Studies on the basal kernel blight disease of barley: pathogenesis and phylogeny of the causal agent Pseudomonas Syringae pv. syringae and its biological control by antagonistic Pantoea agglomerans
by Andrea Braun

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
The pathogenesis and phylogeny of Pseudomonas syringae pv. syringae (Pss), the cause of basal kernel blight of barley, and its biological control by antagonistic Pantoea agglomerans Pa, (syn. Erwinia herbicola, Eh) was investigated. Scanning electron micrographs located bacteria close to stomata and bases of kernel trichomes. Light micrographs revealed Pss in intercellular spaces of aleurone and amyloplast cells. Electron micrographs demonstrated Pss in xylem vessels and cell wall degradation in association with capsule protected bacteria.

Fifty-five Pss strains, isolated from infected kernels of 12 barley cultivars were tested for tobacco hypersensitivity, pathogenicity, toxin production and carbon source utilization. Toxin production correlated low ($r=0.31$; $p=0.046$) with pathogenicity, but 96% of toxin-plus strains were pathogenic, supporting the importance of toxins for virulence. Nutritional analyses revealed a phenotypic variability of Pss strains with similarity coefficients between 76-100%. The intrapathovar variation of Pss populations was analyzed by RFLPs of digested DNA using XbaI, SpeI and pulsed field gel electrophoresis. Macrorestriction fingerprinting supported a heterogeneity of Pss populations (43-77% similarity).

Field experiments (1994, 1995) revealed 45-70% disease control, when Pa was applied to barley heads prior to the Pss infection window. Pa provided 80-100% disease reduction in greenhouse studies. Efficacy of Pa was affected by time and rate of application, with a single application providing control. Survival of formulated Pa was better when stored at 4 C than 22 C. Biocontrol activity was not affected by storage.

Multiple mechanisms including antibiosis, preemptive exclusion, and induced systemic resistance were involved in the Pa-Pss interaction. Antibiotics were polar, thermostable, protease insensitive, but base labile ($pH \geq 6.0$). Antibiotic production correlated weakly ($r = 0.43$) with disease suppression. Nutritional niche overlapping indices revealed a high possibility of preemptive exclusion of Pss. Pss populations on barley kernels were reduced 100-fold in the presence of Pa. $FeCl_3$ amendment to the phyllosphere reduced the biocontrol efficacy of Pa, and 2 out of 3 siderophore-minus mutants were less efficient in disease reduction than the parental strain. Eh 460 and 239 reduced kernel blight through induced systemic resistance, similar to the plant activator benzothiadiazole. This is the first report on the induction of systemic resistance in barley by Pantoea agglomerans.

STUDIES ON THE BASAL KERNEL BLIGHT DISEASE OF BARLEY: PATHOGENESIS AND PHYLOGENY OF THE CAUSAL AGENT *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* AND ITS BIOLOGICAL CONTROL BY ANTAGONISTIC *PANTOEA AGGLOMERANS*

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APPROVAL

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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... DAS EINZIGE, WAS WIR BRAUCHEN,

UM GUTE PHILOSOPHEN ZU WERDEN, IST DIE FÄHIGKEIT,

UNS ZU WUNDERN ...

(Jostein Gaarder aus: Sofies Welt -
Ein Roman über die Geschichte der Philosophie)

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ABSTRACT

The pathogenesis and phylogeny of *Pseudomonas syringae* pv. *syringae* (Pss), the cause of basal kernel blight of barley, and its biological control by antagonistic *Pantoea agglomerans* Pa, (syn. *Erwinia herbicola*, Eh) was investigated. Scanning electron micrographs located bacteria close to stomata and bases of kernel trichomes. Light micrographs revealed Pss in intercellular spaces of aleurone and amyloplast cells. Electron micrographs demonstrated Pss in xylem vessels and cell wall degradation in association with capsule protected bacteria.

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CHAPTER 1

LITERATURE REVIEW

Thesis Preface

Barley is the second largest small grain crop produced in the United States of America and in Montana behind wheat production. In 1997, 13.2 million tons of barley were produced in the US with an average yield of 50.7 dt/ha. North Dakota continued to rank as the number one barley producing state, followed by Montana, Idaho, Washington, and Minnesota; with these five states producing more than three-fourths of the US production in 1997. While yields in North Dakota dropped by 8.7 dt/ha to 39.2 dt/ha in 1997 compared to 1996 due to dry weather conditions in the west and the scab disease in the eastern portion of the state, yields in Montana increased by 8.7 dt/ha to 46.1 dt/ha as a result of timely rains (Anonymous, 1997 a). Montana farmers planted 526315 ha of barley in 1997, unchanged from the last four years. Malting and brewing varieties recommended by the American Malting Barley Association accounted for almost 44 percent of the total barley planted (Anonymous, 1997 b). Harrington remained the top malting variety compared to 1996 followed by B 1202, B 2601, and Morex. Baronesse remained in first place among feed-type varieties followed by Hector, Gallatin, and Bowman (Anonymous, 1997, b).

Bacterial Diseases of Small Grains

Bacterial diseases of small grains are generally considered of minor importance compared to other diseases, however, they can cause significant damage when environment favors disease development (Paul & Smith, 1989; Mathre, 1997). Examples of important bacterial diseases on wheat and barley consist of bacterial leaf and kernel blight caused by *Pseudomonas syringae* pv. *syringae* (Peters et al., 1983; Martinez-Miller, 1994; Martinez-Miller & Braun, 1997), bacterial blight of barley caused by *Xanthomonas translucens* pv. *translucens* (Forster, 1997) and basal glume rot caused by *Pseudomonas syringae* pv. *atrofaciens* (von Kietzell, 1995). Bacteria are almost always present on plant surfaces and may live as epiphytes without causing any host damage (Hirano & Upper, 1983, 1990; Lindow, 1985). However, in some years epiphytic populations can become pathogenic. The mechanisms and stimuli for this change are not fully understood, but recent research indicated that population size, including quorum-sensing autoinduction processes, and plant signal molecules can increase virulence gene expression (Pirhonen et al., 1993; Quigley & Gross, 1994; Beck von Bodman & Farrand, 1995; Fuqua et al., 1996; Farrand et al., 1996). Alternatively, new virulent races which may overcome deployed host plant's resistance can occur as a result of genetic changes in pathogen populations through mutation and/or recombination, in addition to selection and migration (Vera Cruz et al., 1996; Vivian & Gibbon, 1997). Understanding these events is critical to the apprehension and needed for control or management of pathogens, such as *P. s.* pv. *syringae*.

The Kernel Blight Disease of Barley

Kernel blight diseases of barley have been reported to be associated with fungi, such as *Bipolaris sorokiniana*, *Fusarium graminearum*, and *Alternaria* spp. (Miles et al., 1987; Johnston, 1997) and with bacteria in the Northern Great Plains (Peters et al., 1983; Martinez-Miller, 1994). *Pseudomonas syringae* pv. *syringae* was first described as causing bacterial kernel spot of Klages barley in Idaho (Peters et al., 1983). The disease has also been reported in Montana and was characterized as a well defined discolored area with distinct margins on the lemma of the kernel (Martinez-Miller, 1994). Another disease caused by *P. s.* pv. *syringae* in Montana is basal kernel blight, which consists of a dark brown discoloration at the embryo end of the kernel (Martinez-Miller & Braun, 1997). Similar symptoms have been associated with *Bacillus* sp. in South Africa by Basson et al. (1990), who found counts of these bacteria on black-end barley kernels significantly higher than on healthy kernels. In the temperate climates of Europe, *P. s.* pv. *atrofaciens* was regularly isolated from basal glume rot symptoms on wheat, barley (Poschenrieder, 1986; Toben et al., 1989) and rye (Gvozdyak & Pasichnik, 1991). Toben et al. (1989,1991) confirmed its pathogenicity in wheat and barley seedling assays, which correlated with typical disease symptoms obtained when adult wheat plants were inoculated at the milk stage (EC 75). However, wheat spikes also developed basal glume rot symptoms when *P. s.* pv. *syringae* isolates from lilac, bean, or *Pennisetum* sp. or *P. s.* pv. *aptata* isolates from sugar beets were inoculated (von Kietzell, 1995).

These diseases are primarily of concern to malting and brewing industries, who discount or even reject barley lots when more than 4 % of kernels are discolored in a lot.

Kernel blight reduces grain yield and quality (Anderson & Banttari, 1976) and Gebhardt et al. (1992) reported, that protein, malt extract, wort color, beer taste and aroma were affected by kernel discoloration. Furthermore, the suppression of germination seemed to be correlated with the formation of black-end kernels (Basson et al.,1990).

We have reported previously on the etiology and significance of basal kernel blight of barley (Martinez-Miller et al., 1998). Kernel discoloration caused by *P. s.* pv. *syringae* was found to vary in severity with cultivars, environmental conditions, and virulence of the pathogen. The critical period for infection increased from late milk to soft dough stages of kernel development (EC 85), with free moisture being necessary for infection and disease development (Martinez-Miller et al., 1998). A similar sensitivity to *P. s.* pv. *atrofaciens* infection was observed by von Kietzell & Rudolph (1991) on wheat plants. While the use of overhead irrigation at soft dough should be avoided by growers during this time, it was useful in screening experiments, in which the susceptibility of cultivars was tested under field conditions (Martinez-Miller et al., 1998).

The Pathogen *Pseudomonas syringae* pv. *syringae*

The bacterium *Pseudomonas syringae* pv. *syringae* [van Hall 1902](Pss) originally isolated from lilac (*Syringa vulgaris* L.), is a common plant pathogen found throughout the world on widely diverse hosts (Bradbury, 1986; Young, 1992; Georgakopoulos & Sands, 1992; Martinez-Miller & Braun, 1997). The disease symptoms associated with the pathogen are of two types. One symptom is known as bacterial canker or dieback on stone fruits

(cherry, apricot, plum) and citrus trees and the other symptom distinguishes foliar or blossom blights (brown spots) on legumes (snap beans, peas, etc.), tomato, wheat, apple and pear (Klement et al., 1990; Goto, 1992; Sigee, 1993). The one-celled bacteria are Gram-negative, straight or curved rods, 0.5-0.7 x 1.5-3.0 μm in size. Cells are motile with one to seven polar flagella and the mol% G+C of the DNA ranges from 58-71. They are catalase-positive and strict aerobes (Palleroni, 1984). A characteristic feature of all *P. syringae* is the production of fluorescent pigments on media devoid of iron (Hildebrand et al., 1988). Species identification is mainly based on levan production on 5 % sucrose medium, oxidase reaction, pectolytic activity on potato slices or pectate gel, arginine dihydrolase activity, and hypersensitivity reaction (HR) on tobacco leaves (LOPAT) (Klement et al., 1990). While most members of the species are variable in the levan reaction, they are oxidase-negative, arginine dihydrolase-negative, non-pectolytic and HR-positive. The species comprises many pathovars on the infrasubspecific level based on distinctive pathogenicity to one or more plant host species (Dye et al., 1980). Pathovar identification is difficult and relies on host specificity as well as nutritional, serological, plasmid, phage, protein, and fatty acid profiles (Klement et al., 1990) and toxin analysis (Gross, 1991; Quigley & Gross, 1994). Currently, the *P. syringae* species consists of 51 pathovars (Young et al., 1996). Hildebrand et al. (1988) identified strains belonging to the pathovar *syringae* based on biochemical characters, since members are positive in trigonelline, quinate and L-lactate utilization, while strains of other pathovars are variable (*pisi*) or negative in some of these traits (*coronafaciens*, *striafaciens*).

Furthermore, identification can be based on commercially available kits, such as the Biolog GN MicroPlate™ system (Biolog, Inc., Hayward, CA). The system provides a standardized method using biochemical tests to identify a broad range of enteric, non-fermenting, and fastidious Gram-negative bacteria. Biolog's MicroLog™ computer software identifies the bacterium from its metabolic pattern in the GN MicroPlate, which is based on the utilization (oxidation) of 95 different carbon sources. Another commercially available source is the Microbial Identification System (MIDI, Microbial ID Inc., Newark, Delaware) which identifies microbes based on their cellular fatty acid compositions, using fatty acid methyl esters of whole cells and high resolution gas chromatography.

Pathogenesis and Population Dynamics of *P. s.* pv. *syringae* (Pss)

In bacterial pathogenesis the infection process can be divided into three phases: (1) migration or attachment to the host (=contact), (2) recognition, and (3) invasion and establishment. These phases often occur in continuity and are difficult to separate (Goto, 1992; Sigee, 1993). For *P. syringae* motility is accomplished by flagella and motile bacteria frequently have an invasive advantage over nonmotile strains, increasing leaf infection (Panopoulos & Schroth, 1974). However, loss in motility did not result in loss of virulence, as inside the leaf inherent differences between motile and nonmotile isolates in virulence were less apparent and bacterial cells in diseased plant tissue were generally nonmotile (Goto, 1992). Furthermore, Chet et al. (1973) observed that *P. syringae* pv. *lachrymans* was capable of following a non host-specific chemical gradient presented by solutions of sugars, amino acids, guttation fluids, etc. However, there was no conclusive evidence that

chemotaxis was required for plant pathogenic bacteria to enter into stomata or wounds. Instead, the process of invasion was substantially affected by the mode of dispersal and sources of inoculum (Goto, 1992), which are for *P. syringae* pathovars aerosols, wind, dew, fog, rain, as well as seeds, plant debris, dormant tissues, and nonhost plants (Hirano & Upper, 1983). Contact (attachment) between host and pathogen was believed to be established when components of bacterial cell walls, such as lipopolysaccharides (LPS - O-antigens) bind to receptor sites on host cell surfaces, such as lectins or other glycoproteins with carbohydrate-binding sites (Sequeira, 1978; Sigee, 1993). Romantschuk et al. (1993) and Suoniemi et al. (1995) studied the attachment of *P. syringae* pathovars to host and non-host plant surfaces as an epiphytic fitness factor. The attachment of phage-resistant non-piliated mutants to leaf surfaces was lowered by a factor between two and ten compared to the piliated parental strains, but pilus-mediated adhesion was not host-specific. Piliated strains adsorbed well to both host and non-host plants (Romantschuk et al., 1993). The ability of the piliation-mutants to cause symptoms when infiltrated into bean leaf tissue was unaffected, but in spray-inoculation experiments the presence of pili correlated positively with frequency of infection (Romantschuk & Bamford, 1986). However, when piliated and non-piliated bacteria were inoculated together as mixtures, the initial epiphytic cell density of each bacterium was similar to that of the separately inoculated plants and the wild-type strain did not have an advantage over the mutant during undisturbed epiphytic growth, indicating that epiphytic growth per se was not dependent on piliation (Suoniemi, et al., 1995). Furthermore, adherence of *Pseudomonas* species to plant cell surfaces inside and outside the plant or other surfaces was mediated by capsular extracellular polysaccharides

(EPS) and biofilms (Smith & Mansfield, 1982; Costerton, 1987; Sigee, 1993; Suoniemi et al., 1995). In addition, EPS caused water-soaking in susceptible cultivars resulting in bacterial multiplication in compatible interactions (El-Banoby & Rudolph, 1979). Many studies have linked free water and high relative humidity to increased epiphytic *P. syringae* populations on plant surfaces and to a subsequent successful plant invasion by improving migratory pathways through natural openings (stomata, hydathodes), through surface wounds or breaks in fragile trichomes (Hirano & Upper, 1983; Goto, 1992; Sigee, 1993). Recognition in the plant-pathogen interaction either resulted in compatibility (disease) or incompatibility (resistance = HR reaction), which will be discussed in more detail below.

With respect to *P. syringae* population dynamics, ecology and epidemiology the tremendous work by Hirano & Upper (1983, 1990) should be acknowledged. They recognized the significance of *P. syringae* as an epiphyte living on plant surfaces of host and nonhost plants as potential inoculum sources for several diseases. In addition, they reported on host and cultivar preference for some genotypes of *P. s.* pv. *syringae* (Hirano & Upper, 1990) and emphasized that its population size at any given time on any given leaf was a function of growth, death, immigration and emigration (Hirano & Upper, 1983, 1990; Kinkel, 1997). Furthermore, they agreed that the amount of disease in a field was directly related to pathogen population sizes on individual leaves within a canopy (Hirano & Upper, 1990) based on earlier studies in which disease incidence could be predicted by the frequency with which pathogen populations exceeded approx. 10^4 cfu per gram of symptomless leaflets (Lindemann et al., 1984).The authors also speculated that the phenotypic and genotypic variability within the species probably allows many populations

to persist under changing environmental conditions, growth and death cycles, immigration and emigration processes in an open system, finally leading to ecosystem stability and evolution based on fitness (Hirano & Upper, 1990).

Another important mechanism of plant pathogenesis in *P. s.* pv. *syringae* is the production of necrosis inducing-toxins, such as syringomycin, its amino acid derivatives, syringotoxin, syringostatin (Gross & Cody, 1985; Gross, 1991), syringopeptin (Ballio et al., 1991; Iacobellis et al., 1992), and pseudomycin (Ballio et al., 1994). Necrosis-toxin production appears limited in *P. syringae* pathovars and has only been described in pathovar *syringae* (Gross, 1991; Quigley & Gross, 1994) and recently in pathovar *atrofaciens* (Braun & Sands, 1995; Vassilev et al., 1996). Most pathogenic strains produced one or more of these low molecular weight lipopeptide antibiotics, which were not host specific but biocidal to a wide spectrum of microbes (Gross, 1991; Iacobellis et al., 1992). Although not exclusively necessary for pathogenicity, syringomycin contributed to virulence in several diseases caused by different ecotypes of *P. s.* pv. *syringae* (Xu & Gross, 1988; Mo & Gross, 1991 a; Gross, 1991). The primary mode of action of syringomycin was determined to be membrane disruption by forming ion channels, releasing monovalent and divalent cations, leading to uncontrolled ion transport across the plasmalemma and finally cell death (Gross & Cody, 1985; Hutchison et al., 1995). Furthermore, syringomycin was found to have biosurfactant activity, lowering the surface tension of water (Hutchison et al., 1995). It also stimulated ATPase activity and uncoupled the oxidative phosphorylation in maize mitochondria of resistant and susceptible lines (Gross & Cody, 1985). Toxin production was reported to be regulated by nutritional and environmental factors, similar to other secondary

metabolites (Gross & Cody, 1985; Gross, 1991). Low phosphate concentrations (<1 mM), moderate temperatures (< 28 C), a pH near neutrality and high iron concentrations were conducive for toxin production (Gross, 1985). It was calculated that between 4-40 ng of iron were needed to produce 50 units of syringomycin, which was the threshold concentration at which symptoms became visible in maize (Gross, 1985). The magnitude of iron required for syringomycin synthesis (>2μM of Fe^{3+} for max. expression; Mo & Gross, 1991 a) and the unavailability of iron to microbes in their environments suggested that *P. s.* pv. *syringae* acquired iron efficiently by the production of its fluorescent siderophore $pyoverdine_{Pss}$ (Gross & Cody, 1984). Research also demonstrated that plant signal molecules, such as arbutin, salicin and other phenolic glucosides activated toxin gene expression, resulting in an activation of phytotoxin production (Mo & Gross, 1991 b; Quigley & Gross, 1994). Moreover, the analysis of *syrB* and *syrC* genes indicated that the gene products function as peptide synthetases in a thiotemplate multienzyme mechanism of syringomycin biosynthesis, which involves non-ribosomal post-translational peptide chain modifications (Zhang et al., 1995).

In relation to recognition, pathogenicity and host range, the discovery of hypersensitivity reaction and pathogenicity genes (*hrp*), their regulation, function, and interaction with other genes, for instance avirulence (*avr*) genes or gene products, may be one of the most important recent findings in phytopathogenic bacteria (Lindgren, 1997). While *avr* genes limit the host range (race/cultivar specificity), they have been found to improve fitness and therefore confer a selective advantage on their host bacteria (Vivian & Gibbon, 1997). *Hrp* genes control the ability of phytopathogenic bacteria to cause disease

on susceptible cultivars and to elicit HR-reactions on resistant cultivars or nonhost plants (Vivian & Gibbon, 1997). Recent studies have demonstrated that Hrp proteins are components of Type III secretion systems, regulatory proteins, proteinaceous elicitors of the HR, and enzymes needed for synthesis of periplasmic β-1,2-glucans (Alfano & Collmer, 1996; Lindgren, 1997). The Type III secretion system is involved in the secretion of pathogenicity proteins across the inner and outer membranes in Gram-negative bacteria and the delivery of such proteins directly into host cells (Alfano & Collmer, 1996; Vivian & Gibbon, 1997). Furthermore, the transcriptional activation of a number of bacterial avirulence (*avr*) genes is controlled by Hrp regulatory proteins, and data suggest that Avr proteins may be transported by Hrp secretion systems directly into plant cells. Thus, avirulence genes may after all be virulence factors, and plants may have evolved to recognize the virulence factors of the pathogen and to localize the damage inflicted by way of a programmed cell-death (HR) (Taylor, 1996; Vivian & Gibbon, 1997). Interesting was also the discovery of the conservation of Type III secretion systems in animal pathogens (*Yersinia, Shigella, Salmonella*) and plant pathogens (*P. syringae*, *P. solanacearum*, *E. amylovora*, *X. c. vesicatoria*), indicating that fundamental determinants of pathogenicity may be linked or have evolved from a common ancestor in completely different pathogens (Alfano & Collmer, 1996; Vivian & Gibbon, 1997; Finlay & Falkow, 1997)

In addition to *hrp*- and *avr*-genes, *tox*-genes (Gross, 1991) and the *lemA/gacA* regulon (= a two-component sensory signal transduction system) (Rich et al., 1992; Barta et al., 1992; Rich et al., 1994) also represent genes encoding proteins that transcriptionally regulate the expression of pathogenicity/virulence factors which contribute to disease and

symptom development in *P. syringae*. LemA serves as a membrane-bound sensor kinase that, in response to external environmental signals, activates the cytoplasmic GacA by phosphorylation. Activated GacA in turn stimulates the expression of several genes including toxin genes, thus leading to the production of syringomycin, in *P. s.* pv. *syringae* (Rich et al., 1994) or tabtoxin in *P. s.* pv. *coronafaciens* (Barta et al., 1992). Although *hrp*- and *avr*-gene regulation seemed to be linked, it is not clear whether virulence factors may be controlled simultaneously by several regulators and/or several regulatory systems can regulate a single virulence factor (Finlay & Falkow, 1997). However, multiple mechanisms have evidently evolved within phytopathogenic bacteria for the secretion of pathogenicity/ virulence factors (Lindgren, 1997). The multiplicity of such factors may allow the bacterium to encounter host resistance as well as to tolerate mutations in one or more virulence genes without severe loss of pathogenicity. Thus, mutations in only one of these genes generally reduces rather than abolishes pathogenicity (Vivian & Gibbon, 1997).

In conclusion, despite their interkingdom distinctness (e.g. plant and animal pathogens, prokaryotes and eukaryotes), the systems seem to have common/conserved themes with regard to microbial pathogenicity (Finlay & Falkow, 1997). These include virulence factors and their regulation (Rahme et al., 1995), resistance mechanisms, such as programmed cell-death systems and signal transduction pathways (Baker et al., 1997), and protein secretion systems (Alfano & Collmer, 1996; Baker et al., 1997). These findings may provide new areas of research, since new classes of broad-spectrum compounds (e.g. inhibitors of conserved bacterial regulatory systems) may be developed or transferred from one system to the other with respect to disease control, once the interconnections between

regulatory systems have been elucidated. Accordingly, resistance to common virulence factors might be transferred from plant to animal pathogens, or vice versa.

Phenotypic and Genetic Diversity of *P. s.* pv. *syringae*

Over the last decade, nucleic acids research has become of increasing importance for the taxonomic identification and classification of bacteria, especially at the pathovar and strain level. In addition, DNA analyses provided information on the assessment of genetic diversity and phylogenetic relationships among bacterial populations and individuals within a population. The heterogeneity of *P. syringae* strains within and between pathovars has been described using both phenotypic (Sands et al., 1970; Endert & Ritchie, 1984; Hirano & Upper, 1985; Roos & Hattingh, 1987; Denny, 1988) or genetic characters (Denny et al., 1988; Cooksey & Graham, 1989; Louws et al., 1994) or a combination of both (Hendson et al., 1992; Liang et al., 1994). Most authors agreed that strains within the pathovar *syringae* represented very diverse populations while strains of pathovars *tomato* (Denny et al., 1988) or *morsprunorum* (Endert & Ritchie, 1984; Liang et al., 1994) were more host-specific and more similar in their genetic structure. The level of genetic diversity in *P. s.* pv. *syringae* supported the hypothesis that variation was higher among strains from diverse habitats (e.g. wide host range Pss) than among those from a uniform habitat (e.g. *P. s.* pv. *tomato* from tomato) (Denny et al., 1988; Cooksey & Graham, 1989). However, Hendson et al. (1992) found no distinctive profiles that clearly differentiated strains of different pathovars (*tomato*, *maculicola*, *antirrhini*), which were isolated from different hosts but exhibited similar nutritional properties and overlapping host ranges. Genetically they all belonged to a single

group with several subgroups, suggesting a common ancestry for these strains. They concluded that nutritional characterization was a more reliable indicator of genetic relationships among strains than the host of origin. Furthermore, the nutritional versatility of Pss was thought to be responsible for the fast adaptation and survival on a multiplicity of plants, including hosts and nonhosts (Hildebrand et al., 1988; Hendson et al., 1992). The recovery of bean strains of Pss from different locations with an identical haplotype, and different haplotypes from within one region, was reported to be attributed to the introduction of the pathogen on bean seed into the areas, since the pathogen is seed-borne and companies distribute seeds throughout the US (Legard et al., 1993). Due to rapid generation times of approximately 3.5 to 4.5 hours after rain events, Pss populations on snap beans had the potential to evolve quickly (Hirano & Upper, 1985). The authors hypothesized that if selection among genotypes (i. e., strains) occurs in each generation, the possibility of several generations per day provides opportunity for amplification of small changes per generation, resulting in large population differences in a relatively short period. The ecosystem in which Pss was an integral component was variable and dynamic and so was the number of Pss genotypes present (Hirano & Upper, 1985).

Although, most studies employed restriction fragment-length polymorphism (RFLP) and DNA-hybridization for the differentiation of *Pseudomonas syringae* between and within pathovars (Denny et al., 1988; Hendson et al., 1992; Legard et al., 1993; Quigley & Gross, 1994; Martinez-Miller, 1994), similar results were obtained with genomic fingerprinting. Rare-cutting restriction endonucleases generated distinctive patterns of large DNA fragments which could be separated by field inversion (Cooksey & Graham, 1989) or pulsed-

field gel electrophoresis (Grothues & Tümmler, 1991). Such macrorestriction fingerprints of the entire bacterial genome were shown to be more sensitive to mutations, including deletions and insertions at a specific restriction site than RFLP analysis with only one DNA-hybridization probe, thus changing the genome profile and exposing polymorphism. The methods separated *Pseudomonas* species (Grotheus & Tümmler,1991) and classified *P. s.* pv. *tomato* to be distinct from other pathovars of *P. syringae* (Cooksey & Graham, 1989). Recently, PCR-RFLP analysis of rRNA operons and rep-PCR-based amplification of variable DNA regions between conserved bacterial repetitive elements have resulted in the rapid and reproducible identification of plant pathogenic bacteria at the species, pathovar and even strain level (Manceau & Horvais, 1997; Louws et al., 1994). Alternatively, Bragard et al. (1997) employed AFLP (Amplified Fragment Length Polymorphism) DNA fingerprinting to characterize 68 xanthomonad strains from small grains and found the results to be consistent with pathogenicity tests and RFLP analysis, recommending AFLP as a method of choice not only for taxonomy, but also for epidemiological studies. Although each of the above mentioned DNA-techniques alone provided some information on whether different isolates represented a single evolutionary line or were composed of several lines that had converged to a similar pathogenic phenotype, the combination of biochemical, genetic and pathological analyses was recommended for the assessment of phylogeny and evolution of strains in relation to the host plant (Denny, 1988; Hendson et al., 1992). In addition, strains of the same geographical origin and host cultivar should be investigated to study genetic diversity and evolutionary relationships within a field population. Accordingly, the variation of the bacterial rice blight pathogen *Xanthomonas oryzae* pv. *oryzae* was

investigated in only one field, on only one cultivar in the Philippines (Vera Cruz et al., 1996). This analysis, done at the micro geographic scale, provided valuable information on the effects of host resistance on the emergence of new virulent races in the population structure of this pathogen.

The Control of Basal Kernel Blight

Due to the apparent ubiquitous distribution and dissemination of *P. s.* pv. *syringae* on nonhost plants in barley growing areas, which resembled the distribution of Pss in bean growing areas in Wisconsin (Hirano & Upper, 1983), the elimination of the pathogen from seeds and foliage of the host may not be sufficient to control the basal kernel blight disease. In addition, the use of bactericides and antibiotics on cereal grains has not been considered practical. Hence, the only control for barley kernel blight suggested to date has been the avoidance of irrigation during the most susceptible kernel developmental stage at soft dough and the use of less susceptible cultivars (Martinez-Miller et al., 1998). Over the last three years we have evaluated a biological control system based on the application of indigenous antagonistic *Pantoea agglomerans* bacteria prior to the *P. s. syringae* infection window to reduce the kernel blight disease incidence (Braun et al., 1996; Braun et al., 1997). Furthermore, the use of biological control agents to control plant diseases, especially with respect to integrated pest management strategies, has become more practical and acceptable in the United States in the last decade, despite its lack of funding (Cook, 1993; Jacobsen, 1997). IPM strategies, including the commercial use of biological control agents, is of particular significance in minor crops or major crops in which no chemicals are available

to control certain pests or diseases; an example being the IR-4 program, a national project promoting pest control products for minor crops. Market studies indicated a growth rate of 10 % per annum for biocontrol-based crop protection. It is predicted that bio-control products will capture a $2-3 billion market share of the $20-30 billion pesticide market at the producer's level worldwide by the turn of the century (Menn et al., 1993).

The Antagonist *Pantoea agglomerans*

Taxonomically, *Pantoea agglomerans* belongs to the family Enterobacteriaceae. The genus *Pantoea* was created in 1989 by Gavini et al. (1989) based on DNA-DNA hybridization and protein electrophoresis. It included two species, *Pantoea agglomerans* [syn. *Enterobacter agglomerans* (Beijerinck 1888) Ewing & Fife 1972; syn. *Erwinia herbicola* (Löhnis 1911) Dye 1964; syn. *Erwinia milletiae* (Kawakami & Yoshida 1920) Magrou 1937] and *Pantoea dispersa*. *P. agglomerans* is frequently referred to by its synonyms, *Enterobacter agglomerans* (Ewing & Fife, 1972) by human pathologists when isolated as clinical isolates from animal or human sources or *Erwinia herbicola* by phytopathologists when isolated from plant sources (Holt et al., 1994). *E. herbicola* exists as an epiphyte on plant surfaces and as a saprophytic secondary organism in lesions caused by many plant pathogens (Dye, 1983; Lelliot & Dickey, 1984). Some strains were reported to cause galls on table beets (Burr et al., 1991), black spot necrosis on beach peas in Canada (Khetmalas et al., 1996), or damage to garlic crops (Koch et al., 1996). *Erwinia herbicola* pv. *gypsophilae* induced gall formation on *Gypsophila paniculata* (Young et al., 1991).

Because the name *Pantoea agglomerans* has finally entered Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and has been approved by the International Society for Plant Pathology (Young et al., 1996), it will be used throughout this report.

The Use of *P. agglomerans* as Biological Control Agent (BCA)

Pantoea agglomerans has been used in several studies to control fungal as well as bacterial plant pathogens. For instance, Kempf (1988) employed *E. herbicola* bacteria to control *Fusarium culmorum* and *Puccinia recondita* f. sp. *tritici* on wheat. He found about 50-60% *F. culmorum* disease suppression on wheat seedlings in the greenhouse after seed coating with *E. herbicola* isolate B247 based on shoot and root dry weight determinations. Furthermore, 78 % disease suppression of *P. recondita* f. sp. *tritici* was revealed when strain B247 was sprayed on wheat leaves two hours prior to uredospore application. Adetuyi (1992), also working on the antagonistic potential of *E. herbicola* against soilborne pathogens on wheat, discovered that antagonists coated onto seeds protected them from attack of *Fusarium nivale* and *Pythium ultimum*. Rate of emergence, stand counts, average root and shoot height were significantly higher in bacterial treated seedlings than in untreated controls, suggesting protection as well as plant growth promoting effects. A plant growth promoting effect of *P. agglomerans* strain D5/23 isolated from the phyllosphere of winter wheat was also noted by Ruppel et al. (1989). The authors detected nitrogenase- and phytohormone activity in these plant associative bacteria in addition to wheat and barley yield increases of up to 500 kg/ha in field experiments (Ruppel et al., 1989; Scholz-Seidel & Ruppel, 1992). Rosales et al. (1993) isolated 23 bacterial strains including one *E.*

herbicola isolate from rice fields in the tropics and tested their effect on germination, seedling development and inhibition of mycelial growth of *Rhizoctonia solani*. All strains inhibited *Rhizoctonia solani in vitro*, however *Bacillus subtilis* isolates performed the best. Seed bacterization provided a sheath blight protection of 4.5 to 73% in a glasshouse trial depending on the isolate screened. Yuen et al. (1994), who studied the inhibition of *Sclerotinia sclerotiorum* on dry edible beans by *E. herbicola*, noticed that strains applied to beans at fully expanded, mature blossom stage prior to inoculation with ascospores of the pathogen, inhibited ascospore germination and subsequent development of white mold lesions in a bioassay, while field applications were less effective due to unfavorable weather conditions for the antagonists. Furthermore, *E. herbicola* was reported to control *Alternaria solani* on tomato (Sujkowski et al., 1994), *Xanthomonas campestris* on onions (Paulraj et al., 1993) and *Verticillium dahliae* in an *in vitro* assay (Berg & Ballin, 1994).

Another often cited example of excellent biocontrol performance of *Erwinia herbicola* is the fire blight system in apples and pears. In research apple orchards, *E. herbicola* has been demonstrated to be an effective antagonist of *Erwinia amylovora* (Beer et al., 1984 a). The protection from fire blight achieved by spraying apple blossoms with *E. herbicola* one day prior to *E. amylovora* was often equivalent (42-51%) to the protection achieved by spraying streptomycin (61%), the most commonly used control agent in North America. Isenbeck & Schulz (1985) isolated several *E. herbicola* strains from blighted ornamentals in Germany that showed antagonistic activity against *E. amylovora in vitro*. Eh 112 y, an isolate obtained from Cornell University demonstrated effective disease reduction in an immature pear fruit assay and under controlled conditions using *Cotoneaster bullatus*

as a test plant if the antagonist was injected 24 h ahead of the pathogen. The antagonist treatment in fact was as effective as the 200 ppm streptomycin control. The authors reported 20-40% fire blight disease reductions under field conditions following natural infection. Vanneste (1990) studied the control of fire blight by *E. herbicola* strain Eh252 in an experimental orchard in France and got 40% disease control compared to the untreated control. Furthermore, Wilson et al. (1990) demonstrated fire blight protection of hawthorn with *E. herbicola* under controlled conditions in the United Kingdom. In New Zealand an *E. herbicola* strain was sprayed on apple blossoms in the orchard and prevented fire blight disease in apple blossom in glasshouse trials (Kearns & Hale, 1993). Field trials conducted in 1991 and 1992 in Oregon and 1992 in Washington revealed a 87.5% fire blight disease reduction in pear blossoms in Oregon 1991 compared to water controls. In 1992 the disease was reduced by 50% and 56% in Washington. However, the streptomycin control resulted in the best disease prevention over all years and locations with 98.8%, 95.5%, and 72.2% disease reduction, respectively (Johnson et al., 1993).

Another example of the engagement of *E. herbicola* in biocontrol was presented by Lindow (1983), when he successfully employed an antagonistic *E. herbicola* isolate (INA-minus) to control frost injury, caused by ice nucleation active (INA-plus) *P. s.* pv. *syringae* bacteria on corn in the field.

The Use of *P. agglomerans* in Systems other than Plant Pathology

In addition to the use of *P. agglomerans* as biocontrol agents in plant pathology, the bacterium and the compounds it produces have been exploited commercially. For instance,

its tyrosine phenol-lyase activity catalyzed the production of L-tyrosine and dihydroxyphenyl-L-alanine (L-dopa), two essential amino acids that can be synthesized from ammonia, pyruvate, and phenol or catechol, respectively (Lloyd-George & Chang, 1995). Furthermore, *P. agglomerans*' massive and fast gluconic acid production by glucose oxidation enhances mineral-phosphate solubility due to acid dissolution of hydroxyapatite (Liu et al., 1992). This is important, since the world's phosphate resources are limited and phosphate is present but unavailable in most soils, due to its structural insolubility, similar to iron.

Herbicolins were described as new acylpeptide antibiotics with antimycetic activity against dermatophytes in human medicine (Winkelmann & Adam, 1980). Furthermore, outer membrane lipopolysaccharides from *P. agglomerans* (LPSp) have been found to inhibit morphine (Okutomi et al., 1992) and cocaine dependent withdrawal signs (Suzuki et al., 1994), revealing their potential use in pharmacotherapy for the prevention of drug abuse. The administration of LPSp showed a marked inhibition of the jumping of mice on naloxone-precipitate withdrawal, however endogenous β-endorphin and tumor necrosis factor (TNF) levels were induced (Okutomi et al., 1992). In a second study, the treatment with LPSp abolished cocaine- induced locomotor enhancement in mice and the cocaine-induced place preference in rats, suggesting that while LPSp itself may possess neither reinforcing nor locomotor enhancing effects, it blocks both the reinforcing and the locomotor enhancing effects of cocaine (Suzuki et al., 1994). The authors claimed that immediately after LPSp administration, a cytokine cascade begins which produces TNF, followed by ACTH/β-LPH gene product, which generates β-endorphin as an endogenous

opioid over a period of 6 h after the injection. As a result, LPSp induced an antinociceptive effect. In 1994, Kamei and coworkers reported that subcutaneous injection of LPSp suppressed hyperalgesia in streptozotocin-induced diabetic mice and also exerted a therapeutic effect on hyperalgesia in these animals. The data indicated that LPSp may be effective in relieving the pain associated with diabetic neuropathy. Recently, a low molecular weight (5 kDa) LPSp from *P. agglomerans* has been recognized as potent antitumor agent in animal tumor models and its use in human cancer therapy is currently under investigation (Goto et al., 1996). Clinical application of LPSp administered intradermally in combination with chemotherapeutics such as cyclophosphamide appeared even more promising in terms of its antitumor effects, whereas pretreatment by anti-tumor necrosis factor antibody reduced the effect exerted by LPSp, indicating that induced TNF might play a crucial role in antitumor activity and the induction of cell mediated immunity (Iwamoto et al., 1996; Inagawa et al., 1997).

In contrast to these studies, a negative effect of endotoxins, associated with LPS from *P. agglomerans* and *Enterobacter spp.*, was found relevant to pathogenesis of alveolitis in organic dust-induced lung diseases (Milanowski et al., 1995). Wood dust from American basswood contained numerous Gram-negative bacteria, including *P. agglomerans*, that were shedding large amounts of the endotoxin-containing membrane vesicles, suggesting that these environmental "dust-borne" endotoxins represented an increased risk for people exposed to various organic dusts (Dutkiewicz et al., 1992).

Biological Control Mechanisms Associated with *P. agglomerans*

Nitrogen Depletion and Acidification

Nitrogen depletion and acidification of the environment have been reported in the interaction between *E. herbicola* and pathogenic bacteria such as *E. amylovora* or *Xanthomonas oryzae* in vitro (Riggle & Klos, 1972; Hsieh & Buddenhagen, 1974; Beer et al., 1984 b). However, organic acid production had never been demonstrated as a mechanism *in planta* (Gibbins, 1978; Wodzinski et al., 1994) and results of the N-depletion hypothesis were inconsistent (Wodzinski et al., 1987; Wodzinski et al., 1994).

Antibiosis

Since *P. agglomerans* are successful inhibitors of plant pathogens, much attention has been paid to the diversity and role of antimicrobial compounds produced by several strains. Herbicolins A and B were described as lipodepsinonapeptide antibiotics that inhibited sterol-requiring fungi (Winkelmann et al., 1980; Aydin et al., 1985). Antibiotic genes were cloned and transferred from *E. herbicola* to *E. coli*, resulting in expression of antifungal activity in *E. coli* (Tenning et al., 1993). Proteinaceous antibiotics that only kill strains of the same or closely related species to *E. herbicola* had been called bacteriocins (Beer & Rundle, 1980; Vidaver, 1983) or bacteriocin-like if they were non-proteinaceous in nature (Beer & Vidaver, 1978; Stein & Beer, 1980). All other compounds with a wider spectrum of activity, including more distantly related bacteria, were designated simply antibiotics. Their chemical structures, if known, mostly contained monocyclic and bicyclic

β-lactam molecules (Parker et al., 1982; Ishimaru et al., 1988; Bainton et al., 1992; Kearns & Hale, 1996), and as such interfered with the transpeptidation step in the cross-linking of amino acids that connect the glycan chains to form the peptidoglycan of bacterial cell walls. This inhibition of the final stage of murein synthesis somehow triggers the autolytic activities of murein hydrolases, resulting in cell lysis (Jacobs, 1997). Biosynthesis of β-lactam antibiotics in *E. carotovora* was shown to be regulated by cell density-dependent gene regulation processes and autoinducer signal molecules (Bainton et al., 1992; Chhabra, et al., 1993). Furthermore, pyrrolnitrin production by *E. herbicola* strain IC1270, usually produced by *Pseudomonas* species, was reported by Chernin et al. (1996), which generated broad spectrum antagonistic activity towards fungal and bacterial phytopathogens, including *P. s.* pv. *syringae*. Another *E. herbicola* antibiotic from strain Eh 252 shared DNA sequence homologies with microcin C7 from *E. coli*, which is a modified heptapeptide and inhibits translation in protein synthesis of *Enterobacteriaceae* (Vanneste & Yu, 1996). Finally, strain Eh 318 was reported to produce two antibiotics termed pantocin A and B, of which pantocin A's structure and function resembled microcin C7 and pantocin B remains to be sequenced and its mode of action determined (Wright, 1997; Wright & Beer, 1997). Studies on the role of *E. herbicola* antibiotics in biocontrol have demonstrated that non-antibiotic producing strains reduced fire blight as efficiently as antibiotic producing strains, indicating no *in-vivo* role for antibiotics (Beer et al., 1984; Wodzinski & Paulin, 1994). Similarly, Lindow (1988) found no correlation of *in vitro* antibiosis with antagonism of ice nucleation active bacteria on leaf surfaces by non-ice nucleation active bacteria. However, for strains Eh 252 (Vanneste et al., 1992) and Eh 318 (Wodzinski et al., 1994; Wright, 1997), effective