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**Role and *In situ* Regulation of Growth Regulators
Produced in Plant-Microbe Interactions by *Erwinia
herbicola***

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Table of Contents

	Page
Abstract	1
Objectives of the original research proposal	2-4
Relevant data, methodology, results and discussion	5
1. Lichter, A., Manulis, S., Sagee, O., Gafni, Y., Gray, J., Meilan, R., Morris, R.O., and Barash, I. 1995. Production of cytokinins by <i>Erwinia herbicola</i> pv. <i>gypsophilae</i> and isolation of a locus conferring cytokinin biosynthesis. Molec. Plant-Microbe Interactions 8:114-121.	6-13
2. Lichter, A., Barash, I., Valinsky, L., and Manulis, S. 1995. The genes involved in cytokinin biosynthesis in <i>Erwinia herbicola</i> pv. <i>gypsophilae</i> : Characterization and role in gall formation. J. Bacteriol. 177:4457-4465.	14-22
3. Lichter, A., Manulis, S., Valinsky, L., Karniol, B., and Barash, I. 1996. IS1327, a new insertion-like element in the pathogenicity-associated plasmid of <i>Erwinia herbicola</i> pv. <i>gypsophilae</i> . Molec. Plant-Microbe Interactions 9:98-104.	23-29
4. Brandl, M., Clark, E.M., and Lindow, S.E. 1996. Characterization of the indole-3-acetic acid (IAA) biosynthetic pathway in an epiphytic strain of <i>Erwinia herbicola</i> and IAA production <i>in vitro</i> . Can. J. Microbiol. 42:586-592.	30-36
5. Brandl, M., and Lindow, S.E. 1996. Cloning and characterization of a locus encoding an indolepyruvate decarboxylase gene involved in indole-3-acetic acid synthesis in <i>Erwinia herbicola</i> . Appl. Environ. Microbiol. 62: (in press).	37-76
6. Brandl, M., and Lindow, S.E. 1996. <i>ipdC</i> , a gene involved in indole-3-acetic acid synthesis in <i>Erwinia herbicola</i> , responds to plant signals and osmotic stress. Molec. Plant-Microbe Interactions (in preparation).	77-108
7. Brandl, M., and Lindow, S.E. 1996. Contribution of indole-3-acetic acid production to the epiphytic fitness of <i>Erwinia herbicola</i> . Appl. Environ. Microbiol. (in preparation).	109-130
Description of the cooperation	131
Evaluation of the research achievements	132
Conclusions	133-137
List of publications	137-138
Patents	138

ABSTRACT

Role and *in situ* regulation of growth regulators produced in plant-microbe interactions by *Erwinia herbicola*

The main objective of this work was to gain a better understanding of how some strains of *Erwinia herbicola* have evolved into serious plant pathogens while also commonly existing as epiphytes on the surface of healthy plants. The focus of our studies was to determine the nature of, and regulation, of virulence factors, including the phytohormones IAA and cytokinins, which are encoded on a large plasmid (pPATH) found in gall-forming strains of this species. In addition, the *in situ* regulation and contribution to epiphytic fitness of a second, chromosomal, IAA biosynthetic locus (*ipdC*) was determined to ascertain the relative contribution of the two redundant IAA-biosynthetic pathways to the biology of *E. herbicola*. Genes (*pre-etz* and *etz*) conferring production of cytokinins were clustered immediately 3' of the *iaaM* and *iaaH* genes conferring IAA biosynthesis on pPATH. A new insertion-like element, IS1327, was also found immediately 3' of *etz* on pPATH, suggesting that these virulence factors were all introduced onto pPATH from another pathogenic bacterium. Mutants of *E. herbicola* in which *etz*, *iaaH*, and *iaaM*, but not *ipdC*, were disrupted caused smaller galls to form on gypsophila plants. In contrast, *ipdC* but not *iaaH* or *iaaM* mutants of *E. herbicola* exhibited reduced ability to grow and survive on plant surfaces. Transcription of *ipdC* was induced when cells were on plants compared to in culture, suggesting that *ipdC* may play a selective role in fitness on leaves.

Objectives of the original research proposal:

One of the main objectives of the proposal was to gain a better understanding of how some strains of *Erwinia herbicola* have evolved into serious pathogens of plants and alter normal plant development by interacting with plants via phytohormones. To achieve this objective we proposed to make critical tests of the following hypotheses:

1) All the necessary virulence factors such as IAA and cytokinin biosynthesis to render non-pathogenic *E. herbicola* strains capable of gall-formation reside on a transmissible plasmid (pPATH) but redundant mechanisms for producing IAA and separate genes for cytokinin production necessitate the coordinate regulation of these pathways.

As a test of this hypothesis the expression of IAA by the pPATH plasmid-borne IAA biosynthesis genes *iaaH* and *iaaM* (which confer production of IAA via indole-3-acetamide) were to be compared and contrasted with the expression of a chromosomal *ipdC* gene (which is required for the production of IAA via indole-3-pyruvic acid and indole-3-acetaldehyde intermediates) both in culture and on plants. The evolution of pathogens from non-pathogenic bacteria strains was also proposed to be studied by determining the requirement, sufficiency, and identity of other genes found on pPATH that distinguish them from the non-pathogenic strains with which such pathogens are associated. Specifically, it was proposed to isolate and characterize cytokinins and cytokinin-encoding genes in pathogenic *E. herbicola* strains and determine their role in tumor production.

2) The metabolic cost of IAA biosynthesis is mitigated by tight regulation leading to its production only at times when it will benefit the bacteria that produce it.

While both pathogenic and saprophytic *E. herbicola* strains are capable of producing copious quantities of IAA in some culture media, the unregulated production of IAA on plants or in other natural habitats such as on plant debris or in soil, would likely place the producing strain at a relative disadvantage if the cell received no benefit from its production. We therefore proposed to ascertain the transcriptional activity of IAA biosynthetic genes in different habitats and on plants under different physiological conditions to determine whether signals are involved in the regulation of this phenotype.

3) The metabolic and genetic load of IAA production is offset by benefits to the producing strains in their saprophytic phase on plants.

Most saprophytic strains of *E. herbicola* produce IAA in culture and presumably can do so on leaves. This phenotype would not be maintained in the population unless it significantly benefitted the producer. Exogenous IAA of bacteria origin may alter the nature of the leaf surface habitat to make it more suitable for epiphytic colonization. We thus proposed to determine the epiphytic fitness benefits associated with IAA production by comparing the relative competitiveness of isogenic IAA⁺ and IAA⁻ strains produced by marker exchange mutagenesis using disrupted IAA biosynthesis genes produced *in vitro*.

We pursued and completed all of the objectives except for the measurement of the transcriptional regulation of *iaaH* and *iaaM* genes. We conducted expensive studies of the regulation of the *ipdC* gene both in culture and *in situ*. Our extensive work on other parts of the project, including the study of the regulation of *ipdC*, took longer than we originally estimated, and we thus could not complete it during the course of the project period. Because of our interest in determining the regulation of these other IAA-biosynthetic genes we continue to conduct work toward this goal on a time-available basis, using other, discretionary, funding available to both the Israeli and Berkeley scientists, and plan to complete this study before the end of 1996. All other objectives of the project were pursued as originally described and have been completed.

Relevant data, methodology, results and discussion

All of the studies conducted as part of this project have now been published, are in press, or are in preparation for publication. We therefore will report our results in the following 7 research publications. The relationship of the results reported in each publication to the original objectives of the project (as outlined earlier) should be obvious and will not be reiterated.

Description of the cooperation:

A very close cooperation has been established during the course of this study. This collaboration has been facilitated by frequent communication via electronic mail, through exchange of laboratory personnel, and through exchanging genetic materials. Dr. Barash spent 3 months working in the laboratory of Dr. Lindow in Berkeley during the summer of 1993. Dr. Lindow spent several weeks during the summer of 1994 visiting the laboratories of Dr. Barash, Gafni, and Manulis to discuss research results. The Israeli and Berkeley scientists also had the opportunity to meet and discuss results during the International symposium on Molecular Plant-Microbe Interactions held in Edinburgh Scotland in the summer of 1994 as well as the meeting of this society held in 1992 in Seattle, Washington. Dr. Manulis visited the Berkeley laboratory during the summer of 1992, 1993 and 1995. These frequent meetings permitted constant discussions and generation of ideas and experiments throughout the course of this study. The work initiated during the course of this BARD project, and continued using discretionary funding on the relative fitness contribution of different redundant IAA biosynthetic genes in pathogenic *E. herbicola* strains has resulted in a joint publication that is about to be submitted for publication. A significant measure of collaboration in this project was the large amount of genetic material that was exchanged. For example, the *ipdC* gene, required for IAA biosynthesis in saprophytic *E. herbicola* strains was cloned in Berkeley and used by the Israeli collaborators to determine its role in gall formation in pathogenic *E. herbicola* strains.

Evaluation of the research achievement:

The work conducted in this project was far-ranging. The biochemistry of cytokinin and IAA biosynthesis were explored in great depth. In addition, the genetic determinants for both IAA and cytokinin biosynthesis were cloned and characterized. Detailed studies of the regulation of some of these genes was also performed both in culture and *in situ*. Extensive biological and ecological studies of the contribution of IAA and cytokinin production on gall-formation and other phytopathogenic phenotypes as well as on the epiphytic fitness of *Erwinia herbicola* strains was also studied in depth. These later experiments involved both field as well as laboratory studies. Thus we feel we have been quite productive and that our work has addressed all of the original objectives. Our data was sufficiently voluminous and of sufficient quality to generate 7 publications. We thus feel that we have thoroughly met the original objectives of the project, and through these studies have elucidated important features of plant-microbe interactions.

Conclusions:

Considerable information was obtained as a result of this study on the nature and role of genes located on a large plasmid, pPATH that is unique to phytopathogenic strains of *E. herbicola* in virulence and other ecological features. Considerable attention was placed on examining the genes in the vicinity of the *iaaH* and *iaaM* genes on pPATH that confer production of IAA. It was extremely interesting to find that two genes that are required for cytokinin production by pathogenic strains were located immediately 3' of the two genes for IAA production. Sequence analysis revealed that one of these two genes (*etz*) was homologous to other described cytokinin biosynthesis genes. Interestingly, another putative gene (*pre-etz*) was found immediately 5' to *etz*. Northern analysis revealed that a common transcript encoded both the *pre-etz* and *etz* gene product in most cases, indicating that they were part of an operon. These two genes appear to be the sole determinants of cytokinin production in pathogenic *E. herbicola* strains since their inactivation greatly reduced cytokinin content of cell suspensions and the size of galls when mutants were inoculated into gypsophila plants. Thus the IAA and cytokinin biosynthetic loci are clearly physically linked. Their coincident nature on pPATH may be readily explained by another finding on this research. Analysis of DNA sequences immediately 3' of *etz* revealed the presence of IS1327, a new insertion-like element. The finding of this IS element so close to both IAA and cytokinin biosynthesis genes in pathogenic *E. herbicola* strains, and the observation that this IS element was lacking from all non-pathogenic strains, suggests strongly that phytohormones biosynthesis genes (and possibly other genes required for

virulence) were introduced into pPATH from another source. It was also significant to note, that while IS1327 is apparently not currently active in pathogenic *E. herbicola* strains (probably due to internal deletions that have been acquired since its last transposition), it is present in all variant forms of pPATH that have been observed, and may in fact, be responsible for the variation in pPATH that are observed. The ecological consequences of horizontal transfer of phytohormone genes into previously non-pathogenic bacteria are obvious; the results of this study should provide the tools to further elucidate the mechanisms and consequences of such transfer.

Considerable effort was made to study the sole determinants of IAA biosynthesis that are found in non-pathogenic *E. herbicola* strains that are also found in phytopathogenic *E. herbicola* strains (in addition to *iaaH* and *iaaM*). Considerable work was performed to determine the biosynthetic pathway in non-pathogenic *E. herbicola* strains. It was found that IAA was produced from a tryptophan precursor with indole-3-pyruvic acid and indole-3-acetaldehyde as intermediates. Great progress was made in understanding the regulation of this pathway with the cloning of a gene, *ipdC* encoding an indolepyruvate decarboxylase that required for the conversion of indole-pyruvate to indole-acetaldehyde. It appears that in non-pathogenic *E. herbicola* strains that a majority of the IAA that is produced in the presence of exogenous tryptophan is produced via the indole-pyruvic acid pathway since inactivation of the chromosomally-located *ipdC* gene resulted in over a 10-fold reduction in IAA production. This finding contrasts with the pattern of IAA production found in phytopathogenic *E. herbicola* strains; in these strains the inactivation of *ipdC* leads to only a small decrease in IAA biosynthesis, apparently due to the redundant indole acetamide pathway encoded by

iaaH and *iaaM* located on pPATH in these strains.

A very important practical application to plant stress avoidance has resulted from the finding that the indole-pyruvic acid pathway is solely responsible for IAA production in non-pathogenic strains of *E. herbicola* that are common on plants surfaces, including the fruit of apples and pear. We had previously associated the presence of IAA-producing *E. herbicola* strains on young fruit surfaces with severe fruit russetting, apparently caused by changes in normal epidermal cells development caused by exogenous auxin production by bacteria. Since the first step in IAA biosynthesis in non-pathogenic *E. herbicola* strains was found to be the deamination of tryptophan to produce indole-3-pyruvic acid, we expected that this enzymatic step could be inhibited in the presence of excess ammonium ion. In fact, we found that this reaction was strongly inhibited by even small amounts of ammonium. This suggested that application of ammonium ions to the surfaces of young pear fruit could reduce fruit russetting by inhibiting bacterial IAA production. Research funded by other sources has now verified that applications of ammonium ions to fruit can reduce fruit russet by over 3-fold compared to control trees under field conditions. This finding, resulting from the basic research supported here, has the potential to greatly improve fruit quality as well as to reduce pesticide applications designed to reduce fruit russetting.

Studies of the transcriptional regulation of the *ipdC* gene revealed a strong environmental regulation of this gene, consistent with our hypothesis. The transcription of *ipdC*, as revealed by *ipdC::inaZ* gene fusions, was rather low in most culture media and increased over 100-fold when *E. herbicola* cells were introduced onto plants. The maximum apparent transcription of *ipdC* on plants occurred when plant parts were dry.

Interestingly, we found that the expression of *ipdC* could also be greatly enhanced when the water availability of culture media was reduced by the addition of osmolites or non-penetrating solutes such as polyethylene glycol. Thus, *E. herbicola* apparently regulates the expression of *ipdC* not in response to any specific plant compound, but instead, in response to water stress that would be a common environmental signal present when cells existed epiphytically on plant surfaces. Apparently such signals would not be as commonly experienced when cells were in other habits such as water or plant debris. Thus the expression of IAA biosynthetic genes on plants is apparently conservative as we hypothesized. These results also indicate that we need to learn more about the other possible habitats that *E. herbicola* occupies when it is not existing epiphytically on plants.

We obtained strong evidence that IAA production is of significant benefit to *E. herbicola* cells existing epiphytically on plant surfaces. Sensitive measures of the relative fitness of isogenic IAA⁺ and IAA⁻ strains of *E. herbicola* were made by measuring changes in the proportion of cells in mixtures after time after inoculation onto plants both in greenhouse and field experiments. Fore example, we found that within two weeks, when cells had undergone about 10 generations of multiplication on pear flowers in the field, that the population size of the IAA⁺ *E. herbicola* parental strain had increased about 3 fold compared to an isogenic IAA⁻ mutant. These findings are consistent with our hypothesis that IAA production is advantageous to producing strains such as *E. herbicola* by modifying the plant surface to make it more habitable to bacteria epiphytes. These findings have string implications that bacterial epiphytes may have major effects on the physiology of the plants on which they live. Certain aspects of what we consider "normal" plant development such as flower, fruit and leaf abscission may, in

fact, be influenced by the epiphytic bacterial microflora. The magnitude of these effects on plant development need to be further examined. It is possible that this phenomenon may be exploited to benefit agricultural production. For example, synthetic auxins are currently applied to many agricultural crops to thin fruit, and to increase fruit retention before harvest. It is possible that exogenous auxin of epiphytic bacterial origin could be used to achieve these agricultural benefits.

List of publications:

1. Lichter, A., Manulis, S., Sagee, O., Gafni, Y., Gray, J., Meilan, R., Morris, R.O., and Barash, I. 1995. Production of cytokinins by *Erwinia herbicola* pv. *gypsophilae* and isolation of a locus conferring cytokinin biosynthesis. *Molec. Plant-Microbe Interactions* 8:114-121.
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7. Brandl, M., and Lindow, S.E. 1996. Contribution of indole-3-acetic acid production to the epiphytic fitness of *Erwinia herbicola*. Appl. Environ. Microbiol. (in preparation).

Patents

None