BARD

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The Roles of Hrp-Dependent Proteins and *hrp* Gene Regulation ad Determinants of Virulence and Host-Specificity in *Erwinia stewartii* and *E. herbicola* pvs. *Gypsophilae* and *betae*

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Project Title: The roles of Hrp-dependent proteins and *hrp* gene regulation as determinants of virulence and host-specificity in *Erwinia stewartii* and *E. herbicola* pvs. *gypsophilae* and *betae*

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Pantoea stewartii, *Pantoea agglomerans*, maize, gypsophilia, beets, type-III secretion system, Stewart's Wilt, galls, water-soaking

Abbreviations commonly used in the report, in alphabetical order:

Avr, avirulence effector; Dsp, disease specific effector; GFP, green fluorescent protein; Hrp, hypersensitive response and pathogenicity; *hrpSp*, the *hrpS* promoter; *Pnss*, *Pantoea stewartii* subsp. *stewartii*; *Pag*, *P. agglomerans* pv. *gypsophilae*; *Pab*, *P. agglomerans* pv. *betae*; TTSS, type III secretion system;
Wts, water-soaking.

Budget: IS: $ 172,000 US: $ 128.000 Total: $ 300,000

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)		2	4	6
Submitted, in review, in preparation		4		4
Invited review papers		1	2	3
Book chapters				
Books				
Master theses			4	4
Ph.D. theses		1	3	4
Abstracts		3	5	8
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

none

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		2	1	3
Longer Visits (Sabbaticals)		1		1

Description of Cooperation:

Parallel work on all objectives was done in each country with the Israeli groups working on *Pag* and the US group working on *Pnss*. Overall, the collaboration was highly "synergistic".

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				0
Issued (allowed)				
Licensed				

Abstract

Gram-negative plant pathogenic bacteria employ specialized type-III secretion systems (TTSS) to deliver an arsenal of pathogenicity proteins directly into host cells. These secretion systems are encoded by *hrp* genes (for hypersensitive response and pathogenicity) and the effector proteins by so-called *dsp* or *avr* genes. The functions of effectors are to enable bacterial multiplication by damaging host cells and/or by blocking host defenses. We characterized essential *hrp* gene clusters in the Stewart's Wilt of maize pathogen, *Pantoea stewartii* subsp. *stewartii* (*Pnss*; formerly *Erwinia stewartii*) and the gall-forming bacterium, *Pantoea agglomerans* (formerly *Erwinia herbicola*) pvs. *gypsophilae* (*Pag*) and *betae* (*Pab*). We proposed that the virulence and host specificity of these pathogens is a function of a) the perception of specific host signals resulting in bacterial *hrp* gene expression and b) the action of specialized signal proteins (i.e. Hrp effectors) delivered into the plant cell. The specific objectives of the proposal were: 1) How is the expression of the *hrp* and effector genes regulated in response to host cell contact and the apoplastic environment? 2) What additional effector proteins are involved in pathogenicity? 3) Do the presently known *Pantoea* effector proteins enter host cells? 4) What host proteins interact with these effectors?

We characterized the components of the *hrp* regulatory cascade (HrpXY $\rightarrow$ HrpS $\rightarrow$ HrpL $\rightarrow$ *hrp* promoters), showed that they are conserved in both *Pnss* and *Pag*, and discovered that the regulation of the *hrpS* promoter (*hrpSp*) may be a key point in integrating apoplastic signals. We also analyzed the promoters recognized by HrpL and demonstrated the relationship between their composition and efficiency. Moreover, we showed that promoter strength can influence disease expression. In *Pnss*, we found that the HrpXY two-component signal system may sense the metabolic status of the bacterium and is required for full *hrp* gene expression in planta. In both species, acyl-homoserine lactone-mediated quorum sensing may also regulate epiphytic fitness and/or pathogenicity. A common Hrp effector protein, DspE/WtsE, is conserved and required for virulence of both species. When introduced into corn cells, *Pnss* WtsE protein caused water-soaked lesions. In other plants, it either caused cell death or acted as an Avr determinant. Using a yeast-two-hybrid system, WtsE was shown to interact with a number of maize signal transduction proteins that are likely to have roles in either programmed cell death or disease resistance. In *Pag* and *Pab*, we have characterized the effector proteins HsvG, HsvB and PthG. HsvG and HsvB are homologous proteins that determine host specificity of *Pag* and *Pab* on gypsophila and beet, respectively. Both possess a transcriptional activation domain that functions in yeast. PthG was found to act as an Avr determinant on multiple beet species, but was required for virulence on gypsophila. In addition, we demonstrated that PthG acts within the host cell. Additional effector genes have been characterized on the pathogenicity plasmid, $pPATH_{Pag}$, in *Pag*. A screen for HrpL-regulated genes in *Pnss* pointed up 18 candidate effector proteins and four of these were required for full virulence.

It is now well established that the virulence of Gram-negative plant pathogenic bacteria is governed by Hrp-dependent effector proteins. However, the mode of action of many effectors is still unresolved. This BARD supported research will significantly contribute to the understanding of how Hrp effectors operate in *Pantoea* spp. and how they control host specificity and affect symptom production. This may lead to novel approaches for genetically engineering plants resistant to a wide range of bacterial pathogens by inactivating the Hrp effectors with "plantabodies" or modifying their receptors, thereby blocking the induction of the susceptible response. Alternatively, innovative technologies could be used to interfere with the Hrp regulatory cascade by blocking a critical step or mimicking plant or quorum sensing signals.

Achievements

Objective 1. Regulation of Hrp effectors. A major question that has not yet been resolved in phytopathogenic bacteria is how the pathogen perceives an external signal from the host plant?

- In both *Pantoea* spp., we characterized a common signal transduction pathway for controlling the *hrp* regulon. Environmental signaling is also mediated by the HrpX/HrpY two component system, with HrpX functioning as a sensor-kinase and HrpY as a response regulator. HrpY activates *hrpS*, which encodes a transcriptional enhancer. HrpS then activates *hrpL*, which encodes an alternate sigma factor that recognizes "*hrp* box" promoters. Epistasis analysis, expression experiments using gene fusions, and genetic reconstruction of each step in *E. coli* were used to delineate and confirm the pathway.
- Phosphorylation of HrpY by HrpX was shown to be an initial key step in turning on the regulatory cascade in *Pnss* and *Pag,* although some differences were revealed between the two systems. The precise mechanism that triggers the phosphorylation in the apoplast environment is still unknown (5, 8). However, *Pnss* HrpX was required for maximal expression of *hrp* genes in corn plants and in vitro the HrpX PAS domains were shown to respond to Kreb's cycle intermediates.
- HrpY was found to directly regulate *hrpSp* and autoregulate the *hrpXY* operon. The transcriptional start sites for *hrpX*, *hrpS* and *hrpL* were located by primer extension analysis. In addition, a novel transcriptional autoregulatory circuit was characterized in *Pnss* (6).
- Global regulatory circuits were also shown to affect *hrpS* expression. Quorum sensing regulation in *Pag* has been demonstrated and its involvement in epiphytic fitness has been shown and in *Pnss* repression by the EsaI/R quorum sensing system appears to enter the *hrp* regulatory pathway at the level of *hrpS* expression (9), but EsaR does not directly control *hrpSp*. Likewise *hrp* gene expression in *Pnss* was turned on primarily in stationary phase and it was negatively regulated by the Lon protease.
- The "Hrp box" promoter, which is activated by HrpL, is common to *Erwinia, Pseudomonas* and *Pantoea* spp. It has been previously defined on the basis of computational techniques. In the present project we have, for the first time, combined a quantitative *in vivo* assay for analyzing *hrp*-promoter activity with site specific mutagenesis to measure the effect of consensus and non-consensus nucleotides on its activity. We have identified the nucleotides

required for HrpL binding and demonstrated that variation in this promoter's strength in *Pag* can affect disease expression (7).

Objective 2. Are additional effector proteins involved in host specificity and pathogenicity?

- The sequence of $pPATH_{Pag}$ (135 kb) has been completed and provides the full inventory of nine TTSS effectors (1,3). The role of the recently discovered four TTSS effectors in pathogenicity is now under investigation. The entire *hrp* cluster from *Pnss* has also been sequenced and, except for *wtsEF*, the variable effector regions are completely different from those in *E. amylovora* and *Pag*. Several candidate effector genes were present, but they did not appear to contribute to virulence in corn.
- It has been demonstrated that PthG acts as an Avr determinant on multiple beet species (2). Molecular determinants affecting activity of PthG were identified. The C-terminal 225 aa were sufficient for HR elicitation on beet (3). The most important achievement for this objective was the isolation of HsvB, a homolog of HsvG, from plasmids $pPATH_{Pag}$ and $pPATH_{Pab}$. In contrast to HsvG, which determines host specificity of *Pag* and *Pab* on gypsophila, HsvB determines host specificity of both pathovars on beet (1).
- To identify more *Hrp* secreted proteins in *Pnss*, we screened for genes with HrpL-regulated promoters. From a collection of 26,000 Tn*5gusA* insertion mutants, 18 mutants represented possible new effector genes and four of these caused less severe symptoms in corn at low inoculum concentrations. These included homologs of *Pseudomonas syringae* HopPtoC and HopPtoE. We also screened for genes that were down-regulated by HrpL and found a second TTSS with homology to pathogenicity genes in *Salmonella* and *Shigella*. We are currently testing if this system is needed for transmission of *Pnss* by the corn flea beetle vector.

Objective 3. Translocation into the host cells.

- Bombardment experiments of GFP-labeled *Pag* PthG have demonstrated that this TTSS effector functions in plant cells (2).
- Two systems have been used to deliver *Pnss* WtsE into plant cells. An *Escherichia coli* strain carrying the *Erwinia chrysanthemi hrp* cluster was able to translocate WtsE into various leaf tissues. In sweet corn, WtsE caused water-soaked lesions within 12 h, but in beet and *Nicotiana benthamiana*, it caused an HR-like response. We are very excited about the discovery that translocation of WtsE is indeed able to cause water-soaking in corn. This is the first time an effector protein from erwinias has been shown to cause this symptom,

which is characteristic of many bacterial diseases. We have also succeeded in transiently expressing *wtsE* in *N. benthamiana* and *N. tabacum* using an *Agrobacterium tumefaciens* system, where it causes necrosis. *Pnss* strains were also able to cause an HR in beet and water-soaking in Arabidopsis. Both responses were depending on WstE.

- In the course of the yeast-two hybrid experiments, we also discovered that intact WtsE is toxic to cells of the budding yeast, *Saccharomyces cerevisiae*. These results suggest that the targets of WtsE and DspE are conserved between cells from different kingdoms and they may be generally toxic to cukaroytes.
- WtsF is predicted to be a chaperone for WtsE. However, *Pnss wtsF* mutants retain significant pathogenicity, so it does not appear to be required for WtsE translocation. Western blots revealed that the amount of WtsE present in both the cell pellets and culture supernatants of *wtsF* mutants was greatly reduced. We therefore believe that the major role of this chaperone is to stabilize WtsE inside the bacterium and protect it from degradation by the Lon protease.

Objective 4. What host proteins interact with *Erwinia* Hop effectors?

- We have used a yeast two-hybrid system to screen for the target of *Pnss* WtsE in maize cells. Almost all of the candidate interacting proteins identified appear to be involved with signal transduction that leads either to PCD or disease resistance. Specifically, the N-terminal half of WtsE interacted with LRR receptor kinases that form a major class of R-proteins and the C-terminal half interacted with a separate set of proteins that may also regulate resistance signaling by controlling ubiquitination and subsequent degradation of proteins.

Cooperation: Parallel work on all objectives was done in each country with the Israeli groups working on *Pag* and the US group working on *Pnss*. Overall, the collaboration was highly "synergistic". The Israeli scientists specialized in characterizing the secreted proteins and the US scientists concentrated more on *hrp* gene regulation. During the first year of this grant, Dr. Barash spent two months in Dr. Coplin's laboratory studying the regulation of *hrp* genes in *Pag* by a quorum sensing mechanism. On May, 2004 Drs Manulis and Barash visited Coplin's lab to discuss the obtained results.

Reviewed Publications:

1. Barash I. and Manulis S. 2005. Hrp-dependent biotrophic mechanism of virulence - how it has evolved in tumorigenic bacteria? *Phytoparasitica* 33:317-324.

2. Ezra D, Barash I, Weinthal DM, Gaba V, Manulis S. 2003. *pthG* from *Pantoea agglomerans* pv. *gypsophilae* encodes an avirulence effector that determines incompatibility in multiple beet species. *Mol. Plant Pathol.*: 5:105-113.

3. Manulis S, Barash I. 2003. Contribution of virulence determinants from *Pseudomonas* and other bacteria to *hrp*-dependent gall formation by *Erwinia herbicola* pv. *gypsophilae*. pp.373-381 in *Pseudomonas syringae and related pathogens.*, ed. NS Iacobellis, Kluwer Academic Publisher.

4. Manulis S, Barash I. 2003. *Pantoea agglomerans* pvs. *gypsophilae* and *betae*, a recently-evolved pathogen? *Molecular Plant Pathology* 4:307-314.

5. Merighi, M., D. R. Majerczak, E. H. Stover, and D. L. Coplin. 2003. The HrpX/Y two-component system activates *hrpS* expression, the first step in the regulatory cascade controlling the *Hrp* regulon in *Pantoea stewartii* subsp. *stewartii*. *Mol. Plant-Microbe Interact.* 16:238-248.

6. Merighi, M., Majerczak, D.R., and Coplin, D.L. 2005. A novel transcriptional autoregulatory loop enhances expression of the *Pantoea stewartii* subsp. *stewartii* Hrp type III secretion system. FEMS Microbiol. Lett. 243:479-487.

7. Nissan G., Manulis S., Weinthal D.M., Sessa, G. and Barash I. 2005. Analysis of promoters recognized by HrpL, an alternative sigma factor protein from *Pantoea agglomerans* pv. *gypsophilae* .Mol. Plant –Microbe Interact. 2005, 18: 634-643.

8. Nizan-Koren R, Manulis S, Mor H, Iraki NM, Barash I. 2003. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophilae*. *Mol. Plant-Microbe Interact.* 15:249-60.

9. von Bodman, S., Bauer, W.D., and Coplin, D.L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.

APPENDIX

Table of Contents

Publication List

Peer Reviewed Publications:

1. Merighi, M., D. R. Majerczak, E. H. Stover, and D. L. Coplin. 2003. The HrpX/Y two-component system activates *hrpS* expression, the first step in the regulatory cascade controlling the *Hrp* regulon in *Pantoea stewartii* subsp. *stewartii*. *Mol. Plant-Microbe Interact.* 16:238-248.
2. Nizan-Koren R, Manulis S, Mor H, Iraki NM, Barash I. 2003. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophilae*. *Mol. Plant-Microbe Interact.* 15:249-60.
3. Manulis S, Barash I. 2003. *Pantoea agglomerans* pvs. *gypsophilae* and *betae*, a recently-evolved pathogen? *Molecular plant Pathology* 4:307-314
4. Manulis S, Barash I. 2003. Contribution of virulence determinants from *Pseudomonas* and other bacteria to *hrp*-dependent gall formation by *Erwinia herbicola* pv. *gypsophilae*. In *Pseudomonas syringae and related pathogens.*, ed. NS Iacobelis, pp.373-381. Kluwer Academic Publisher
5. Ezra D, Barash I, Weinthal DM, Gaba V, Manulis S. 2003. *pthG* from *Pantoea agglomerans* pv. *gypsophilae* encodes an avirulence effector that determines incompatibility in multiple beet species. *Mol. Plant Pathol.*: 5:105-113.
6. von Bodman, S., Bauer, W.D., and Coplin, D.L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.
7. Barash I. and Manulis S. 2005. Hrp-dependent biotrophic mechanism of virulence - how it has evolved in tumorigenic bacteria? *Phytoparasitica* 33:317-324.
8. Nissan G., Manulis S., Weinthal D.M., Sessa, G. and Barash I. 2005. Analysis of promoters recognized by HrpL, an alternative sigma factor protein from *Pantoea agglomerans* pv. *gypsophilae* .Mol. Plant –Microbe Interact. 2005, 18 :634-643.
9. Merighi, M., Majerczak, D.R., and Coplin, D.L. 2005. A novel transcriptional autoregulatory loop enhances expression of the *Pantoea stewartii* subsp. *stewartii* Hrp type III secretion system. FEMS Microbiol. Lett. 243:479-487.

Presentations at Meetings:

1. Merighi, M. and Coplin, D. L. 2003. The HrpX/HrpY two-component signal transduction system is a dual activator/repressor of the Hrp/type III secretion regulon in *Pantoea stewartii* subsp. *stewartii*. Abstr. Mol. Genet. of Bacteria and Phages Mtg., Univ. of Wisconsin, Madison.
2. Merighi, M., Majerczak, D. R., and Coplin, D. L. 2003. HrpY response regulator is a dual activator/repressor of *hrp* gene expression in *Pantoea stewartii* subsp. *stewartii*. Abstr. Phytopathology 93(6):S61.
3. Ham, J. H., and Coplin, D. L. 2003. Lon protease is a negative regulator of the Hrp type II protein secretion system in *Pantoea stewartii* subsp. *stewartii*. Abstr. Phytopathology 93(6):S32.

4. Nissan G, Manulis S, Barash I. 2003. Molecular determinants affecting activity of the virulence effectors, PthG and HsvG of *Erwinia herbicola* pv. *gypsophilae*. *11-International Congress of Molecular Plant-Microbe Interactions, p152, St. Petersburg, Russia.*
5. Weinthal, D.M., Ezra, D., Barash, I., Gaba, V. And Manulis, S. 2004. *pthG* from *Pantoea agglomerans* pv. *gypsophilae* encodes an avirulence effector that determines incompatibility in multiple beet species. Phytoparasitica 32, 185.
6. Barash, I., Panijel, M., Gurel, F., Chalupowicz, L. and Manulis, S. 2005. Molecular events in the transformation of *Pantoea agglomerans* from an endophyte-epiphyte into a tumorigenic bacterial pathogen. Proceeding of the First International Conference on Plant-Microbe Interactions: Endophytes and Biocontrol Agents. Saariselka, Finland.
7. Chalupowicz, L., Itkin, M., Barash, I. and Manulis, S. 2005. Quorum sensing *in Pantoea agglomerans* pv *gypsophilae*: involvement in epiphytic fitness and identification of target genes. Proc. of the 12th International Congress on Molecular Plant-Microbe Interactions. Cancun, Mexico.
8. Nissan, G., Manulis, S., Weinthal, D. M., Mor, H. and Barash, I. 2005. HsvG and HsvB are two homologous type III effector proteins that determine host specificity of *Pantoea agglomerans* on gypsophila and beet. Proc. of the 12th International Congress on Molecular Plant-Microbe Interactions. Cancun, Mexico.

In Preparation:

Merighi M., Majerczak, D.R., Zianni, M., Kimberly, T.K., and Coplin, D.L. 2005. Molecular characterization of *Pantoea stewartii* subsp. *stewartii* HrpY, a conserved response regulator of type III secretion systems, and its interaction with the *hrpS* promoter. In revision for resubmission to J. Bacteriol.

Ham, J.H., Majerczak, D.R., Arroyo-Rodriguez, A.S., and Coplin, D.L. WtsE, an AvrE-family effector protein from *Pantoea stewartii* subsp. *stewartii*, causes cell death in corn and requires a chaperone protein for stability. To be submitted to Mol. Plant-Microbe Interact. in July.

Merighi, M. and Coplin, D.L. Genetic analysis of the role of the HrpX PAS kinase in signal sensing and activation of *hrp*/type III secretion gene expression in *Pantoea stewartii* subsp. *stewartii*. To be submitted to J. Bacteriol. in August.

Merighi, M., Majerczak, D.R., von Bodman, S., and Coplin, D.L. N-acylhomoserine lactone-dependent quorum-sensing controls expression of the Hrp-type III secretion system of the biotrophic plant pathogen *Pantoea stewartii* subsp. *stewartii*. To be submitted to Microbiology in August.

Introduction: Gram-negative plant pathogenic bacteria employ specialized type-III secretion systems (TTSS) to deliver an arsenal of pathogenicity proteins directly into host cells. These secretion systems are encoded by *hrp* genes and the effector proteins by so-called *dsp* or *avr* genes. Both classes of elicitors have been designated "Hrp-outer-proteins" (Hops). The functions of Hops are to enable bacterial multiplication by damaging host cells and/or by blocking host defenses. We have characterized essential *hrp* gene clusters in the Stewart's Wilt pathogen, *Erwinia stewartii* (synon. *Pantoea stewartii* subsp. *stewartii Pnss*) and the gall-forming bacterium, *E. herbicola* (synon. *P. agglomerans*) pvs. *gypsophilae* (*Pag*) and *betae* (*Pab*). We have proposed that the virulence and host specificity of these pathogens is a function of a) the perception of specific host signals resulting in bacterial *hrp* gene expression and b) the action of specialized signal proteins (i.e. Hops) delivered into the plant cell. We are characterizing the components of the *hrp* regulatory cascade, have shown that they are conserved in both *Pnss* and *Pag*, and discovered that the regulation of *hrpS* may be a key point in integrating apoplastic and cell contact signals. A common Hop protein, DspE/WtsE, is conserved and required for virulence in both species. In *Pag* and *Pab*, the effector proteins HsvG, HsvB and PthG determine host specificity and other Hops may be involved in gall formation.

Objective 1. How is the expression of the *hrp* and *hop* genes regulated in response to cell contact and apoplastic environment?

Under a previous BARD grant, we found that the transcription of the *hrp/wts* systems of both *Pnss* and *Pag* are controlled by at least four genes that constitute a regulatory cascade. Environmental signaling appears to be mediated by the HrpX/HrpY two component system, with HrpX functioning as a sensor-kinase and HrpY as a response regulator. HrpY activates *hrpS*, which encodes a transcriptional enhancer. HrpS then activates *hrpL*, which encodes an alternate sigma factor that recognizes "*hrp* box" promoters. In both the US and Israeli laboratories, an initial objective was to finish the genetic analysis of this pathway in both bacteria.

***Pantoea agglomerans* (formerly *Erwinia herbicola*) pv. *gypsophilae* (Israel)**

a. The regulatory cascade that activates the Hrp regulon in *Erwinia herbicola* pv. *gypsophilae* (Mol. Plant –Microbe Interact. 2003. 16: 249-260).

The pathogenicity of *Erwinia herbicola* pv. *gypsophilae* (*Ehg*) is dependent on a plasmid ($pPATH_{Ehg}$) which harbors the *hrp* gene cluster and additional virulence genes. The *hrp* regulatory cascade of *Ehg* comprises an *hrpX*Y operon encoding a two-component system; *hrpS* encoding a transcriptional factor of the NtrC family and *hrpL* encoding an alternative sigma factor. Results obtained suggest the following signal transduction model for activating the Hrp regulon: phosphorylated HrpY activates *hrpS*, HrpS activates *hrpL* and HrpL activates genes containing "*hrp* box" promoter. This model was supported by studies on the effects of mutations in the regulatory genes on pathogenicity and complementation analysis. Nonpolar mutations in *hrpX* did not affect virulence or transcription of downstream genes. Site-directed mutagenesis of

the conserved aspartate 57 in HrpY suggested that its phosphorylation is crucial for activating the *hrp* regulatory cascade. Studies on the effects of mutations in the *hrp* regulatory genes on transcriptional activity of downstream genes or of their isolated promoters *in planta* showed dependency of *hrpS* expression on active HrpY, of *hrpL* expression on active HrpS and of *hrpN* or *hrpJ* expression on active HrpL. These results were also partially supported by over-expression of regulatory genes under *in vitro* conditions. The *hrpXY* is constitutively expressed with high basal level under repressive conditions in contrast to *hrpS* and *hrpL*, which exhibit low basal expression levels and are environmentally regulated.

b. Analysis of promoters recognized by HrpL, an alternative sigma factor protein from *Pantoea agglomerans* pv. *gypsophilae* (Mol. Plant –Microbe Interact. 2005. 18: 634-643).

HrpL, an alternative σ factor, activates the transcription of the Hrp regulon by its binding to a common '*hrp* box' promoter. Based on computational techniques the '*hrp* box' was previously defined as a consensus bipartite *cis* element, namely, 5'-GGAACC- N_{15-16}- CCACNNA-3'. The present report combines a quantitative *in vivo* assay for measuring Hrp promoter activity with site-specific mutagenesis, to analyze the effect of consensus and non-consensus nucleotides (nt) on promoter efficiency. The analysis was carried out with Hop effectors of the tumorigenic bacterium, *Pantoea agglomerans* pv. *gypsophilae (Pag)*, in which HrpL is indispensible for gall formation. Mutational analysis indicates that the '*hrp* box' consensus can be divided into crucial and non-crucial nt. The first five nt of the –35 consensus motif (GGAAC) and the three nt of the –10 motif (ACNNA) are crucial whereas other consensus and adjacent non-consensus nt exert a significant effect on the promoter's strength. With spacing of 13 or 17 nt between the two motifs significant activity was still retained. Gel shift assays indicated that deletion of GG from the –35 consensus motif eliminated HrpL binding whereas mutations in the –10 consensus motif or modification of the spacing, which eliminates promoter activity, did not elicit any effect. The degeneracy in Hrp promoters of four *hrp* and type III effector genes of *Pag* indicated significant differences in promoter efficiency, while increasing the promoter efficiency of the Hop effector, HsvG, resulted in over-expression of gall formation.

c. Quorum sensing regulation in *Pag* (abstract submitted to the 12th MPMI Congress).

Previous studies demonstrated that multiplication of *P. agglomerans in planta* was *hrp*-dependent and the bacterium was found within the developing gall in cell aggregates. These observations led us to investigate the contribution of cell density dependent - quorum sensing (QS) system in pathogenicity and epiphytic fitness of this bacterium. QS signaling has not been previously characterized in *P. agglomerans*. By using *Chromobacterium violaceum* CV026 mutant and *E. coli lux* reporters, we have shown that *Pag* synthesizes an acyl-homoserine lactone (AHLs) in which N-butyryl –D-homoserine lactone is the predominant signal identified by mass spectral analysis. We have cloned and sequenced the gene that confers AHLs biosynthesis; *pagI*, and a related gene, *pagR* that encodes a transcriptional regulator. In this strain, as in other plant pathogenic bacteria, *pagI/pagR* are linked and convergently transcribed. However, PagR protein does not control the expression of *pagI* gene that appears to be constitutive as demonstrated by an *in vivo* assay. A mutant of *pagI* defective in AHLs production significantly reduced the

epiphytic fitness on bean leaves but so far did not seem to affect virulence on gypsophila. By transposition of the *pagI* mutant with a promoterless *GUS*, in the presence and absence of AHL, we recently identified a putative QS target gene that encodes dihydroorotate dehydrogenase (DHODH), an essential enzyme for *de novo* biosynthesis of pyrimidines. The transcriptional activity of DHODH was highly enhanced in *pagI* mutant culture with exogenous addition of AHL. This evidence suggests that the production of AHL in *Pag* might modulate metabolic traits that affect its survival on leaves. Further work will focus on the role of *pagR* in epiphytic growth as well as virulence of *Pag*.

Pantoea stewartii (USA)

a. The regulatory cascade. Epistasis analysis, expression experiments using gene fusions, and genetic reconstruction of each step in *E. coli* were used to delineate and confirm the above pathway. (The difference in the approach of the US lab to that of the Israeli lab, described below, was that most of the expression studies were done in IM inducing medium with GUS reporter gene fusions, rather than in planta with *inaZ* fusions.) In contrast to a previous report in *E. amylovora*, HrpY was shown to directly regulate *hrpSp* and autoregulate the *hrpXY* operon. The transcriptional start sites for *hrpX*, *hrpS* and *hrpL* were located by primer extension analysis. HrpL has an IM inducible σ54 promoter as well as a weaker σ70 promoter. This model was published in Mol. Plant-Microbe Interact.

Merighi, M., Majerczak, D. R., Stover, E. H., and Coplin, D. L. 2003. The HrpX/HrpY two-component system activates *hrpS* expression, the first step in the regulatory cascade controlling the Hrp regulon in *Pantoea stewartii* subsp. *stewartii*. Mol. Plant-Microbe Interact. 16:238-248.

After 2002, the biochemistry of HrpX, HrpY and HrpS and part of the transcriptional analysis of the *Hrp* regulatory region was continued under a separate USDA NRI supported project. This was primarily the work of a student, Massimo Merighi, who completed his Ph.D. degree in December, 2003. He also worked on the role of HrpX in sensing plant and environmental signals and the regulation of *hrp* genes by quorum sensing (see below). Unfortunately, Dr. Merighi had to graduate early due to health concerns and his research was continued part time by a technician in our lab, which has delayed its publication. Dr. Merighi presented three posters at national meetings that included data from our BARD grants. A paper, acknowledging BARD support, on a novel transcriptional autoregulatory loop was published in FEMS and a second paper on the biochemistry of HrpY was submitted to J. Bacteriol. However, the reviewers of the later paper asked that we include site directed mutagenesis of the HrpY binding site. This work almost done and the paper will be resubmitted in July, 2005.

Merighi, M., Majerczak, D.R., and Coplin, D.L. 2005. A novel transcriptional autoregulatory loop enhances expression of the *Pantoea stewartii* subsp. *stewartii* Hrp type III secretion system. FEMS Microbiol. Lett. 243:479-487.

Merighi M., Doris R. Majerczak, D.R., Michael Zianni, M., Kimberly Tessanne, K., and David L. Coplin, D.L. 2005. Molecular characterization of *Pantoea stewartii* subsp.

stewartii HrpY, a conserved response regulator of type III secretion systems, and its interaction with the *hrpS* promoter. In revision for resubmission to J. Bacteriol.

b. Regulation of *hrp* gene expression by external and environmental signals and the role of HrpX. Although the some of the work on the biochemistry of HrpY was part of a concurrent USDA NRI grant, our research on the mechanisms by which *Pnss* senses environmental and plant signals via HrpX continued to be part of the objectives of this BARD project.

To determine the role of the HrpX sensor kinase in the activation of HrpY, non-polar, in-frame deletion mutations in *hrpX* were constructed. The Δ*hrpX* mutants were unable to express the *hrp/wts* genes in IM inducing-medium and were greatly reduced in the ability to elicit an HR in tobacco, which requires preinduction of the *hrp* regulon in IM. This indicated that HrpX senses the nutritional and/or environmental signals present in IM. In pathogenicity assays on sweet corn seedlings, the Δ*hrpX* mutants could still cause significant disease, suggesting that either a second plant-specific pathway or non-specific cross-talk with an alternate kinase activates HrpY in planta. However, both disease severity and percent infection were highly variable with the mutants indicating that HrpX is needed to ensure consistent infection in response to variability in host nutrition or physiology. During the last year, Δ*hrpX* mutants missing either or both PAS domains were constructed by allele exchange of unmarked deletions. We have found that deletion of either PAS domains negatively affects signal transduction in vitro under Hrp-inducing conditions. Moreover, these mutations are much less virulent than mutants that are missing the entire *hrpX* gene. This indicates that both PAS domains are needed to stimulate kinase activity, whereas deletion of either PAS domain may convert it to a phosphatase that counteracts other means of phosphorylating HrpY. Alternatively, these PAS mutants proteins might sequester HrpY.

A manuscript on the ΔhrpX mutants and the role of the PAS domains in signal sensing has been written and will be submitted to J. Bacteriol. as soon as the paper on HrpY is accepted. (Merighi, M. and Coplin, D.L. Genetic analysis of the role of the HrpX PAS kinase in signal sensing and activation of *hrp*/type III secretion gene expression in *Pantoea stewartii* subsp. *stewartii.)*

Using various *hrp-gfp* reporter fusions and laser confocal microscopy, we have shown that the *Pnss hrp* regulon is activated within 2 h of inoculation into corn and cell contact did not appear to be required. However in IM, only a subpopulation of cells expresses GFP. Using a different GFP derivative with a short half-life, we also found that the percentage of wild-type *Pnss* cells expressing an *hrpJ::gfp-aav* fusion is much greater if the cells are grown on the surface of cut corn stalks for two days than if they are grown in IM. Moreover, this in planta frequency drops from 84% to 11% in a *hrpX* mutant and to zero in a *hrpYD57N* mutant. These results clearly indicate that HrpX plays and important role regulating *hrp* genes in planta.

The response of a *hrpJ-lacZ* fusion to cultural conditions was extensively studied in our lab. We previously knew that maximal expression was obtained at pH 5.5 in an apoplast-mimicking minimal medium (IM) that was low in nitrogen and phosphate. As part of the BARD project, we found that anaerobiosis, higher pHs (>6-7), and high osmolarity strongly represses *hrpS* and downstream genes. Over-expression of HrpS, but not HrpYD57N, bypassed all of these signals.

Preliminary experiments also showed that HrpYD57N over-expression moderately bypasses repression by some Krebs cycle intermediates (succinate and citrate) and nitrogen sources. In addition, *hrpSp*, *hrpLp* and *hrpJp* were most strongly expressed in stationary phase.

c. Quorum sensing regulation of the Hrp TTSS. Exopolysaccharide production in *Pnss* is under the control of the EsaI/R quorum sensing system, which produces a 3-oxo-hexanoyl homoserine lactone autoinducer. Mutations in the *esaI* gene, encoding AHL-synthetase, were avirulent and reduced in HR elicitation, suggesting involvement of quorum sensing in the regulation of *hrp* genes. Plasmid-borne *hrp-uidA* fusions were strongly down-regulated in an *esaI* strain, but slightly upregulated in a strain lacking the cognate EsaR repressor. In an *esaR esaI* double mutant, *hrp-uidA* expression was intermediate between the wild type and the *esaI* mutant. The EsaR repression appears to enter the pathway at the level of *hrpS* expression. The time course for expression of a *hrp* secretion gene was shown to have a typical quorum sensing response curve, with a threshold at 3 x 10^8 CFU per ml. However, addition of exogenous AHL did not result in early expression of the reporter genes. Likewise they were not up-regulated significantly at low cell density in *esaR* mutants. This observation that addition of exogenous HSL can restore *hrp* expression in an *esaI* mutant only at high cell density suggests that a second layer of regulation involving entry into late exponential stationary phase signals may be involved. Our colleague, Susanne vonBodman at the Univ. of Connecticut, found that the purified EsaR repressor does not bind to a potential "Esa" box promoter upstream of *hrpS*. We confirmed this using an *E. coli* reconstruction system. This indicates that quorum sensing regulation does not directly control *hrpSp*.

This work was summarized an article in the Annual Review of Phytopathology and a note is in preparation for J. Bacteriology.

von Bodman, S., Bauer, W.D., and Coplin, D.L. 2003. Quorum sensing in plant-pathogenic bacteria. Annu. Rev. Phytopathol. 41:455-482.

d. Negative regulation of *hrp* genes by the Lon protease. In an attempt to identify novel negative regulators of the Hrp regulon, we screened about 40,000 mini-Tn*5*Cm transposon mutants of *P. stewartii* for the enhanced expression of an *hrpJ::uidA* reporter gene fusion in *hrp*-inducing medium. The insertions from three up-regulated mutants were cloned and sequenced. They were found to have inserted in homologs of *lon*, *recA* and *nikR*, all of which are known regulatory genes in *E. coli*. The *P. stewartii* Lon protein displayed 90% amino acid identity to other enteric Lon homologs. In *E. coli*, *lon* encodes a protease that is a global regulator of stress responses. In contrast to wild-type *P. stewartii* strains, *hrpJ* gene expression in the *lon* mutant was increased and it was not repressed by high pH and high salt concentrations. On the other hand, *hrpS* expression was not drastically altered. Mucoidy and *cps* gene expression was also elevated in the *lon* mutant. Most importantly, the *lon* mutant was frequently able to cause an HR on tobacco plants without the normal need for preinduction of inocula in *hrp*-inducing medium and it was intermediate in virulence on corn. We were able to complete molecular Koch's postulates to verify the Hrp phenotype of *lon* mutants by recombining the wild-type gene back into the mutants.

This work was presented at the 2003 Annual Meeting of the American Phytopathological Society and as soon as we determine the stability of HrpS and HrpL in *lon* mutants, we will submit at a paper to J. Bacteriol.

Objective 2: What additional effector proteins are involved in host specificity and pathogenicity?

***Pantoea agglomerans* (Israel)**

a. Searching for additional virulence effectors. (Two papers in the proceedings of a *Pseudomonas* meeting and an endophytes meeting)

The sequence of $pPATH_{Pag}$ (135 kb) has been almost completed and provides a fingerprint for molecular events that led to the transition of *Pag* from a commensal bacterium into a host-specific tumorigenic pathogen. A pathogenicity island (PAI) on the $pPATH_{Pag}$ of ~90 kb has been identified that accommodates *hrp* gene cluster, an inventory of nine characterized and four putative genes encoding type III virulence effectors, phytohormones and IS elements. The virulence genes on the PAI are interposed with various homologous sequences and pseudogenes of other bacteria implying an early stage of evolution. The DNA region outside the PAI harbors genes encoding for plasmid replication and maintenance, including a repA that shows homology to IncN incompatibility group.

b. Molecular determinants affecting activity of the virulence effectors, PthG and HsvG of *Erwinia herbicola*. (Abstract submitted to the 11th MPMI congress).

Erwinia herbicola (*Pantoea agglomerans*) pv. *gypsophilae* (*Ehg*) induces gall formation in gypsophila whereas *E. herbicola* pv. *betae* (*Ehb*) elicits galls in beet and gypsophila. Pathogenicity of both pathovars is dependent on plasmids (i.e., $pPATH_{Ehg}$ and $pPATH_{Ehb}$). These plasmids harbor an *hrp* gene cluster, genes encoding type III virulence effectors and biosynthetic genes of phytohormons. *hsvG*, which is present in both plasmids, determines host specificity of *Ehg* and *Ehb* on gypsophila while functional *pthG* is present only in $pPATH_{Ehg}$ and acts as a virulence effector in gypsophila and HR elicitor in beet. Both genes are *hrp*-regulated and type III-secreted. A chimeric fusion between the N-terminus of *hsvG* (1-323 codons) and part of the C-terminus of *pthG* (247-472 codons) induced HR in beet when introduced *in trans* into *Ehb*. Motifs scan of the 247-472 codons suggests the presence of two putative myristolation sites, putative phosphorylation sites and a putative DNA binding domain. Using the C-terminus of *pthG* as a reporter, the type III translocation signals for HsvG and PthG were determined as the first 40 and 43 aa of the N-terminus, respectively. Various deletions performed between 43 and 247 aa of PthG caused loss of pathogenicity on gypsophila. Studies are underway to pinpoint domains in PthG and HsvG that are responsible for gall formation on gypsophila as well as their interactions with the host plants.

c. PthG acts as an Avr determinant on multiple beet species (Molecular Plant Pathology. 2004. 5: 105-113).

We have previously isolated and partially characterized a pleiotropic gene from the $pPATH_{Pag}$ designated as *pthG* that encodes a virulence factor in gypsophila and an elicitor of a hypersensitive-like response in beet roots. The present study was undertaken to characterize *pthG* further as an *avr* gene. Infiltration of beet leaves with strains expressing PthG (i.e., *Pag* or *Pab* containing *pthG in trans*) caused an HR response within 48 h, whereas strains lacking intact *pthG* (i.e., *Pab* or *Pag* mutated in *pthG*) resulted in gall formation after 5 days. HR was elicited by PthG on multiple beet species, whereas a marker exchange mutant of *Pag* in *pthG* extended its host range on these beet species. A marker exchange mutant of *Pag* in *hrpJ*, encoding a component of the Type III secretion system, prevented HR elicitation. Mutations in each of the *hrp* regulatory genes (*hrpY*, *hrpS* and *hrpL*) substantially reduced the transcriptional activity of *pthG* in gypsophila cuttings.

d. HsvG and HsvB are two homologous type III effector proteins that determine host specificity of *Pantoea agglomerans* on gypsophila and beet (abstract submitted to the 12th MPMI congress).

Pantoea agglomerans (*Erwinia herbicola*) pv. *gypsophilae* (*Pag*) and pv. *betae* (*Pab*) are two related tumorigenic pathogens. *Pag* elicits gall formation on gypsophila and HR on beet, whereas *Pab* induces galls on beet and gypsophila. Pathogenicity of both pathovars is determined by an indigenous plasmid ($pPATH_{Pag}$ or $pPATH_{Pab}$) bearing a pathogenicity island (PAI). The HR caused by *Pag* on beet is governed by *pthG*, an *avr* gene that is inactive in *Pab*. Insertional mutant in *pthG* (*Pag*B45) causes the latter to incite galls also on beet. Two homologous type III (TTSS) effectors, HsvG and HsvB that exhibit 74% aa identity were found to determine host specificity in both pathovars on gypsophila and beet respectively. The two genes are present on the PAIs of *Pag*B45 and *Pab*. HsvG is composed of 672 aa with 2 direct repeats: RI=74 aa (379-453) and RII=71aa (453-524) with 88% identity. In contrast, RI is missing in HsvB of *Pab* and partially deleted in HsvB of *Pag*. The reciprocal genes could not complement mutations in either HsvG or HsvB in both pathovars. Mobilization of RI-deleted *hsvG* into either *Pab* or *Pag*B45 mutants of *hsvB* restored pathogenicity of both mutants on beet. Alternatively, insertion of intact RI into HsvB and its mobilization into either *Pab* or *Pag*B45 mutants of *hsvG* restored their pathogenicity on gypsophila. Yeast two hybrid system with *hsvG* as a bait for screening cDNA library of *Pag*-infected gypsophila seedlings showed strong activation of the reporter *lacZ* in the yeast strain EGY48 [p8op-*lacZ*) lacking the plasmid containing activation domain. Disruption of RI in HsvG abolished the activation of p8op-*lacZ*. Experiments are in progress to determine whether HsvG is localized to the nucleus and whether it acts as DNA-binding protein in order to support the hypothesis that HsvG might act as a transcription factor.

***Pantoea stewartii* (USA)**

a. The Hrp PAI. We finished sequencing the 40-kb *hrp* pathogenicity island (PAI) and it appears that the central *hrp/hrc* region contains the same genes in the same order as found in

Pag, *E. amylovora*, *E. chrysanthemi (Ech)* and *E. carotovora* subsp. *atroseptica*. *Pnss* produces a single harpin, but the two pantoeas are missing several ORFs that are present in *E. amylovora* and *Ech*, including *hrpW* and some *avr*-like genes. Sequence information from the variable regions to the left and right of the *hrp* cluster in *Pnss* indicates that it is completely different from the same regions in *E. amylovora* and *Pag*. These flanking regions primarily contain sequences that match DNA from plasmids, transposons and IS elements, including several transposase genes. This raises the possibility that the *Pnss hrp* cluster could be on one of the many large plasmids that are characteristic of this species. The region to the right of *wtsF* contains a pseudogene, *wtsG*, that contains a premature frame-shift, which destroys what would otherwise be 86% aa identity to AvrPpiB from *P. syringae* pv. *pisi* . Although this ORF is present in a dozen wild-type *Pnss* strains, all of them contain the same mutation. *wtsG* mutants remained fully virulent, so it is unlikely that it is involved in pathogenicity on corn, even if translational frameshifting does occur. The region to the left of the *hrp* cluster contains homologues of *E. amylovora* ORF12, *virK* and *ipx47*. Mutations in these genes likewise did not affect virulence.

b. Additional Hrp effectors. To identify more *Hrp* secreted proteins, we completed an extensive screen for genes with *hrp*-regulated promoters in *Pnss*. Strain DC283 was mutagenized with transposon mini-Tn*5gusA*, which creates ß-glucuronidase (GUS) reporter gene fusions. Over 26,000 mutants that did not express GUS activity in rich media were screened for increased GUS activity following introduction of a *hrpL*$^+$ plasmid by conjugation. The DNA adjacent to one or both ends of each transposon insertion that was up-regulated by HrpL was directly sequenced from genomic DNA. Of 107 up-regulated mutants, 38 contained insertions in known *hrp*, *hrc* and *wts* genes, 27 had no hits in the databases, and 18 represented possible new effector genes. These included homologues of HopPtoE, HopPtoC and two candidate TTSS effector proteins from *P. syringae* DC3000, an Avr-like protein from *X. campestris*, two inner membrane proteins from *R. solanacearum* and *P. syringae*, a catalase, *Salmonella mucB*, and a hypothetical transcriptional regulator from *E. coli*. In preliminary pathogenicity assays, all of these mutants caused lesions and wilting, however, in subsequent tests at a low inoculum dosage, mutants lacking HopPtoC, HopPtoE, a candidate *P. syringae* effector homolog, or catalase caused less severe symptoms.

c. A second TTSS. We also screened for mini-*Tn5gusA* insertions that were down-regulated by HrpL, i.e. genes that would be turned off in planta and this screen revealed the presence of a second TTSS with homology to pathogenicity genes in *Salmonella* and *Shigella*. Insertions were obtained in two genes that could be part of the secretion apparatus and in two candidate effector genes. The latter are homologs of *Salmonella prgI*, located in SPI-1, and *sicA*, an invasin. All of the HrpL down-regulated mutants were still virulent in corn. Since *Pnss* does not have a free-living phase, we hypothesize that these genes and the second TTSS are only expressed in the insect vector and that they may be involved in insect transmission. HrpL may therefore activate a repressor of genes that are needed in the insect. We are currently using GFP labeled bacteria and field collected corn flea beetles and will determine if these genes and others are potential insect colonization factors.

Objective 3. Do the presently known effector proteins enter the host cell?

Pantoea agglomerans (Israel)

a. PthG operates within the host cell. This part is included in the paper entitled "*pthG* from *Pantoea agglomerans* pv. *gypsophilae* encodes an avirulence effector that determines incompatibility in multiple beet species" (Molecular Plant Pathology 2004. 5: 105-113).

Infiltration of crude PthG protein preparations into beet leaves did not cause HR, which suggests that PthG does not operate through the external cell membrane. Further studies were therefore undertaken to test the operation of PthG within the host cell by means of particle bombardment. Initial attempts to utilize the ß-glucuronidase (GUS) reporter were unsuccessful with beet leaves since addition of chromogenic ß-glucuronidase substrate 5-bromo-4-chloro-3-indolyl- ß –D-glucuronic acid (X-gluc) caused leaf blackening. Consequently derivatives of pGreenII0029 containing GFP were employed for introducing *pthG* into beet leaves. Particle bombardments with different constructs containing active or inactive PthG were carried out on beet leaves and the results were compared with those on non-host (melon) leaves. Particle bombardment with inactive PthG on beet leaves, or any of the bombardments on melon leaves resulted in approximately 1000 sites of GFP activity per leaf, whereas the active PthG (with or without GFP) produced only between 1 to 60 sites of diffuse autofluorescent activity per bombarded beet leaf.

Pantoea stewartii (USA).

a. WtsE. For our initial studies on the transfer of Hops to and their activity within host cells we originally proposed to use *wtsG*, but switched emphasis when we discovered it was a pseudogene. Consequently, we shifted the emphasis of this objective to WtsE, which is a 201-kDa (1835 aa) protein that is conserved in *Pag* and several other phytopathogenic erwinias (DspE) and pseudomonads (AvrE). This is one of the very few effector proteins that appears to be required for virulence in many plant pathogenic bacteria. Non-polar mutations in *wtsE* completely abolished pathogenicity, but they did not affect HR elicitation in tobacco. The *wtsE* mutant was complemented for pathogenicity by a clone containing *E. amylovora dspEF*, indicating that the two effectors have a similar function in corn. However, *dspEF* from *Pag* only weakly complemented a *wtsE* mutant for virulence on corn and *wtsEF* from *Pnss* did not complement an *E. amylovora dspEF* mutant (S.V. Beer, pers. commun.). This suggests that these proteins may have some host specificity. Western blots showed that WtsE is secreted into the supernatant in a *hrp*-dependent manner when *hrpS* is over-expressed.

WtsE contains multiple putative amino acid sequence motifs identified by PSORT II and other programs, including a leucine zipper at aa 539-560, a coiled-coil region at 996-1057, a nuclear localization signal at aa 1358-1361, and an endoplasmic reticulum membrane retention signal (ERR) in the last five aa. In our analysis of DspE from *E. amylovora* and *Pag* and AvrE from *P. syringae* DC300 and B728a, only the C-terminal ER retention signal was conserved, although AvrE has a C-terminal leucine zipper and they all have potential NLSs. We have a mini-Tn*5uidA* insertion only 36 bp from the end of *wtsE* that abolishes pathogenicity, so it is likely that integrity of the C-terminus, including the ERR signal, is required. Proteins with ERR signals can regulate vesicular traffic in the ER-Golgi network, including the release of transport vesicles that may be involved in non-host resistance.

b. WtsE causes water-soaking in corn leaves. Ham et al. (PNAS 95:10206-11) demonstrated that *E. coli* strain MC4100 carrying cosmid pCPP2156 that contains the entire *Ech hrp* cluster can promiscuously deliver effector proteins from other bacteria into a variety plant cells and secrete them in culture. We have used this system to translocate WtsE in host cells. When vacuum-infiltrated into young leaves of sweet corn seedlings, MC4100 (pCPP2156/pAA008 *wtsEF*$^{+}$) caused Wts-like symptoms within 12 h. We are very excited about our discovery that translocation or transient expression of WtsE is indeed able to cause water-soaking. This is the first time an effector protein from erwinias has been shown to cause this symptom, which is characteristic of many bacterial diseases.

c. The WtsF molecular chaperone. *wtsF* is downstream of *wtsE* in the same operon, although we have verified that it has its own σ^{70} promoter. By virtue of its small size (15.6 kDal) and physical properties WtsF is predicted to be a chaperone for WtsE. We previously concluded that *wtsF* was required for virulence. This was based on transposon mutants that were mapped by sizing restriction fragments. We recently sequenced the insertions in these strains and found that the ones used in our complementation tests were actually at the very end of *wtsE*. Consequently, we repeated this work with additional *wtsF*::Tn*5* mutants that were mapped by sequencing and found that they were only slightly reduced in virulence. This implied that they could still translocate WtsE in planta from *Pnss*, although WtsF was required for the above *E. coli* MC4100(pCPP2156) delivery system. Western blots indicated that *wtsF* mutants secreted greatly reduced amounts of WtsE into culture supernatants and their cell pellets contained also contained less WtsE. We therefore hypothesize that the major role of the WtsF chaperone is to stabilize WtsE inside the bacterium. This notion was supported by the observation that cell pellets from *wtsF lon* mutants contained normal levels of WtsE.

The above results on the sequence analysis of *wtsE*, delivery of WtsE into corn cells, and the role of WtsF will be submitted to Mol. Plant-Microbe Interact. in July.

d. Effects of WtsE in non-hosts.

The ability of *E. coli* MC4100 (pCPP2156/pAA008 *wtsEF*$^{+}$) to cause cell death on other hosts was also tested. In addition to corn, it also caused rapid tissue collapse in infiltrated tobacco, *Nicotiana benthamiana*, and beet leaves starting within 6 h, indicating that WtsE can cause cell death in many other plants. We have also succeeded in transiently expressing *wtsE* in *N. benthamiana* and *N. tabacum* using *Agrobacterium tumefaciens* and the pGD expression vector series (Goodin et al. 2002. Plant J. 31:375-383). Infiltration of *A. tumefaciens* cells carrying *wtsE* into *Nicotiana* leaves resulted in water-soaking and cell death after 48 h. Neither *wtsF* nor the first 236 aa of WtsE, containing the N-terminal secretion signal, was necessary for elicitor activity using "agroinfiltration". However, deletion of the first 492 aa inactivated WtsE. In the course of the yeast-two hybrid experiments, we also discovered that intact WtsE is toxic to cells of the budding yeast, *Saccharomyces cerevisiae*. These results suggest that the targets of this family of effectors are conserved between cells from different kingdoms and they may be generally toxic to eukaroytes.

A wild-type *Pnss* strain also caused cell death in *N. benthamiana*, beet and *Arabidopsis*, but a *wtsE* mutant did not. At this point we are determining whether the wtsE induced cell death we observe in non-hosts a typical HR or similar to the toxicity seen in corn. Preliminary experiments, where we attempted to block the HR with cycloheximide, suggest that WtsE causes a water-soaking-like response in corn and *Arabidopsis*, but an HR in *N. benthamiana* and beet. In the latter hosts, the HR may mask the underlying toxicity of WtsE. This finding is significant because we may be able to use different hosts and inhibitor treatments to study both the virulence and avirulence functions of this important conserved effector protein.

Objective 4. What host proteins interact with *Erwinia* Hop effectors?

***Pantoea stewartii* (USA)**

We have used a yeast two-hybrid system to screen for the target of WtsE in maize cells. pAS1 was used as a bait vector for the WtsE constructs and we obtained Hybridzap (Stragene) maize prey cDNA libraries from Dr. E. Grotewold's lab at OSU. Due to the toxicity of full-length WtsE to the yeast cells, we had to screen separately with the N-terminal and C-terminal halves of WtsE. The WtsE receptor/target proteins were sequenced and the BLAST hits with known genes in rice and *Abrabidopsis* are shown in Table 1. Almost all of the candidate interacting proteines are involved with signal transduction that leads either to PCD or disease resistance. Specifically, the N-terminal half of WtsE interacted with LRR receptor kinases that form a major class of R-proteins and the C-terminal half interacted with a separate set of proteins that may also regulate resistance signaling by controlling ubiquitination and subsequent degradation of proteins.

Table 1. WtsE-interacting proteins (WIPs) identified from yeast-two-hybrid assays using a maize seedling cDNA prey library and the C- and N-terminal halves of WtsE as bait.

Description	Homologs found in[a]	Possible Function
N-terminal		
PP2A regulatory subunit B`	Rice, *Arabidopsis*, yeast	Signal transduction (PCD)
PP2A B` kappa subunit	Rice, *Arabidopsis*	Signal transduction (PCD)
LRR receptor kinase	Rice, *Arabidopsis*	Signal perception (Dis. resist.)
LRR transmembrane receptor kinase	Rice, *Arabidopsis*	Signal perception (Dis. resist.)
LRR receptor kinase	*Arabidopsis*	Signal perception (Dis. resist.)
Putative Ser/Thr protein kinase catalytic domain	Rice, *Arabidopsis*	Signal transduction
20S proteasome subunit, F-box proteins	Rice, *Arabidopsis*	Ubiquintination and ethylene signaling
Protein phosphatase 2C	Rice, *Arabidopsis*	Signal transduction
C-terminal		
PRLI-interacting factor	Rice, *Arabidopsis*	Controls ubiquitination
Ankyrin repeat protein	Rice, *Arabidopsis*	Protein-protein interactions
F-box family protein	Arabidopsis	Controls ubiquitination