**BARD Final Scientific Report
Cover Page**

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BARD Project Number: US-2816-96

Project Title: The role of proteins secreted by the Hrp-pathways of *Erwinia stewartii* and *E. herbicola* pv. *gypsophila*e in eliciting water-soaking symptoms and initiating galls.

Investigators

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The Ohio State University _____

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

Pantoea, maize, gypsophilia, beets, type III secretion system, Stewart's Wilt

Abbreviations commonly used in the report, in alphabetical order:

Avr, Dsp, Ehg, Hsv, Hrp, GUS, PAS, PCR, Wts

Budget: IS: \$194,000

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	2	1	2	5
Submitted, in review, in preparation		1 in prep.	2 submitted 2 in prep.	5
Invited review papers				
Book chapters				
Books				
Master theses		1	2	3
Ph.D. theses			2	2
Abstracts		3	7	10
Not refereed (proceedings, reports, etc.)		1		1

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

Ming Guo _____

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	2	2	5
Longer Visits (Sabbaticals)				

Description of Cooperation:

Parallel work on all objectives was done in each country with the Israeli groups working on *Ehg* and the US group working on *Es*. Overall, the collaboration could be described as “synergistic”. The Israeli scientists specialized in characterizing the secreted proteins and the US scientists concentrated more on *hrp* gene regulation.

Patent Summary (numbers)

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

Abstract

Many bacterial pathogens of plants can inject pathogenicity proteins into host cells using a specialized type III secretion system encoded by *hrp* genes. This system deliver effector proteins, into plant cells that function in both susceptible and resistant interactions. We have found that the virulence of *Erwinia stewartii* (*Es*; syn. *Pantoea stewartii*) and *Erwinia herbicola* pv. *gypsophila* (*Ehg*, syn. *Pantoea agglomerans*), which cause Stewart's wilt of corn and galls on *Gypsophila*, respectively, depends on *hrp* genes. The major objectives of this project were: To increase expression of *hrp* genes in order to identify secreted proteins; to identify genes for proteins secreted by the type-III systems and determine if they are required for pathogenicity; and to determine if the secreted proteins can function within eukaryotic cells.

We found that transcription of the *hrp* and effector genes in *Es* and *Ehg* is controlled by at least four genes that constitute a regulatory cascade. Environmental and/or physiological 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 upregulates *hrpS*, which encodes a transcriptional enhancer. HrpS then activates *hrpL*, which encodes an alternate sigma factor that recognizes "*hrp* boxes". All of the regulatory genes are essential for pathogenicity, except HrpX, which appears only to be required for induction of the HR in tobacco by *Es*. In elucidating this regulatory pathway in both species, we made a number of significant new discoveries. HrpX is unusual for a sensor-kinase because it is cytoplasmic and contains PAS domains, which may sense the redox state of the bacterium. In *Es*, a novel methyl-accepting protein may function upstream of *hrpY* and repress *hrp* gene expression in planta. The *esaIR* quorum sensing system in *Es* represses *hrp* gene expression in *Es* in response to cell-density.

We have discovered six new type III effector proteins in these species, one of which (DspE in *Ehg* and WtsE in *Es*) is common to both pathogens. In addition, *Es* wtsG, which is a homolog of an *avrPpiB* from *P. syringae* pv. *pisi*, and an *Ehg* ORF, which is a homolog of *P. syringae* pv. *phaseolicola* AvrPphD, were both demonstrated to encode virulence proteins. Two plasmid-borne, *Ehg* Hop proteins, HsvG and PthG, are required for infection of gypsophila, but interestingly, PthG also acts as an Avr elicitor in beets. Using a calmodulin-dependent adenylate cyclase (*cyaA*) reporter gene, we were successful in demonstrating that an HsvG-CyaA fusion protein can be transferred into human HeLa cells by the type-III system of enteropathogenic *E. coli*. This is a highly significant accomplishment because it is the first direct demonstration that an effector protein from a plant pathogenic bacterium is capable of being translocated into a eukaryotic cell by a type-III secretion system.

Ehg is considered a limiting factor in *Gypsophila* production in Israel and Stewart's Wilt is a serious disease in the Eastern and North Central USA, especially on sweet corn in epidemic years. We believe that our basic research on the characterization of type III virulence effectors should enable future identification of their receptors in plant cells. This may lead to novel approaches for genetically engineering resistant plants by modifying their receptors or inactivating effectors and thus blocking the induction of the susceptible response. Alternatively, *hrp* gene regulation might also provide a target for plant produced compounds that interfere with recognition of the host by the pathogen. Such strategies would be broadly applicable to a wide range of serious bacterial diseases on many crops throughout the USA and Israel.

Achievements

Significant achievements were made on all objectives. We discovered new complexity in the regulation of *hrp* genes and, most significantly, we characterized six new virulence genes encoding Hrp-dependent outer proteins (Hops). Our original proposal allowed for alternate approaches to finding Hrp-secreted proteins, so we made a decision to emphasize the regulation work, when it became apparent that most the proteins of interest would not be secreted into the milieu unless we better understood *hrp* gene regulation in both *Es* and *Ehg* and how to manipulate it.

The transcription of the *hrp* system and effector genes in *Es* and *Ehg* is controlled by at least four genes that constitute a regulatory cascade. Environmental and/or physiological 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 upregulates *hrpS*, which encodes a transcriptional enhancer. HrpS then activates *hrpL*, which encodes an alternate sigma factor that recognizes "*hrp* boxes". All of the regulatory genes are essential for pathogenicity, except HrpX, which appears only to be required for induction of the HR in tobacco by *Es*. Hrp genes are induced in an apoplast-mimicking medium in an *hrpX*-dependent manner. In elucidating this regulatory pathway in both species, we made a number of significant new discoveries: 1) By site-directed mutagenesis we located the phosphorylation site of HrpY and have shown that phosphorylation is required for activation of *hrpS*. 2) HrpX is unusual for a sensor-kinase because it is cytoplasmic and contains PAS domains, which may sense the redox state of the bacterium. An alternate kinase may activate HrpY in planta. 3) In *Es*, a novel methyl-accepting protein may function upstream of *hrpY* and repress *hrp* gene expression in planta. 4) The arrangement of all of the regulatory genes into one large operon (*hrpL-hrpXY-hrpS*) with multiple internal promoters provides an elegant transcriptional feedback amplification mechanism. 5) The *esaIR* quorum sensing system represses *hrp* gene expression in *Es* in response to cell-density. 6) Information from environmental and plant signals, quorum sensing, and the redox state of the cell is integrated through transcriptional control of *hrpS*. 7) Using green fluorescent protein and ice nucleation genes as reporters, we have found that the *hrp* genes and effector genes, such as *hsvG*, are induced in planta as early as 2 h after infection. We have also developed a method for screening for Hrp regulated genes that should be useful in identifying genes encoding additional effector proteins.

Both *Ehg* and *Es* carry a fully functional *hrp* cluster and we have sequenced most of the important *hrp* genes. In the case of *Ehg*, the cluster is located on a plasmid (pPATH) and we believe it may be on a plasmid in *Es* as well. The *hrp* cluster in *Ehg* is especially interesting because this is the first tumorigenic (and non-necrogenic) bacterium shown to depend on a Hrp secretion system for pathogenicity. To search for new virulence genes, we screened *Ehg* transposon mutants for loss of pathogenicity as well as sequenced the regions flanking the *hrp* clusters in each species. Work on this objective resulted in the discovery of six new Hop proteins, one of which (DspE in *Ehg* and WtsE in *Es*) is common to both pathogens and is a homolog of *Pseudomonas syringae* AvrE. In addition, *Es* WtsG, which is a homolog of an AvrPpiB from *P. syringae* pv. *pisi*, and *Ehg* AvrPphD, which is a homolog of *P. syringae* pv. *phaseolicola* AvrPphD, were both demonstrated to be virulence proteins. Two plasmid-borne, *Ehg* Hop proteins, HsvG and PthG, are required for infection of gypsophila, but interestingly, PthG also acts as an Avr elicitor in beets. Using a calmodulin-dependent adenylate cyclase (*cyaA*) reporter gene, we were successful in demonstrating that an HsvG-CyaA fusion protein

can be transferred into human HeLa cells by the type-III system of enteropathogenic *E. coli*. This is a highly significant accomplishment because it is the first direct demonstration that an effector protein from a plant pathogenic bacterium is capable of being translocated into a eukaryotic cell by a type-III secretion system. Moreover, it proved that a CyaA translational fusion does not interfere with translocation, so that we can now feel confident in extending this approach to plant cells.

We have found that *hrp* gene regulation and the plethora of secreted proteins in these species is more complex than originally imagined. We hypothesize that virulence and host specificity are determined both by the perception of specific plant signals and by the arsenal of pathogenicity proteins that are delivered into host cells. Once inside a plant cell, we believe these proteins suppress host defenses and trigger release of nutrients, thereby enabling bacterial multiplication. The discovery of the six effector proteins described above will enable us to determine how these proteins function inside plant cells. We will attempt to transfer them into plant cells and search for their cellular targets/receptors. For this purpose, we have successfully prepared a yeast-two-hybrid library of infected *Gypsophila* cDNA to use in identifying proteins that interact with HsvG, PthG and DspE.

In the process of searching for new effector genes, we discovered that both *hrp* clusters include and are flanked by multiple transposon genes, IS elements and plasmid DNA. This indicates that they are truly pathogenicity islands that were acquired by horizontal gene exchange. Further analysis of these clusters in different strains may therefore shed light on how normally epiphytic or endophytic bacteria, such as those in the *Pantoea* group, can emerge as new pathogens.

Potential benefits to agriculture. *Ehg* is considered a limiting factor in *Gypsophila* production in Israel and Stewart's Wilt is a serious disease in the Eastern and North Central USA, especially on sweet corn in epidemic years. *Es* is also of considerable international quarantine importance to the maize seed industry. We believe that our basic research on the characterization of type III virulence effectors should enable future identification of their receptors in plant cells. This may lead to novel approaches for genetically engineering resistant plants by modifying their receptors and thus blocking the induction of the susceptible response. Moreover, innovative technologies may be used to inactivate the virulence effectors inside the cells prior to their binding to targets by creating novel disrupting partners. These strategies might include the production of single chain (scFvs) antibodies to Hops by transgenic plants. Alternatively, *hrp* gene regulation might also provide a target for plant produced compounds that interfere with recognition of the host by the pathogen. Such strategies would not only help to control the two pathogens studied in this project, but would be broadly applicable to a wide range of serious bacterial diseases on many crops throughout the USA and Israel. Molecular biologists are already capable of engineering plant proteins and making "plantabodies" so that these findings could be utilized within 8 years.

Collaboration. Parallel work on all objectives was done in each country with the Israeli groups working on *Ehg* and the US group working on *Es*. Overall, the collaboration could be described as "synergistic". The Israeli scientists specialized in characterizing the secreted proteins and the US scientists concentrated more on *hrp* gene regulation.

LIST OF PUBLICATIONS

Refereed Publications

- Ahmad, M., Majerczak, D. R., Pike, S., Hoyos, M. E., Novacky, A., and Coplin, D. L. 2001. Biological activity of harpin produced by *Pantoea stewartii* subsp. *stewartii*. Mol. Plant Microbe Interact. Accepted for publication.
- Ezra, D., Barash, I., Valinsky, L., and Manulis, S. 2000. The dual function in virulence and host range restriction of a gene isolated from pPATHEhg plasmid of *Erwinia herbicola* pv. *gypsophila*. Mol. Plant-Microbe Interact. 13:693-698.
- Frederick, R. D., Ahmad, M., Majerczak, D. R., Arroyo-Rodriguez, A. S., Manulis, S. and Coplin, D. L. 2001. Genetic organization of the *Pantoea stewartii* subsp. *stewartii* *hrp* gene cluster and sequence analysis of the *hrpA*, *hrpN* and *wtsE* operons. Mol. Plant-Microbe Interact. Accepted for publication.
- Mor, H., Manulis, S., Zuc, M., Nizan, R., Coplin, D. L., and Barash, I. 2000. Genetic organization of the *hrp* gene cluster and *dspAE/BF* operon in *Erwinia herbicola* pv. *gypsophila*. Mol. Plant-Microbe Interact. 14:431-436.
- Valinsky, L., Manulis, S., Nizan, R., Ezra, D., and Barash, I. 1998. A pathogenicity gene isolated from the pPATH of *Erwinia herbicola* pv. *gypsophila* determines host specificity. Mol. Plant-Microbe Interact. 11:753-762.

Non-referred Publications.

Papers presented at meetings.

- Barash, I., Valinsky, L., Ezra, D., Mor, H. and S. Manulis. 1998. Host specificity of *Erwinia herbicola* pv. *gypsophila* is determined by interaction between positive and negative-acting virulence genes. Phytopathology. 88:S6.
- Coplin, D. L., Majerczak, D. R., and Stover, E. 1998. Regulation of *hrp* genes in *Pantoea stewartii*. Phytopathology 88:S18.
- Ezra, D., Barash, I., Valinski, L., and Manulis, S. 1999. The dual function of a gene isolated from the pPATH of *Erwinia herbicola* pv. *gypsophila* in virulence and host range restriction. 9th Int. Cong. Mol. Plant-Microbe Interact., Amsterdam, July 25-30.
- Mor, H., Manulis, S., Nizan-Koren, R., Zuk, M., and Barash, I. 1999. Characterization of the *hrp* gene cluster and the *dspEF* operon on the pPATH of *Erwinia herbicola* pv. *gypsophila*. 9th Int. Cong. Mol. Plant-Microbe Interact., Amsterdam, July 25-30.
- Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N. M., and Barash, I. 1999. Characterization of the *hrp* regulon in *Erwinia herbicola* pv. *gypsophila*. 9th International Cong. Mol. Plant-Microbe Interact., Amsterdam.
- Merighi, M., Majerczak, D. R., and Coplin, D. L. 2000. The *hrp* genes of *Pantoea stewartii* are regulated by a complex system that senses environmental signals. 10th Int. Conf. Plant Path. Bacteria. Charlottetown, PEI.
- Merighi, M., Majerczak, D.R., and Coplin, D.L. 2001. A two-component signal transduction system and quorum sensing regulate the Hrp-type III secretion system in *Pantoea stewartii* subsp. *stewartii*. 10th Internatl. Cong. Mol. Plant-Microbe Interact. Madison, WI.

- Nizan-Koren, R., Manulis, S., and Barash, I. 2001. The *hrp* regulatory cascade of *Erwinia herbicola* pv. *gypsophilae*. 10th Internatl. Cong. Mol. Plant-Microbe Interact. Madison, WI.
- Valinsky, L., Nisan, I., Rosenshine, I., Hanski, E., Barash, I. and Manulis, S. 2000. A virulence protein from a phytopathogenic bacterium is translocated into HeLa cells via the type III secretion system of enteropathogenic *Escherichia coli*. Proc. of the 6th International Congress of Plant Molecular Biology. Quebec, Canada.

Chapter in Published Proceedings

- Merighi, M., Majerczak, D. R., and Coplin, D. L. 2000. The *hrp* genes of *Pantoea stewartii* are regulated by a complex system that senses environmental signals. Proc. 10th Int. Conf. Plant Path. Bacteria. In Press.

Submitted Manuscripts

- Valinsky, L., Nisan, I., Tu, X., Nisan, G., Rosenshine, I., Hanski, E., Barash, I., and Manulis, S. 2001. A virulence protein of the plant pathogen *Erwinia herbicola* pv. *gypsophilae* is translocated into human epithelial cells by the type II secretion system of enteropathogenic *Escherichia coli*. Submitted to J. Bacteriol.
- Valinski, L., Barash, I., Chalupowicz, L., Ezra, D. and Manulis, S. 2001. Expression of *hsvG*, a host-specific virulence gene from *Erwinia herbicola* pv. *gypsophilae*, is plant induced and *hrp* regulated. Submitted to Physiological and Molecular Plant Pathology.

Manuscripts in preparation

- Merighi, M., Majerczak, D. R., and Coplin, D. L. Regulation of *hrp* genes in *Pantoea stewartii* subsp. *stewartii* by *hrpL*, *hrpS* and *hrpXY*. J. Bacteriol.
- Guo, M., Manulis, S., Mor, H. and Barash I. 2001. Multiple different IS elements and *avrPphF* are present on the pathogenicity plasmid of *Erwinia herbicola* pv. *gypsophilae*. MPMI.
- Nizan-Koren, R., Manulis, S. and Barash I. 2001. The *hrp* regulatory cascade of *Erwinia herbicola* pv. *gypsophilae*. J. Bacteriol.

APPENDICES

- A. Technical Summary of Results
- B. Reprints of Published Manuscripts
 - 1. Ezra et al. 2000. *Mol. Plant-Microbe Interact.*
 - 2. Mor et al. 2000. *Mol. Plant-Microbe Interact.*
 - 3. Valinsky et al. 1998. *Mol. Plant-Microbe Interact.*
- C. Preprints of Accepted Papers
 - 1. Ahmad et al. 2001. *Mol. Plant-Microbe Interact.*
 - 2. Frederick et al. 2001. *Mol. Plant-Microbe Interact.*
- D. Preprints of Book Chapter
 - 1. Merighi et al. 2001. *Proc. 10th Internatl. Conf. Plant Path. Bacteria*
- E. Abstracts of Submitted Manuscripts
 - 1. Valinsky et al. *J. Bacteriol.*
 - 2. Valinsky et al. *Physiological and Molecular Plant Pathology.*

Technical Summary of Results

Characterization of Hrp pathogenicity islands. The *hrp/dsp* gene clusters of *Es* and *Ehg* are required for Wts ability and gall initiation, respectively, as well as the ability of both species to elicit a hypersensitive response (HR) in tobacco. Nucleotide sequencing of the entire *hrp* clusters is nearly complete for both species. It appears that the central *hrp/hrc* region contains the same genes, in the same order, and they share homology with *hrp* and *dsp* genes from *E. amylovora*, *E. chrysanthemi* and *P. syringae* pathovars. However, the Pantoeas are missing several ORFs that are present in *E. amylovora* and *E. chrysanthemi*, including *hrpW* and some *avr*-like genes. In *Es*, site-directed mutagenesis and complementation analysis defined seven transcription units. Putative *hrp* consensus promoter sequences were identified upstream of *hrpA*, *hrpF*, *hrpN* and *wtsE* in both species and transposon mutations in all of the *hrp* operons abolished pathogenicity and HR elicitation, except for *hrpN* and *hrpV* mutants that were still pathogenic. *hrpS*, *hrpXY* and *hrpL* regulatory mutations abolished harpin production, whereas secretory mutations in the *hrpC*, *hrpA* and *hrpJ* operons permitted synthesis and intracellular accumulation of harpin. *wtsEF* mutants were not pathogenic, but still produced harpin and elicited an HR, indicating that they have a Dsp phenotype.

Sequencing of the regions ca. 8-12 kb to the left and right of the *hrp/dsp* clusters in both *Ehg* and *Es*, revealed only sequences that matched DNA from many different plasmids, transposons and IS elements, including several transposase genes. We did not find any housekeeping or tRNA genes or that usually border chromosomally located pathogenicity islands. This was expected in *Ehg* because the *hrp* cluster is located on the pPATH plasmid, but it raises the strong possibility that the *Es* *hrp* cluster could likewise be on one of the many large plasmids that are characteristic of this species. The finding suggests that these two pantoea group species may have started out as epiphytes or endophytes and later become pathogenic by horizontal gene transfer involving transposable elements and plasmids.

Regulation of *hrp* genes. Sequence analysis of the regulatory regions in *Es* and *Ehg* has revealed four open reading frames, in order, *hrpL*, *hrpX* and *hrpY* and *hrpS*, all transcribed in the same direction (Fig. 1). In *Es*, these correspond to three complementation groups, with *hrpX* and *hrpY* together in one operon. HrpL showed homologies to a sub-family of alternative sigma factors; HrpX and HrpY constitute a putative two component signal transduction system, with HrpX as cytoplasmic sensor-kinase and HrpY as response regulator; and HrpS showed homology to the NtrC output domain, but it is missing the receiver domain. Sequence inspection failed to identify good *rho*-independent terminators at the end of the individual operons in either species and RT-PCR analysis of induced *Es* revealed that the intergenic regions are transcribed (Fig. 1). The only differences between the regulatory regions of each species were a small insertion upstream of *hrpS* in *Es* that had homology to IS elements and an adjacent opposing promoter directed toward *hrpY*.

Similar mutagenesis and complementation experiments were done in both *Es* and *Ehg*. Polar mutations in *hrpX*, *hrpY*, *hrpS* and *hrpL* decreased expression of chromosomal *lacZ* reporter gene fusions in *hrpJ* and *hrpN*, which have *hrp* boxes; mutations in *hrpY* and *hrpS* decreased expression of *hrpL::lacZ* fusions, and a mutation in *hrpY* decreased expression of *hrpS::lacZ*. Plasmids expressing *hrpY*, *hrpS* and *hrpL* from a constitutive *lacp* promoter were tested for their ability to complement polar insertion mutations in each regulatory gene. *hrpX*, *hrpY*, *hrpS*, and *hrpL* polar mutations (all showing a Hrp- phenotype) could be overridden by *lacp-hrpL*; *lacp-*

hrpS complemented *hrpX*, *hrpY* and *hrpS* mutations but not *hrpL* insertions; finally *lacp-hrpY* could complement *hrpY* mutations but not *hrpX*, *hrpS* and *hrpL* insertions. Individual steps of the *Es* regulatory cascade were reconstructed in *E. coli* using reporter fusions and regulatory elements located on separate plasmids. High copy number *lacp-hrpL* stimulated *hrpJp-uidA* fusions 1500-fold; *lacp-hrpS* could turn up *hrpLp::uidA* expression by 250-fold; and *lacp-hrpY* stimulated *hrpSp::uidA* expression by 22-fold. *hrpXp* was up-regulated 25-fold by *lacp-hrpY* indicating that the *hrpX* operon is autoregulated. Mutants containing an alanine or glutamine substitutions at the conserved D57 of HrpY, which is the predicted phosphorylation site, had a Hrp- phenotype as well in both *Es* and *Ehg*, suggesting that phosphorylation is required for activation of HrpY. From these observations, we postulate a basic regulatory cascade (Fig. 2) starting with the HrpX sensor that operates as follows: HrpX phosphorylates the HrpY response regulator and converts it to an active form, HrpY directly or indirectly activates *hrpS*, HrpS activates *hrpL*, and HrpL in turn enables RNAP to recognize *hrp* boxes. Additional experiments in *Es* with the cloned promoters of *hrpJ*, *hrpL* and *hrpS* in various backgrounds confirmed this model and showed that the *hrpX* promoter has moderate constitutive activity. Our basic model is consistent with those proposed for *E. amylovora* and *P. syringae*. The regulation of *hrpS* is unknown in these other systems and our study is the most complete to date concerning upstream regulation. At this point we know that constitutive expression of *hrpS* seems to override most plant and environmental signals and *hrpSp* responds to changes in pH and osmotic pressure, so we hypothesize that the *hrpS* promoter is a key point in the regulatory cascade where various inputs come together.

Non-polar mutations in *hrpL*, *hrpS* and *hrpY* were Hrp-, but non-polar insertions and deletions in *hrpX* did not decrease virulence of either *Es* or *Ehg* on corn or gypsophila. However, Δ *hrpX* mutants of *Es* were unable to elicit an HR in tobacco when grown on inducing-medium. Apparently, tobacco fails to induce sufficient *hrp* gene expression and harpin production, so that bacterial inocula must be preinduced and infiltrated at high cell concentrations to cause an HR. Our inducing medium is low pH and low salt and starves the cells for nitrogen and phosphate. HrpX contains PAS domains, which in some regulators are involved with sensing internal redox potential through the use of an FAD cofactor, so it may be involved with sensing the poor nutritional conditions and low pH found in the plant apoplast. The fact *hrpX* is not required by *Es* in corn further suggests that an alternate kinase, perhaps responding to specific plant signals or host cell contact, phosphorylates HrpY in planta.

Dr. Beck von Bodman at the Univ. of Connecticut has determined that the autoinducer N-3 oxohexanoyl-L-HSL (AHL) controls exopolysaccharide (EPS) synthesis in *Es*. The *esaI* gene encodes the enzyme responsible for AHL production and *esaR* encodes a LuxR-like response regulator that requires AHL to function. The system is unique in that EsaR is a transcriptional repressor that is inactivated by AHL. In the absence of AHL production in the *esaI* mutant, the *cps* genes are highly repressed by EsaR and subsequent deletion of the *esaR* gene removes this repression. We have shown that an *esaI* mutant is avirulent and non-mucoid and that a Δ *esaIR* mutant is super-mucoid and only slightly reduced in virulence. With BARD support, we have found that plasmid-borne *hrpSp-uidA*, *hrpLp-uidA* and *hrpJp-uidA* fusions are turned down 12-, 14- and 50-fold, respectively in an *esaI* background, but are fully expressed in Δ *esaR* and Δ *esaIR* mutants. Most significantly, overexpression of HrpS, but not HrpY, will restore virulence to the *esaI* mutant. EsaR may therefore either repress the *hrpS* promoter or act upstream of HrpS in the regulatory cascade. This is the first report of quorum sensing controlling *hrp* gene expression.

The lack of transcriptional terminators between operons meant that two novel autoregulatory loops were possible (Fig. 1). These would result from the autoregulation of *hrpXY* and the activation of *hrpLp* by HrpS; the resulting read-through transcription from each promoter into *hrpS* would thereby amplify the effect of any signals feeding into the cascade. This model is supported by the observation that *lacp-hrpS* stimulated a *hrpS::lacZ* fusion, but the latter effect was blocked by polar insertions in *hrpL* or *hrpY*, indicating that this apparent autoregulation was due to transcription originating far upstream of *hrpS*.

At left end of the *Es* cluster, we found an ORF, designated *mapA*, that has high aa homology to methyl-accepting chemotaxis proteins (MCPs). However, MapA represents only the cytoplasmic half of an MCP, which contains the methylation site. We have obtained two mutants in this gene; they are pathogenic but differ from wild-type *Es* in being able to elicit an HR in tobacco when grown on rich media. We are postulating that this gene is a negative regulator that is active in tobacco and rich media, but not in corn or an apoplast-mimicking medium.

Hrp-Secreted Proteins. Research under our current BARD grant has yielded the following results on Hops in *Es* and *Ehg*:

hsvG (host-specific virulence gene for gypsophila) determines the ability of *Ehg* and *Ehb* to incite galls on gypsophila. *hsvG* is present in both pPATHEhg and pPATHEhb and encodes a 72 kDal protein. Mutation of *hsvG* eliminated the ability of both *Ehg* and *Ehb* to cause galls on gypsophila, but did not affect virulence of *Ehb* on beets. HsvG is predominately hydrophilic and lacks transmembrane domains and a signal peptide. It is not homologous to any known peptides in Genbank. A truncated *hsvG* gene was still able to partially complement an *hsvG* mutant, indicating that the C-terminal 30% of the protein is not essential. As described above, *hsvG* is Hrp regulated. In Western blots, the HsvG protein was not detected in culture supernatants and could only be found in cell sonicates when *hrpL* or *hrpS* was overexpressed. An *hsvG* mutant could grow well inside gypsophila stems as compared to a *hrpJ* mutant that did not. Using a calmodulin-dependent adenylate cyclase (CyaA) reporter system, we showed that HsvG can be injected into human HeLa cells via the type III secretion system of an enteropathogenic *E. coli* (EPEC) strain. As controls, we used an *escV* mutant, which is deficient in the ability to secrete and inject proteins, and an *espB* mutant, which is deficient in translocation but can still secrete proteins into the milieu. The former failed to secrete and translocate the HsvG-CyaA and the later could only secrete it into the medium. The injection of HsvG into HeLa cells is the first direct demonstration in which a virulence effector from a phytopathogenic bacterium has been shown to be translocated into a eukaryotic cell via the type III machinery. It remains, however, to demonstrate that HsvG can be translocated into plant cells through the Hrp pathway of *Ehg*.

pthG. (pathogenicity gene on gypsophila). *pthG* was isolated from pPATHEhg and encodes a 59-kDal protein. *Ehb* transconjugants receiving *pthG* became incompatible on beets while retaining full pathogenicity on gypsophila. Interestingly, an inactive copy of *pthG* is present in pPATHEhb, suggesting that it was the loss of this gene that enabled pv. *betae* to become a pathogen of beets (18). When mutations in *pthG* were marker-exchanged into *Ehg*, the gypsophila pathovar became pathogenic on beet, but suffered a substantial reduction in pathogenicity on gypsophila. This dual function in virulence (on gypsophila) and host restriction (on beets) is typical of certain Avr proteins. *pthG* is *hrp*-regulated and its HR phenotype on beets is dependent on a functional Hrp secretion system. Injection of purified PthG proteins into beet leaves did not cause HR, suggesting that this effector acts within the host cell rather than through

the cell membrane. Thus, we have found that host specificity of *E. herbicola* can be determined by the interaction between positive-acting (HsvG) and negative-acting (PthB) factors .

An AvrPphD-like effector protein in *Ehg*. . Sequence analysis of the left *hrp*-flanking region of *Ehg*824-1 (cosmid pLA150 of pPATH*Ehg*) revealed an ORF, which exhibited high homology (81% in aa) to *AvrPphD* of *Pseudomonas syringae* pv. *phaseolicola*. This was found only in *Ehg* and not in pPATH from pv. *betea*. Insertional inactivation of *AvrPphD* reduced gall size in gypsophila.

WtsE and DspE. *wtsE* of *Es* and *dspE* of *Ehg* encode 201-kDa proteins that are very similar to DspE in *E. amylovora* and AvrE in *P. syringae* pv. *tomato*. Non-polar mutations in *wtsE* and *dspE* completely abolish virulence, as compared to the moderate to minor effects reported for *E. amylovora dspE* and *P. syringae* pv. *tomato avrE* mutations, but they do not affect HR elicitation. WtsE and DspE could therefore be major virulence factors in determining the ability of *Es* to cause Wts and the ability of *Ehg* to grow in gypsophila. Western blots showed that both WtsE and DspE are secreted into the supernatant when *hrpS* is overexpressed. *wtsF* and *dspE* are downstream of *wtsE* and *dspE* in the same operon and they are also required for pathogenicity. By virtue of their small size (15.6 kDa) and physical properties, these proteins are likely to be chaperones that target WtsE and DspE for secretion and/or stabilize them in the bacterial or host cytoplasm.

WtsG. Sequence information from the 10-kb region to the left of *wtsF* in *Es* indicates that it is completely different from the same region in *E. amylovora* and *Ehg*. A gene for a potential Hop protein was found and this has been designated *wtsG*. It is preceded by a *hrp* box and encodes a 279 aa protein that has 86% aa identity to AvrPpiB from *P. syringae* pv. *pisi*. Aside from AvrPpiB, WtsG has no other homologs in Genbank. A *wtsG::aphA3* mutant was greatly reduced in virulence (disease severity = 0.5 on a scale of 0-3) and could be complemented with a subclone of *wtsG*.

Fig. 1. Possible mRNA transcripts and feedback loops in the *hrp* regulatory region.

Fig. 2. Model for the regulation of *hrp* genes in *P. stewartii*.