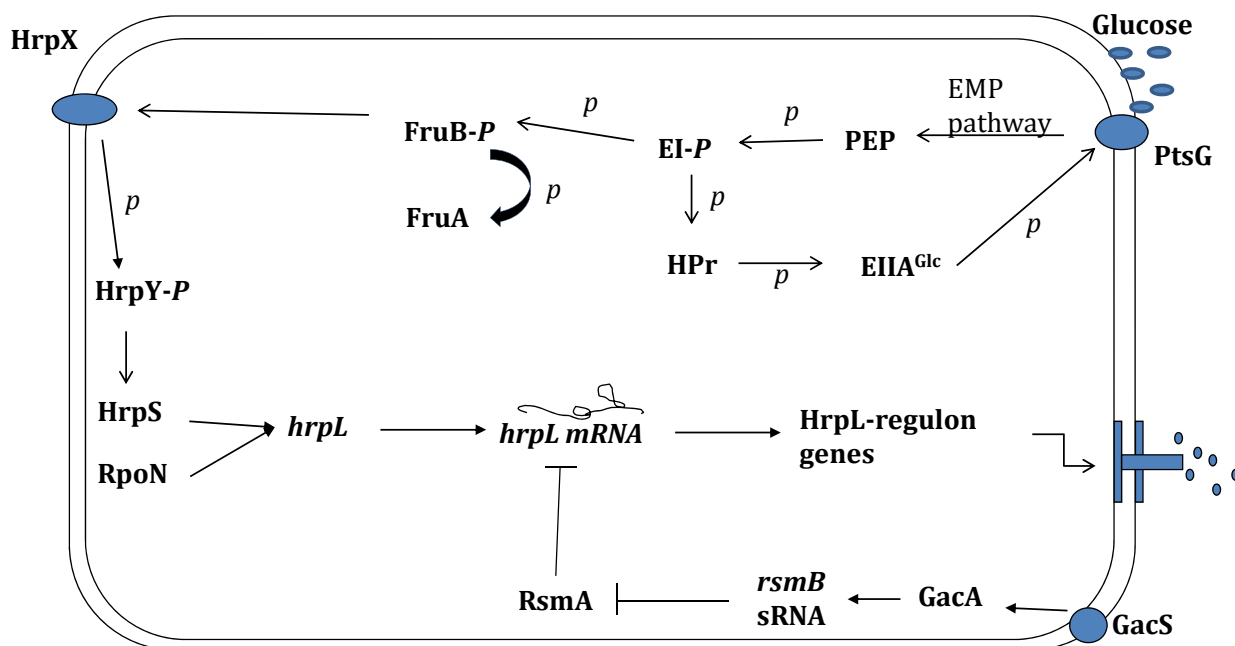


Fig 18. Proposed regulatory model of the fructose PTS on the type III secretion system of *D. dadantii* 3937. Glucose is transported through the cell membrane by PtsG, and metabolized via the Emden-Meyerhof-Parnas pathway of glycolysis, producing phosphoenolpyruvate (PEP). PEP then transfers a phosphate to Enzyme I (EI) of the PTS. EI then transfers its phosphate to various Enzyme IIA (EIIA) proteins of specific carbon transporters. The *D. dadantii* 3937 T3SS is regulated by the HrpX/HrpY-HrpS-HrpL and the GacS/GacA-*rsmB*-HrpL regulatory pathways. Low level transcription from the fructose operon allow production of FruB, FruA, and FruK. The absence of fructose in the media allows an accumulation of FruB, which is able to compete with EIIA^{Glc} for phosphotransfer from EI. FruB then interacts with the T3SS at the level of HrpX, and promotes increased phosphorylation of HrpY. FruB may affect the T3SS through altering the autophosphorylation activity of HrpX. When FruA and fructose is present, phosphorylated FruB is unable to accumulate in the cytoplasm. Straight arrows indicate positive regulation, blocked lines indicate negative regulation. p , phosphate.

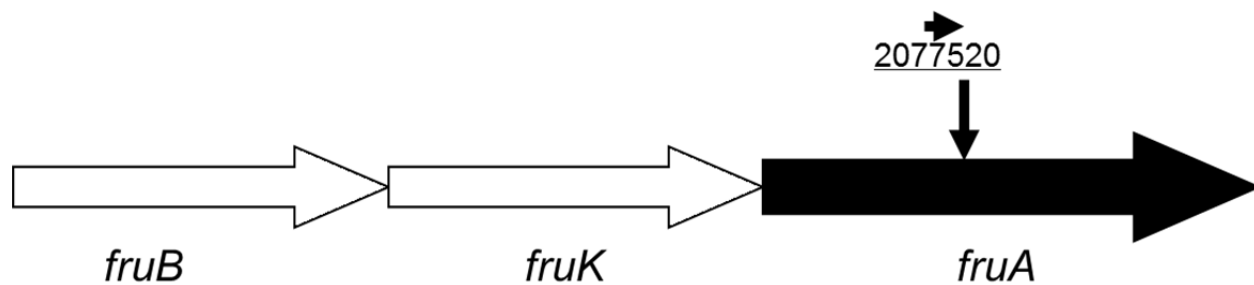


Fig .19. Transposon mutagenesis in *fruA* gene (ASAP ID: 0020028)in *D. dadantii* 3937. 2077520 indicate base insertion site, and orientation of kanamycin cassette transcription.

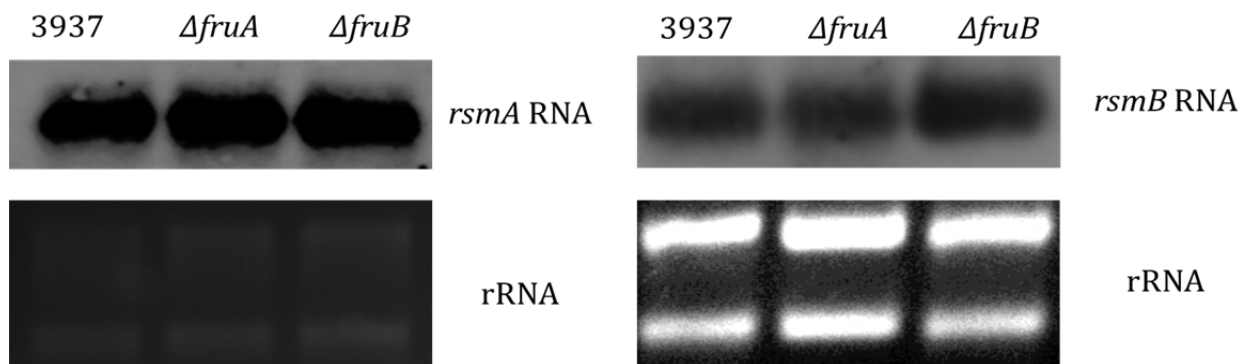


Fig. 20. RNA levels of *rsmA* (left) and *rsmB* (right) in *D. dadantii* 3937 vs. *fruA* and *fruB* mutants. Northern blot analysis of *rsmB* in *D. dadantii* 3937 (lane 1), *fruA* mutant (lane 2), and *fruB* mutant (lane 3). Similar results were obtained in two independent experiments.

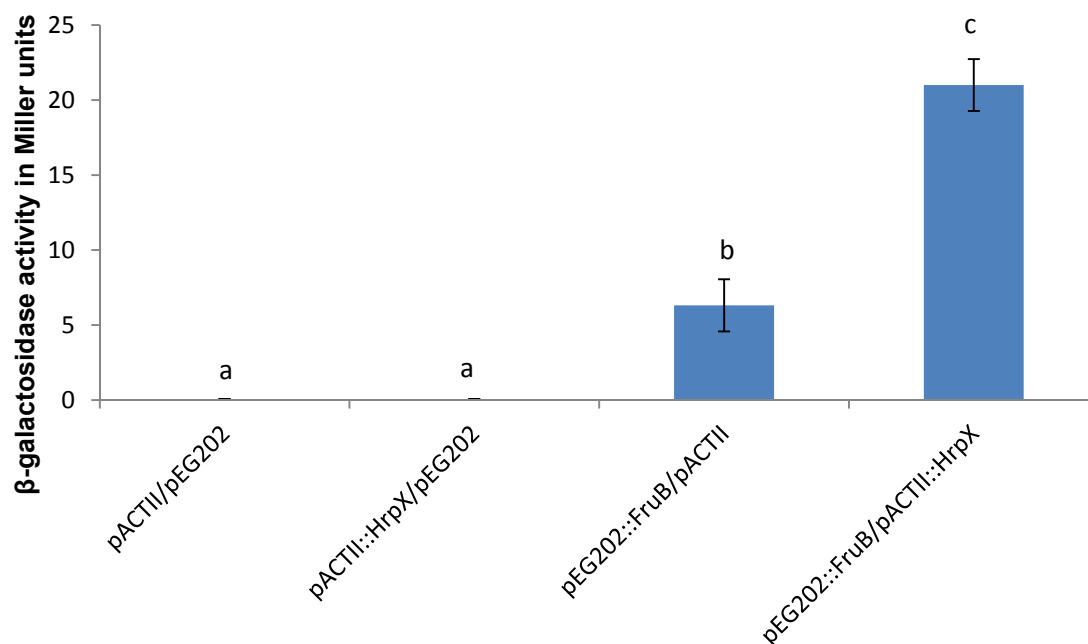


Fig 21. β-galactosidase activity of FruB and HrpX yeast two-hybrid constructs in *S. cerevisiae*. β-galactosidase activity of interactions between pACTII and pEG202, pEG202 and pACTII::HrpX, pACTII and pEG202::FruB, pACTII::HrpX and pEG202::FruB. Similar results were obtained between two independent experiments, with three replicates per experiment. Results of one of the experiments are shown here. Lower case letters denote statistically significant differences between interactions ($P < 0.01$, Student's t-test)

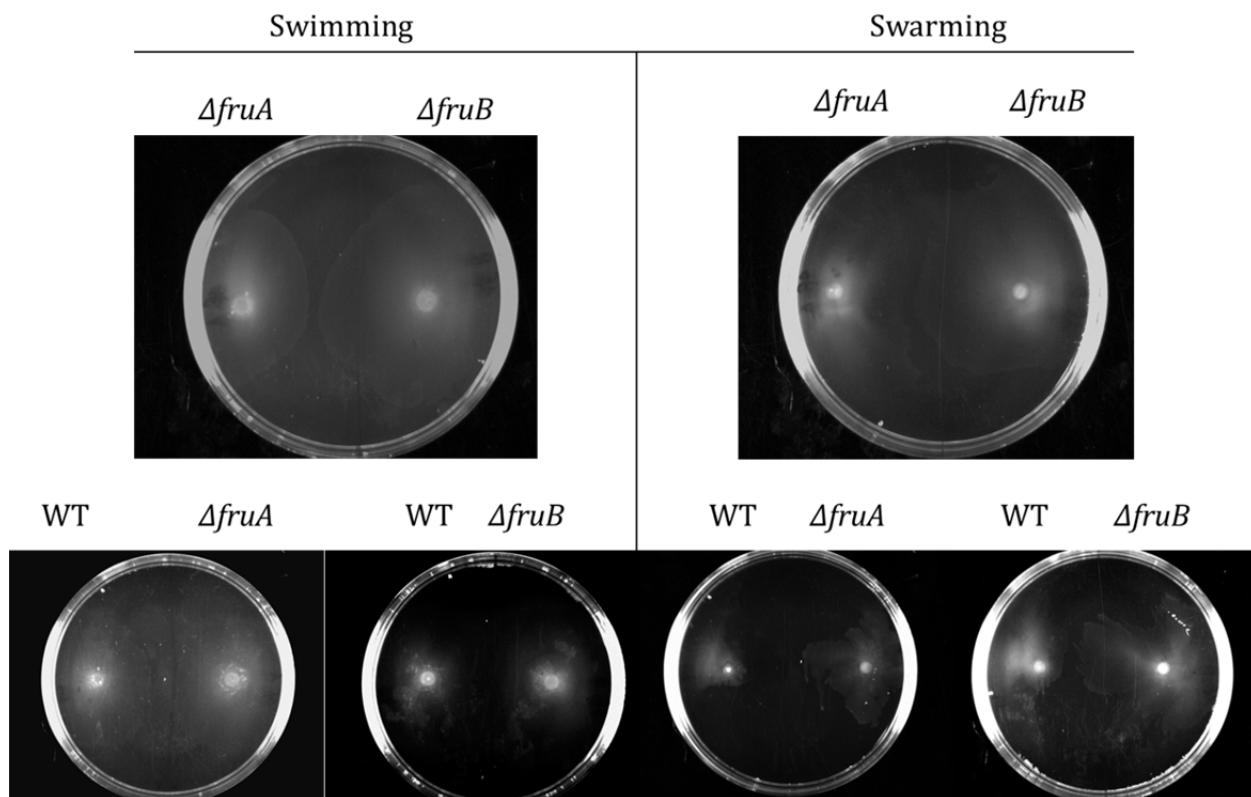


Fig 22. Swimming and swarming of wild type, *fruA* and *fruB* mutants. Swimming was performed on 0.2% MG agar plates. Swarming was performed on 0.4% MG agar plates. 10 μ L was deposited on the center of the plates, and allowed to incubate for 24 hours. Similar results were obtained between duplicate experiments.

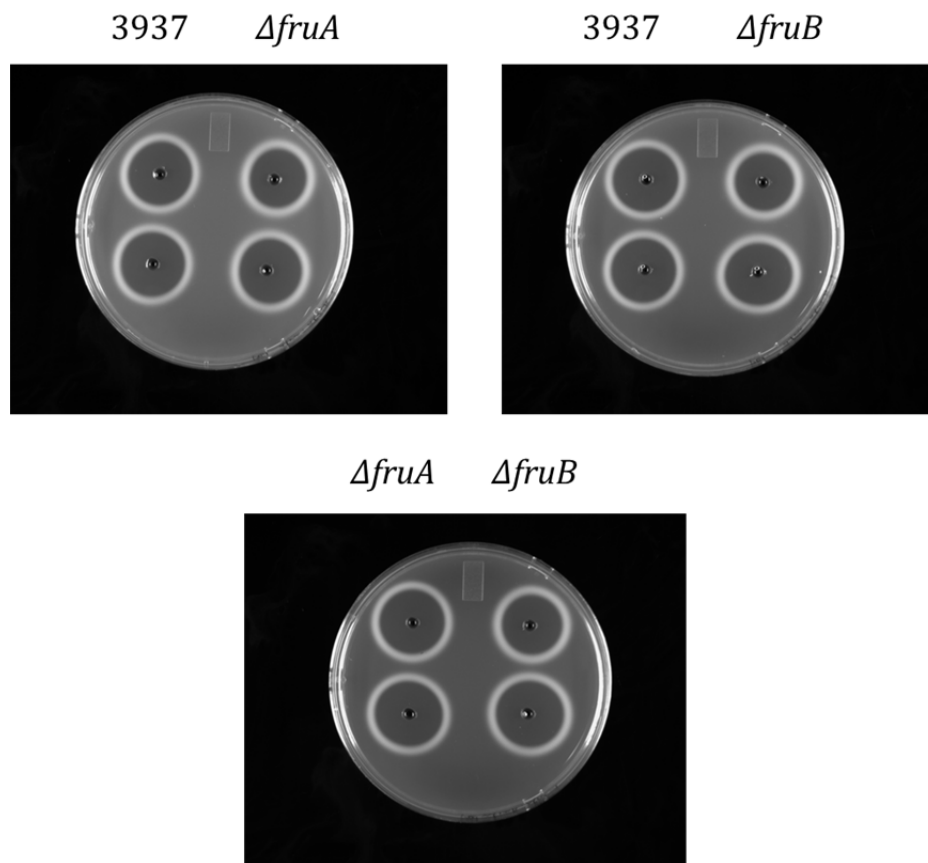


Fig 23. Pectate lyase activity of the total cell extract between *D. dadantii* 3937 (3937), *fruA* and *fruB* mutants. 10 μ L was deposited into a premade, agarose-sealed hole. The plates were allowed to incubate for 24 hours at 28°C, developed with 5N H₂SO₄ and photographed. Kanamycin was added to prevent bacterial growth. Similar results were obtained between duplicate experiments.

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assigned by the course professor. Implementation of enhanced
techniques for improving student experimental outcomes.

Teaching Assistant—Anatomy and Physiology II

2007-2012

Developed laboratory syllabus and overall laboratory structure,
including weekly lab practicum, and administered all grading for
laboratory sections.

ACADEMIC RESEARCH EXPERIENCE

- Explication of specific proteins of the carbon phosphotransferase system
was found to modulate specific type III secretion functionality in *D.*

dadantii 3937 processes. Determined how and where the PTS system regulates the virulence and pathogenicity of this bacterium within the T3SS regulatory pathway.

- Analysis of genes located within central metabolism (glycolytic and TCA cycle) and their effect on induction of the type III secretion system. Elucidated that potential metabolic effectors directly or indirectly played a role in the modulation of the pathogenicity of the bacterium *in vivo* and *in vitro*.
- Investigation of how mammalian bacterial pathogens exist within the phyllosphere of leafy greens. Discerned that the propagation and multiplication of the human pathogen *Escherichia coli* O157:H7 is partially dependent on a functional relationship with a specific plant pathogen, *D. dadantii* 3937.

LABORATORY EXPERIENCE

Department of Pediatrics, Medical College of Wisconsin

Research Technologist III/Lab Manager

2005-2007

Performed *in vivo* experimental procedures in determining detrimental effects of sleep deprivation on various body systems.

Maintained experimental animal colonies and budgetary expenses within the lab.

Constructed and sustained various data acquisition systems.

Instructed undergraduate and medical students on scientific methodology.

Department of Surgery, Medical College of Wisconsin

Research Technologist II

2002-2005

Designed and performed *in vivo* and isolated heart experiments in order to determine phenotypic, genetic, and molecular differences between different rat and rabbit strains.

Designed and constructed mechanical/chemical preparations for laboratory research protocols.

Data acquisition systems.

Instruction of undergraduate students on scientific methods.

Program for Genomic Applications, Dept. of Physiology, Medical College of Wisconsin

Research Technologist I

2000

Performed *in vitro* Langendorff procedures to determine phenotypic differences in consomic rats during myocardial ischemia.

Performed *in vivo* renal preparations to assess vascular differences and salt-sensitivity between consomic rats.

General laboratory duties: maintaining inventory, preparing solutions, etc

Froedtert Memorial Lutheran Hospital Milwaukee, WI

Clinical Research Assistant

1998-2000

Reviewed research protocols for investigational trial implementation at Froedtert Memorial Lutheran Hospital's Investigational Pharmacy Division.

Preparation and dispensation of investigational medications to the respective services within the hospital.

Counseled health care professionals on investigational drug trials.

Perform medical and clinical research analysis for various services within the hospital (Infection Control Committee, Pharmacy and Therapeutics Committee).

PUBLICATIONS AND PAPERS

Hutchins, W., Li, Y., Wu, X., Khokhani, D., Maziarz, M., Kuchin, S., Zhou, L., Ibekwe, A.M., Yang, C.H. The fructose phosphotransferase system functionally modulates the T3SS of *Dickeya dadantii*. (In preparation and will be submitted in July 2013)

Khokhani, D., Zhang, C., Li, Y., Wang, Q., Zeng, Q., Yamazaki, A., **Hutchins, W.**, Zhou, S.S., Chen, X., Yang, C.H. Discovery of plant phenolic compounds that act as type three secretion system inhibitors or inducers of fire blight pathogen *Erwinia amylovora*. Applied and Environmental Microbiology, 2013 (ePub)

Guo W., Zou, L.F., Li, Y.R., Cui Y.P., Ji, Z.Y., Cai, L.L., Zou, H.S., **Hutchins, W.C.**, Yang, C.H., Chen, G.Y. Fructose-bisphosphate aldolase exhibits functional roles between carbon metabolism and the hrp system in rice pathogen *Xanthomonas oryzae* pv. *Oryzicola*. PLoS One 2012, 7(2) e31855

Yamazaki, A., Li, J., Zeng, Q., Khokhani, D., **Hutchins WC**, Yost, A.C., Biddle, E., Toone, E.J., Chen, X., Yang, C.H. Derivatives of Plant Phenolic Compound Affect the Type III Secretion System of *Pseudomonas aeruginosa* via a GacS-GacA Two-Component Signal Transduction System. Antimicrobial Agents and Chemotherapy,

Yamazaki, A., Li, J., **Hutchins, W.C.**, Wang, L., Ma, J., Ibekwe, A.M., Yang, C.H. Commensal Effect of Pectate Lyases Secreted from *Dickeya dadantii* on Proliferation of *Escherichia coli* O157:H7 EDL933 on Lettuce Leaves. Applied and Environmental Microbiology, 2010. 77(1): 156-62

Nandedkar S., Feroah T., **Hutchins W.**, Weihrauch D., Konduri K., Wang J., Strunk R., DeBaun M., Hillery C., Pritchard Jr., K.A. Histopathology of Experimentally-induced Asthma in a Murine Model of Sickle Cell Disease. Blood, 2008. 1126 (6): 2529-2538

Nandedkar, S.D., Feroah, T.R., **Hutchins, W.**, Weihrauch, D., Konduri, K.S., Wang, J., Strunk, R.C., Debaun, M.R., Hillery, C.A., Pritchard, K.A. Histopathology of experimentally induced asthma in a murine model of sickle cell disease. *Blood*, 2008. 112(6): 2529-38.

Manconi, M., **Hutchins, W.**, Feroah, T., Zucconi, M., Ferini-Strambi, L. On the pathway of an animal model for restless legs syndrome. *Neurological Sciences*, 2007. 28(Supp1): S53-S60

Shi, Y., **Hutchins, W.C.**, Su, J., Siker, D., Hogg, N., Pritchard, K.A Jr, Keszler, A., Tweddell, J.S., Baker, J.E. Delayed cardioprotection with isoflurane: role of reactive oxygen and nitrogen. *American Journal of Physiology, Heart Circulation and Physiology*, 2005. 288(1): H175-84

Fitzpatrick, C.M., Shi, Y., **Hutchins, W.C.**, Su, J., Gross, G.J., Ostadal, B., Tweddell, J.S., Baker, J.E. Cardioprotection in chronically hypoxic rabbits persists on exposure to normoxia: role of NOS and K_{ATP} channels. *American Journal of Physiology, Heart Circulation and Physiology*, 2005. 288(1): H62-68

LANGUAGES

[English – native language]

MEMBERSHIPS

American Society of Microbiology