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BARD

FINAL REPORT

PROJECT NO. US-2505-94

**Relationship of Genes Conferring Epiphytic
Fitness and Internal Multiplication in Plants
in *Erwinia herbicola***

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הספריה המרכזית
למדעי החקלאות
בית דגן

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Project Title: Relationship of genes conferring epiphytic fitness and internal multiplication in plants in *Erwinia herbicola*

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Abstracts		1	1	2
Not refereed (proceedings, reports, etc.)				

Patent Summary (numbers)

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

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Visits/Meetings	1	2	5	8
Sabbaticals	0	1	0	1
Postdoctorates	0	0	0	0

- **Cooperation**, briefly explain whether synergistic, complementary or supportive.

Cooperation was quite synergistic. The US and Israeli partners prepared and characterized different types of mutants (Israel – pPATH mutants; US- Chromosomal mutants) and the mutants were shared to enable additional phenotypic and genetic characterization by specialized techniques in the respective labs. Genetic constructs were shared to enable a better

measure of the transcriptional regulation of genes involved in plant hormone and plant virulence while on plants. Genetic tools such as specialized transposons were shared.

Abstract

Relationship of Genes Conferring Epiphytic Fitness and Multiplication *in planta* in *Erwinia herbicola*

Most bacterial plant pathogens colonize the surface of healthy plants as epiphytes before colonizing internally and initiating disease. The epiphytic phase of these pathogens is thus an important aspect of their epidemiology and a stage at which chemical and biological control is aimed. However, little is known of the genes and phenotypes that contribute to the ability of bacteria to grow on leaves and survive the variable physical environment in this habitat. In addition, while genes such as *hrp avr* and others which confer pathogenicity and *in planta* growth ability have been described, their contribution to other aspects of bacterial epidemiology such as epiphytic fitness have not been addressed. We hypothesized that bacterial genes conferring virulence or pathogenicity to plants also contribute to the epiphytic fitness of these bacteria and that many of these genes are preferentially located on plasmids. We addressed these hypotheses by independently identifying genes that contribute to epiphytic fitness, *in planta* growth, virulence and pathogenicity in the phytopathogenic bacterium *Erwinia herbicola* pv *gypsophilae* which causes gall formation on gypsophila. This species is highly epiphytically fit and has acquired a plasmid (pPATH) that contains numerous pathogenicity and virulence determinants, which we have found to also contribute to epiphytic fitness. We performed saturation transposon mutagenesis on pPATH as well as of the chromosome of *E.h. gypsophilae*, and identified mutants with reduced ability to grow in plants and/or cause disease symptoms, and through a novel competition assay, identified mutants less able to grow or survive on leaves. The number and identity of plasmid-borne *hrp* genes required for virulence was determined from an analysis of pPATH mutants, and the functional role of these genes in virulence was demonstrated. Likewise, other pPATH-encoded genes involved in IAA and cytokinin biosynthesis were characterized and their pattern of transcriptional activity was determined in planta. In both cases these genes involved in virulence were found to be induced in plant apoplasts. About half of avirulent mutants in pPATH were also epiphytically unfit whereas only about 10% of chromosomal mutants that were avirulent also had reduced epiphytic fitness. About 18% of random mutants in pPATH were avirulent in contrast to only 2.5% of random chromosomal mutants. Importantly, as many as 28% of pPATH mutants had lower epiphytic fitness while only about 10% of random chromosomal mutants had lower epiphytic fitness. These results support both of our original hypotheses, and indicate that genes important in a variety of interactions with plant have been enriched on mobile plasmids such as pPATH. The results also suggest that the ability of bacteria to colonize the surface of plants and to initiate infections in the interior of plants involves many of the same traits. These traits also appear to be under strong regulatory control, being expressed in response to the plant environment in many cases. It may be possible to alter the pattern of expression of such genes by altering the chemical environment of plants either by genetic means or by additional chemical antagonists of the plant signals. The many novel bacterial genes identified in this study that are involved in plant interactions should be useful in further understanding of bacterial plant interactions.

Relationship of Genes Conferring Epiphytic Fitness and Multiplication *in planta* in *Erwinia herbicola*

E. herbicola (*Eh*) is highly epiphytically fit. Some strains have acquired a plasmid (pPATH) containing pathogenicity determinants that elicit gall formation like *Eh* pv. *gypsophilae* (*Ehg*) or *Eh* pv. *betae* (*Ehb*). The host range of the former is restricted to gypsophila whereas the latter attacks beet and gypsophila. Most bacterial plant pathogens colonize the surface of healthy plants before colonizing internally (apoplast) and initiating disease. A transition from surface colonization to growth *in planta* (endophytic growth) is characteristic of all biotrophic phytopathogenic bacteria and requires the presence of *hrp* genes. In the present project we examined the hypotheses that bacterial genes conferring virulence or pathogenicity to plants also contribute to epiphytic fitness to these bacteria and that many of these genes are preferentially located on plasmids. These hypotheses were addressed by independently identifying genes in *Ehg* that contribute to: a) epiphytic fitness b) *in planta* growth and c) virulence or pathogenicity. The report is presented according to published papers.

The presence of *hrp* gene cluster on the pPATH of *Ehg* ((Nizan et al 1997)).

A *hrp* gene cluster has been reported in most major Gram-negative phytopathogenic bacteria including *Erwinia*, *Psuedomonas*, *Xanthomonas* and *Ralstonia solanacearum*. All these pathogens cause symptoms which eventually lead to necrosis. In contrast to these "necrogenic" bacteria, no *hrp* genes were found in *Agrobacterium tumefaciens* nor have there been any reports of *hrp* genes in any other tumorigenic bacteria. Therefore, until this study it was generally accepted and documented in review articles that tumorigenic bacteria lack *hrp* genes and their pathogenicity is achieved by different mechanisms from necrogenic bacteria. Results obtained in the current project suggest that *hrp*-dependent biotrophic pathogenicity is common to necrogenic and tumorigenic bacteria. Recently we have also detected *hrp* genes in *P. syringae* pv. *savastanoi*, another well known tumorigenic bacterium which suggests that *hrp* function in tumorigenic bacteria with the exception of *Agrobacterium* might be a general phenomenon. It is also noteworthy that the *hrp* cluster in *Ehg* resides on a plasmid whereas all the identified *hrp* gene clusters have been reported to be localized on the chromosome with the exception of *R. solanacearum* in which it is on a megaplasmid.

This study was recently extended beyond this BARD project and a paper demonstrating a full functional *hrp* cluster in *Ehg* has been recently submitted ((Mor et al 2000)). Furthermore, the mode of action of the *hrp* regulon in *Ehg* is being characterized ((Nizan-Koren et al 1999)). It appears to be transcriptionally induced in the apoplast of gypsophila as well as the apoplast of a non-host plant (bean) but not on leaf surfaces.

Differential involvement of IAA biosynthetic pathways in pathogenicity and epiphytic fitness of *Ehg*

Non-pathogenic *Eh* as well as pathogenic *Ehg* produce equivalent amounts of IAA in culture. However, in contrast to *Eh* strains that harbor only the indole-3-pyruvate pathway (IPyA) on the chromosome, all the pathogenic *Ehg* strains carry, in addition to the IPyA, the indole-3-acetamide (IAM) pathway as well as cytokinin biosynthetic genes on pPATH. Mutants were generated in which the various biosynthetic routes were disrupted separately or jointly in order to assess the contribution of IAA of various origins and cytokinins to pathogenicity and epiphytic fitness. Inactivation of the IAM pathway or cytokinin biosynthesis caused the largest reduction in gall size. Inactivation of the IPyA pathway caused a minor, nonsignificant decrease in pathogenicity. No further reduction was observed by the simultaneous inactivation of both IAA pathways only or in combination with that of cytokinin production. However, inactivation of the IPyA pathway caused a 14-fold reduction in the population of *Ehg* on bean plants. Inactivation of the IAM pathway or cytokinin production did not affect epiphytic fitness.

The transcriptional activity of the IPyA and IAM pathways on bean or gypsophila leaves was compared to that of gypsophila stems (apoplast environment). While the apparent transcriptional activity of *iaaM-inaZ* fusion (IAM pathway) increased slightly in cells of *Ehg* on bean and gypsophila leaves, compared with that in culture, very high levels of induction were observed in cells injected into gypsophila stems. In

contrast, moderate levels of induction of *ipdC-inaZ* (IPyA pathway) in *Ehg* were observed on leaves of these plants and in gypsophila stems, when compared with that in culture. These results suggest that the IAM pathway is involved primarily in gall formation and support the main contribution of IPyA pathway to the epiphytic fitness of this bacterial species. These results complement the data of Brandl and Lindow (1998), which also demonstrated that the *ipdC* gene was required for epiphytic fitness of *Eh* on plants under field conditions. In this study, the relative fitness of *IpdC*⁻ mutants in mixtures with a parental strain of *Eh* was directly measured on pear flowers, revealing a substantial contribution of IAA via this pathway to these epiphytic strains. The different pattern of regulation of *iaa* and *ipdC* genes in *Ehg* is also consistent with the findings of Brandl and Lindow (1997) in which it was found that the *ipdC* gene was induced not only on plant surfaces, but also in response to water stress to the bacteria cells. Interestingly, the use of *ipdC:gfp* fusions enabled Brandl and Lindow (1999) to show that while some cells of *Eh* on leaves were strongly induced for *ipdC*, others cells nearby were not induced, suggesting that the local environment of the leaf is very heterogeneous, and that specific signals are sensed by individual cells. This result suggests that the expression of IAA in epiphytic *Eh* strains is triggered by a physical environment typified by occasional lack of free water on plant surfaces, while *iaa* genes involved in intercellular colonization of plants, and not apparently of the leaf surface, is triggered by conditions such as specific chemicals found in the interior of plants.

Two plant-inducible promoters are present in the cytokinin biosynthetic genes of *Erwinia herbicola* pv. *gypsophilae* ((Guo et al 2000)).

In contrast to IAA, cytokinin production was observed only in pathogenic but not in non-pathogenic *Ehg* strains. We have previously shown that the cytokinin biosynthesis gene (*etz*) was preceded by another ORF (*pre-etz*) and the two genes constitute an operon. Mutations in either gene affected cytokinin production and significantly reduced gall formation. Northern hybridization indicated the presence of 2 transcripts, one of which was assigned to the operon whereas the other was assigned to *etz*. Using *inaZ* as a reporter gene, 2 promoters were identified in the present study, one upstream to the operon whereas the other upstream to the *Etz* within the ORF of the *Pre-etz*. Both promoters showed similar transcriptional activity in either minimal or rich media. However, a transcriptional increase of 100 to 1000-fold as compared to LB medium was observed with the 2 promoters when the bacteria was present either on gypsophila leaf surface or injected into the stems. Similar results were obtained with bean plants. These studies reveal that like *iaa* genes, all the tested genes associated with virulence or pathogenicity of *Ehg* are induced by the apoplast environment. The adaptation to the apoplast is achieved either via the *hrp* regulon which exhibits specificity to the apoplast (versus plant surface), and/or addition of a special pathway (IAM) or non-specific induction by the plant (cytokinins).

Relation of genes encoding virulence and epiphytic fitness.

We performed saturation transposon mutagenesis on pPATH as well as of the chromosome of *E.h. gypsophilae*, and identified mutants with reduced ability to grow in plants and/or cause disease symptoms, and through a novel competition assay, identified mutants less able to grow or survive on leaves (Lindow et al. 2000). The number and identity of plasmid-borne *hrp* genes required for virulence was determined from an analysis of pPATH mutants, and the functional role of these genes in virulence was demonstrated by performing virulence assays. 182 randomly produced mutants in pPATH were tested for both virulence and epiphytic fitness. About half of the mutants tested that were avirulent were also epiphytically unfit whereas only about 10% of chromosomal mutants that were avirulent also had reduced epiphytic fitness. About 18% of random mutants in pPATH were avirulent in contrast to only 2.5% of the 297 random chromosomal mutants that were tested. Importantly, as many as 28% of pPATH mutants had lower epiphytic fitness while only about 10% of random chromosomal mutants had lower epiphytic fitness. These results support both of our original hypotheses – namely that genes involved in plant-microbe interactions are enriched on plasmids such as pPATH, and that genes involved in virulence are derived from

genes involved in epiphytic fitness and will be required for both functions. The results also indicate that genes important in a variety of interactions with plants have been enriched on mobile plasmids such as pPATH. The results also suggest that the ability of bacteria to colonize the surface of plants and to initiate infections in the interior of plants involves many of the same traits. It is clear, however, that some genes involved in virulence do not play a role in epiphytic fitness. It seems likely that such genes encode functions that are involved in later stages in plant-microbe interactions, long after bacteria have either entered or multiplied in the apoplastic spaces in plants. Since we now have a large library of such mutants, these virulence genes, the function of which are largely unknown, can now be better understood by investigating their temporal and spatial patterns of expression. That is, it would be expected that genes involved in both epiphytic fitness and virulence would be expressed at different times and places in the plant than those involved only in virulence. Likewise, our results associate particular genes for virulence with epiphytic fitness, which should help elucidate the process of "invasion" of plants since such genes would likely be involved in such a process. These traits also appear to be under strong regulatory control, being expressed in response to the plant environment in many cases. It may be possible to alter the pattern of expression of such genes by altering the chemical environment of plants either by genetic means or by additional of chemical antagonists of the plant signals. The many novel bacterial genes identified in this study that are involved in plant interactions should be useful in further understanding of bacterial plant interactions.

Publications resulting from the present BARD project:

1. Nizan, R., Barash, I., Valinsky, L., Lichter, A., Manulis, S. 1997. The presence of *hrp* genes on the pathogenicity-associated plasmid of the tumorigenic bacterium *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 10:677-682.
2. Brandl, M.T., and S.E. Lindow. 1997. Cloning and characterization of a locus encoding an indolepyruvate decarboxylase involved in indole-3-acetic acid synthesis in *Erwinia herbicola*. *Appl. Environ. Microbiol.* 62:4121-4128.
3. Brandl, M.T., and S.E. Lindow. 1997. Environmental Signals modulate the expression of an indole-3-acetic acid biosynthetic gene in *Erwinia herbicola*. *Molecular Plant-Microbe Interactions* 10:499-505.
4. Brandl, M.T., and S.E. Lindow. 1998. Contribution of indole-3-acetic acid production to the epiphytic fitness of *Erwinia herbicola*. *Appl. Environ. Microbiol.* 64:3256-3263.
5. Lindow, S.E., C. Desurmont, R. Elkins, G. McGourty, E. Clark, and M. Brandl. Occurrence of IAA-producing bacteria on pear trees and their association with fruit russet. *Phytopathology* 88:1149-1157.
6. Manulis, S., Haviv-Chesner, A., Brandl, M. T., Lindow, S. E., Barash, I. 1998. Differential involvement of indole-3-acetic acid biosynthetic pathways in pathogenicity and epiphytic fitness of *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* 11:634-642.
7. Lindow, S.E., Manulis, S., and Barash, I. 2000. Relationship of genes involved in virulence and epiphytic fitness in *Erwinia herbicola*. *Molec. Plant-Microbe Interactions*. (In preparation).
8. Guo, M., Manulis, S., Barash, I., Lichter, A. 2000. Two plant-inducible promoters are present in the cytokinin biosynthetic genes of *Erwinia herbicola* pv. *gypsophillae*. *Mol. Plant-Microbe Interact.* (Submitted).

Other relevant reports

9. Mor, H., Manulis, S., Zuc, M., Nizan, R., Coplin, D. L., Barash, I. 2000. Genetic organization of the *hrp* gene cluster and *dspEF* operon in *Erwinia herbicola* pv. *gypsophillae*. *Molec. Plant-Microbe Interact.* (Submitted)
10. Nizan-Koren, R., Manulis, S., Mor, H., Iraki, N. M., Barash, I. 1999. Characterization of the *hrp* regulon in *Erwinia herbicola* pv. *gypsophillae*. *abstr. 16.7. In abstract book of the 9th International Congress on molecular-Plant Microbe Interactions, Amsterdam, The Netherlands.*

11. Brandl, M., and S.E. Lindow. 1999. Heterogeneity of the transcription of *ipdC* in *Erwinia herbicola* revealed by in situ hybridization and fluorescence microscopy of cells harboring *ipdC:gfp* fusions on plant surfaces. Abstr. 16.65 in abstract book of the 9th International Congress on Molecular Plant-Microbe Interactions. Amsterdam, The Netherlands.