Etiology and characterization of two Pseudomonas syringae pathovars causing two bacterial kernel blights of barley
by Concepcion Martinez-Miller

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

Abstract:
Kernel blight of barley, an important disease for malting and brewing industry, exhibits two types of symptoms in Montana. The most common is basal kernel blight consisting of dark brown discoloration on the embryo end of the kernel. Spot kernel blight, on the other hand, has a well defined spot on the lemma of the kernel. Two different pathovars of Pseudomonas syringae were associated with the different Symptom types. The first group, P. s. pv. syringae, produced syringomycin and utilized L-Iactate and trigonelline. The second group, an undefined pathovar designated as P. syringae 554, did not produce syringomycin nor utilize L-Iactate or trigonelline. P. s. , pv. syringae strains were strongly associated with basal kernel blight while P. syringae 554 was associated with spot kernel blight. Strains were characterized by RFLP analysis using three different probes. A dendrogram showed that most of the P. s. pv. syringae strains were distributed in closely related clusters while P. syringae 554 strains were in a completely divergent clusteri Along with the genetic analysis of these two pathovars, aspects of disease development were also studied. A different window of infection was observed for the two types of blight. The window of infection for basal kernel blight was during late milk and dough stages while that of spot kernel blight was at early milk. High moisture was determined to be necessary during the. window of infection for successful development of the disease. Screening methods revealed that screening for resistance to kernel blight must be performed separately for each type of blight.

ETIOLOGY AND CHARACTERIZATION OF TWO *Pseudomonas syringae* PATHOVARS CAUSING TWO BACTERIAL KERNEL BLIGHTS OF BARLEY

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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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TABLE OF CONTENTS

TABLE OF CONTENTS (CONTINUED)

LIST OF TABLES

LIST OF TABLES (CONTINUED)

LIST OF FIGURES

LIST OF FIGURES (CONTINUED)

LIST OF FIGURES (CONTINUED)

ABSTRACT

Kernel blight of barley, an important disease for malting and brewing industry, exhibits two types of symptoms in Montana. The most common is basal kernel blight consisting of dark brown discoloration on the embryo end of the kernel. Spot kernel blight, on the other hand, has a well defined spot on the lemma of the kernel. Two different pathovars of *Pseudomonas syringae* were associated with the different symptom types. The first group, *P. s.* pv. *syringae*, produced syringomycin and utilized L-lactate and trigonelline. The second group, an undefined pathovar designated as *P. syringae* 554, did not produce syringomycin nor utilize L-lactate or trigonelline. *P. s.* pv. *syringae* strains were strongly associated with basal kernel blight while *P. syringae* 554 was associated with spot kernel blight. Strains were characterized by RFLP analysis using three different probes. A dendrogram showed that most of the *P. s.* pv. *syringae* strains were distributed in closely related clusters while *P. syringae* 554 strains were in a completely divergent cluster. Along with the genetic analysis of these two pathovars, aspects of disease development were also studied. A different window of infection was observed for the two types of blight. The window of infection for basal kernel blight was during late milk and dough stages while that of spot kernel blight was at early milk. High moisture was determined to be necessary during the window of infection for successful development of the disease. Screening methods revealed that screening for resistance to kernel blight must be performed separately for each type of blight.

CHAPTER 1

LITERATURE REVIEW

Kernel Blight of Barley.

Importance of the Disease. 'Kernel blight' and 'kernel discoloration' have been used to describe a barley disease of importance to the malting and brewing industries. Both will be referred to as kernel blight in this study. Since malting is a germination and growth process, and good malt can only be obtained by rapid and uniform germinating barley, any reduction in germination produced by diseased kernels will ultimately result in lower brew-house extract and may adversely affect quality of the final product (Burger and LaBerge, 1985). Microbial infection of kernels can also affect the brewing process by excretion of substances such as germination inhibitors which, besides affecting the malting process, also produce off-flavor metabolites that carry over to the beer (Burger and LaBergue, 1985). Another brewing problem known as gushing involves the overfoaming of beer caused by a sudden release of CO_2 when packed beer is opened which seems to be directly attributable to microbial infection of barley seed (Burger and LaBergue, 1985). Severely affected malting barley can be downgraded or rejected by the industry. It can then only

be sold for animal feed resulting in large economic losses for growers. Kernel blight is commonly present in the upper Midwestern region of the United States and has recently become a problem in the state of Idaho and in the North central part of Montana, varying in frequency and severity from year to year. The Northcentral part of Montana includes a large region where malting barley is one of the primary crops and in some years such as 1989, the disease caused large economic losses.

Disease Description and Causal Agents. Kernel blight in Montana has been characterized by the appearance of two symptom types. The most common type consists of a dark brown discoloration of the embryo end of the kernel (hereafter referred to as **basal kernel blight**) (Fig. 1a). This type of symptom has been termed 'black point' in wheat, barley and rye (Machaceck and Greaney, 1938) and is also known as 'kernel discoloration' for barley (Anderson and Banttari, 1976). *Alternaria* sp. and *Cochliobolus sativus* (Ito and Korib) have been reported as the most common causal agents of black point (Machaceck and Greaney, 1938) with *C. sativus* as the primary causal agent of kernel discoloration in barley in the midwest (Anderson and Banttari, 1976). Up to now no one has reported bacteria as one of the causal agents of this type of symptom.

The second type of blight, reportedly caused by the bacterium *Pseudomonas syringae* pv. *syringae* (Peters *et al.*,

1983), occurs more sporadically and is characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel (hereafter referred to as **spot kernel blight**) (Fig. 1b). This symptom type is called 'bacterial kernel spot of barley' and was first observed in large quantities on sprinkler irrigated "Klages" barley in southern Idaho in 1977 (Peters *et al.*, 1983). Spot kernel blight has been observed to develop mainly in two-row barley cultivars rather than in six-row cultivars (personal communication Bob Miller, 1993 and Darryl Wesenberg, 1994) but the reason for this phenomenon is unknown. A very similar symptom to spot kernel blight has been reported to occur in wheat, barley, rye and grasses and is called "basal glume rot" reportedly caused by *P. syringae* pv. *atrofaciens* McCulloch 1920 (Toben *et al.*, 1989).

The Bacterium *Pseudomonas syringae* van Hall 1902.

One of the main groups of phytopathogenic pseudomonads are those that produce diffusible fluorescent pigments, particularly in iron-deficient media such as King's B (King *et al.*, 1954). Strains in this group usually do not accumulate poly-β-hydroxybutyrate (PHB) and do not utilize D-arabinose (Hildebrand *et al.*, 1988). *Pseudomonas syringae* belongs to this group, and it is characterized as a gram-negative rod of *ca.* 0.7 to 1.2 by 1.5 to 3 μm, occurring singly or in long chains or filaments. It is an obligate aerobe and is motile with polar multitrichous flagella.

Cultures of most strains produce slime in media containing two to four percent sucrose as a result of levan formation. Cytochrome *c* is not detectable and it is arginine dehydrolase negative. Optimal temperature for growth is *ca.* 25 to 30 C with no growth at 41 C. *P. syringae* is found on various plants and almost all strains produce a hypersensitive reaction in tobacco. The G + C content of the DNA is *ca.* 59-61 mole percent (Buchanan and Gibbons, 1974; Hildebrand *et al.*, 1988).

Toxin Production. Plant pathogenic bacteria are equipped with an arsenal of virulence factors that condition the host for colonization as well as being involved in symptom expression. Nonhost-specific phytotoxins are generally acknowledged to be an element of virulence for many bacteria and this is especially evident among the pathovars of *P. syringae* (Gross, 1991). *P. syringae* represents a wide range of plant associated bacteria, including pathogens, weak pathogens or saprophytes. The nonhost-specific phytotoxins produced by pathovars of this bacterium are a family of structurally diverse compounds, usually peptide in nature, that in some cases display a wide-spectrum of antibiotic activity (Mitchell, 1981). Several structurally distinct classes of toxins that cause either chlorotic or necrotic symptoms in infected plant tissue are known to be produced by pathovars of *P. syringae* (Gross 1991). The chlorosis-inducing toxins are the most

common and are classified broadly as either tabtoxin (Barta *et al.*, 1992), phaseolotoxin (Staskawicz and Panopoulos, 1979), coronatine (Palmer and Bender, 1993; Young *et al.*, 1992) or tagetitoxin (Mitchell *et al.*, 1989). The necrosis-inducing toxins seem to be restricted to the pathovar *syringae* and encompass a family of structurally related lipopeptides including, syringomycin (Bidwai and Takemoto, 1987; Fukuchi *et al.*, 1990; Gross, 1991; Iacobellis *et al.*, 1992;), syringotoxin (Ballio *et al.*, 1990), syringopeptin (Ballio *et al.*, 1991; Iacobellis *et al.*, 1992), and pseudomycins (Harrison *et al.*, 1991). This discussion will be limited to syringomycin due to the apparent association of syringomycin producing strains with kernel blight.

Syringomycin Production by *P. s.* pv. *syringae*. Toxin synthesis and secretion are prevalent among the pathovars of *P. s.* pv. *syringae*, suggesting that strong selective pressure for toxigenic strains exists in the plant environment (Gross, 1991). One of these toxins, syringomycin, is the most common and most studied. Syringomycin is a lipodepsinopeptide consisting of a macrocyclic ring of nine amino acids with a fatty acid side chain. Three of the amino acids are uncommon: 4-chlorothreonine, 3-hydroxyaspartic acid, and 2,3-dehydrothreonine (Serge *et al.*, 1989; Fukuchi *et al.*, 1990). Although the biosynthetic pathway for syringomycin production has not been elucidated, it is expected to be

complex due to the unusual amino acids and the macrocyclic ring structure (Xu and Gross, 1988a; Hrabak and Willis, 1993). The mode of action of syringomycin is thought to involve increased plant cell uptake of calcium, resulting in disruption of ion transport across the plasmalemma and activation of a cascade of physiological events leading to cell death (Takemoto, 1992). Studies to determine syringomycin involvement in virulence or pathogenicity were performed by isolation of non-toxigenic mutants generated with the transposon Tn5. Mutants were quantitatively evaluated for their ability to multiply and cause disease in immature sweet-cherry fruits (Xu and Gross, 1988a). Results of these studies indicate that syringomycin is not essential for pathogenicity, but significantly contributes to virulence (Xu and Gross, 1988a). Following the genetic characterization of syringomycin production by *P. s.* pv. *syringae*, Xu and Gross (1988b) reported the existence of two genes, *syrA* and *syrB*, that are involved in syringomycin production and are required for the formation of proteins SR4 and SR5. These proteins are believed to be components of the syringomycin synthase complex. Another gene, *syrD* is thought to encode a protein that belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins (Quigley *et al.*, 1993). It is proposed that the *syrD* gene product is embedded in the bacterial cytoplasmic membrane and functions as an ATP-driven efflux pump for the secretion of

syringomycin (Quigley and Gross, 1994). The *lemA* gene has also been reported to be involved in production of syringomycin (Hrabak and Willis, 1993).

Characterization of Phytopathogenic Bacteria.

Biochemical and physiological tests. More than one criterium is necessary for the genetic characterization of phytopathogenic bacteria such as *P. syringae*. The use of pathogenicity and biochemical tests are important in the systematics of phytopathogenic bacteria (Hildebrand *et al.*, 1988). Systems such as Biolog MicroStation™ (Biolog, Inc. Hayward, CA, 94545), based on the utilization of 95 carbon sources and fatty acid profiles, that combine cellular fatty acid analysis with computerized high resolution gas chromatography such as that performed by Microbial Identification Inc. (MIDI, Newark, DE) have been useful tools in the characterization of some pathogenic bacteria. Although these tests are useful in distinguishing most pathovars (Hildebrand *et al.*, 1988), they cannot distinguish strain differences within a given pathovar (Legard *et al.*, 1993).

RFLP Analysis. Combining biochemical and pathogenicity tests with analysis of restriction fragment length polymorphism (RFLP) of chromosomal DNA has become more and more useful in determining phylogenetic relationships among phytopathogenic bacteria. Hartung and Civerolo (1991)

utilized *in vitro* aggressiveness, RFLP analysis, and carbon source utilization profiles to study variation among strains of *Xanthomonas campestris* causing citrus bacterial spot. These studies showed that most aggressive strains of *X. campestris* belonged to a single RFLP group although members of this RFLP group varied in carbon source utilization (Hartung and Civerolo, 1991). The less aggressive strains comprised a continuum of RFLP types and usually could be separated from the more aggressive strains by carbon source utilization profiles. Similar results were obtained with *Xanthomonas campestris* pv. *citrumelo* Gabriel pv. Nov. using virulence tests, reaction to a panel of monoclonal antibodies and RFLP analysis. The highly aggressive strains of *Xanthomonas campestris* pv. *citrumelo* were correlated with RFLP and serological reaction patterns (Gottwald *et al.*, 1991). Similarly, races and biovars among *Pseudomonas solanacearum* strains could be distinguished by RFLP analysis (Cook *et al.*, 1989).

In the case of *P. syringae*, several studies have utilized RFLP analysis to differentiate strains both within and between pathovars, (Hendson *et al.* 1992; Legard *et al.*, 1993, Denny *et al.*, 1988; Quigley and Gross, 1994). Hendson *et al.*, (1992) compared strains of *P. syringae* pathovars *tomato*, *maculicola*, *antirrhini*, and *apii* by RFLP patterns, nutritional characteristics, host origin and host ranges. Results of this study indicated that phylogenetic

relationships cannot be assessed solely on the basis of pathogenicity, nutritional characteristics alone, or RFLP analysis alone. The combination of multiple criteria would provide better characterization of bacterial strains (Hendson *et al.*, 1992).

Genes involved in toxin production may be useful as probes for characterizing and identifying strains of *P. syringae* by RFLP analysis. Toxin synthesis and secretion are prevalent among the pathovars of *P. syringae*, and it appears that toxigenesis may reflect overall genetic differences (Hildebrand *et al.*, 1982; Gross, 1991). Pathovars of *P. syringae* that produce a particular toxin appear to constitute distinct taxonomic clusters that share a high degree of genomic DNA relatedness (Hildebrand, 1982; Denny *et al.*, 1988). For instance, genes required for phaseolotoxin production by *P. s.* pv. *phaseolicola* and coronatine production by *P. s.* pv. *tomato* and related pathovars have been used as specific probes for disease diagnosis (Cuppels *et al.*, 1990; Shaad *et al.*, 1989; Prosen *et al.*, 1993). Sections of the tabtoxin biosynthetic region have been used in studies on the mechanisms of toxin regulation, and as specific probes for tabtoxin-producing strains of *P. syringae* including pathovars *tabaci*, *coronafaciens*, and *syringae* (Kinscherf *et al.*, 1991). Quigley and Gross (1994) postulated that *syrB* and *syrD* genes encode proteins that function in the synthesis and export of

syringomycin, respectively, and are conserved among a broad spectrum of *P. s.* pv. *syringae* strains that produce syringomycin or one of its analogs, syringotoxin and/or syringostatin. These genes were absent in all *P. syringae* strains tested that did not produce syringomycin or syringomycin analogs (Quigley and Gross 1994). RFLP analyses were used to construct a dendrogram that revealed subclusters of strains that appear to share specific qualities relevant to plant-pathogen interactions (Quigley and Gross, 1994). A large proportion of the strains originally obtained from dicots exhibited the same RFLP profile while the few strains tested from monocots fell into two genetically distinct clusters that were quite divergent from the dicot strains (Quigley and Gross, 1994). Similarly, Denny *et al.* (1988) assessed the genetic diversity among a sampling of monocot and dicot strains of *P. s.* pv. *tomato* by RFLP analysis and found two clusters that were restricted to either monocot or dicot strains.

Another class of genes that appear useful in RFLP analysis to study genetic relationships among *P. syringae* strains are the *hrp* (hypersensitive reaction and pathogenicity) genes. The hypersensitive response (HR) is a rapid, localized necrosis of plant tissue that is observed when many phytopathogenic bacteria are inoculated into nonhost plant species or resistant varieties of susceptible plant species (Klement *et al.* 1990). Very little is known

about the biological mechanisms involved in this plant-microbe interaction, stimulating several studies on the genes, which have been designed *hrp*, that are involved in these mechanisms (Willis *et al.*, 1991). The *hrp* genes have been found in many phytopathogenic bacteria including *Pseudomonas solanacearum* (Boucher *et al.*, 1987), *Xanthomonas campestris* pathovars (Bonas *et al.*, 1991; Daniels *et al.*, 1988), *Erwinia amylovora* (Bauer and Beer, 1987) and several pathovars of *P. syringae* (Willis *et al.*, 1991). Additional studies have shown that the *hrp* genes are conserved with respect to homology and function within *P. syringae* pathovars. Liang *et al.* (1993) compared *hrp* gene restriction maps of *P. s.* pv. *morsprunorum*, *P. s.* pv. *phaseolicola* and *P. s.* pv. *syringae*. This comparison revealed that there are greater similarities in restriction sites between DNA from pathovars *phaseolicola* and *morsprunorum* than between DNA from pathovars *syringae* and *morsprunorum*. Relatedness of *hrp* genes has also been reported among other pathogenic bacteria (Arlat *et al.*, 1991). The use of low-stringency hybridization conditions revealed hybridization between the chromosomal inserts within pCPP430 and pHIR11, which contain the *hrp* clusters from *Erwinia amylovora* and *P. s.* pv. *syringae* (Laby and Beer, 1992). It has also been reported that predicted protein sequences of three *hrp* genes from *P. solanacearum* show remarkable sequence similarity to key virulence

determinants of animal pathogenic bacteria of the genus *Yersenia* (Cough *et al.*, 1992). Similarly, Huang *et al.* (1993) characterized the *Pseudomonas syringae* pv. *syingae* 61 *hrpJ* and *hrpI* genes and the predicted encoded proteins and found that HrpI belongs to a superfamily of proteins that are found in *Yersenia petis* Lcr D.

In *P. s.* pv. *syringae* the *hrp* genes were identified by obtaining mutants of *P. s.* pv. *syringae* strain 61 that failed to induce HR in tobacco. A cosmid clone pHIR11 which had a 31-Kb chromosomal insert was reported to contain all genes necessary to restore HR not only in the mutants but also in non pathogenic bacteria such as *P. fluorescens* and *Escherichia coli* (Huang *et al.*, 1988). This cosmid was used along with other random genomic fragments as probes in RFLP analysis for the construction of a dendrogram to study genetic relationships among *P. s.* pv. *syringae* strains (Legard *et al.* 1993). By these means, Legard *et al.* (1993) were able to show that strains of *P. s.* pv. *syringae* pathogenic to beans belonged to a taxonomic group that was distinct from strains of *P. syringae* pv. *syringae* pathogenic to other hosts.

Factors Involved in Development of Kernel Blight.

The uncertainty of the presence or absence of kernel blight and its variation in severity from year to year

suggest that environmental conditions and the physiological stage of the host may have an effect on this disease.

Kernel Developmental Stage. Basal kernel blight is normally difficult to observe in barley heads in the field and symptoms can usually only be observed after harvest. In contrast, spot kernel blight symptoms can be observed much easier and earlier during kernel development in the field. This may be due to the morphology of the barley head or to the time of onset of infection. Studies on wheat seed infection by *Pyrenophora tritici-repentis* (Died) indicated that the kernel developmental stage of the plant affected the incidence of infection (Schilder and Bergstrom, 1994). Wheat seeds were susceptible to infection throughout most of their development, from the end of anthesis through the soft dough stage. However, an increase in the incidence of *P. tritici-repentis*-infected seeds occurred when inoculation took place at milk stage (Schilder and Bergstrom, 1994). Teviotale and Hall (1976) reported that barley seeds were susceptible to *Pyrenophora graminea* (Ito and Kurib) from head emergence through the soft dough stage, but there was less transmission of fungus through infected seed the later the infection took place. In the case of kernel discoloration of barley, caused by *C. sativus*, the stage of kernel development when infection occurred did not appear to be an important factor (Lutey, 1962) and infection was observed to take place from the end of flowering through

maturity (Stevenson, 1981). However, some studies on the microbial population in barley heads have shown that the number of bacteria and filamentous fungi increase greatly from milk to late dough stage and that the greatest increase of infection by bacteria and yeast comes just before maturity (Follstad 1961). Peters *et al.* (1983) working with bacterial kernel spot (spot type of blight) caused by *P. syringae*, found that infection occurred at the beginning of kernel development, just before the lemma was attached to the seed. The possibility that the presence of a different window of infection for the different symptoms of kernel blight observed in Montana seemed likely and research in that area was included in this study.

Environmental Conditions. The amount of available moisture after heading has often been associated with kernel blight. Kernel discoloration and bacterial kernel spot have always been reported to be most severe after abundant rain or in fields with overhead irrigation (Follstad 1961, Lutey 1962, Stevenson 1981, Wilcoxson, *et al.*, 1980; Miles *et al.*, 1987; Wesenberg, 1994, personal communication; Peters *et al.*, 1983). It has also been reported that the frequency with which fungi were isolated from barley kernels was increased during years with above normal rainfall (Follstad 1961, Lutey 1962). In Montana, the presence of both symptom types of kernel blight has also been associated with wet years but specific experiments have not been done in this

area. While the effect of moisture on the development of kernel blight has been studied extensively in other states, no information is available about the effect of temperature. Since *P. syringae* is known to have an optimum growth temperature around 28 C, temperature may also have an effect on the development of this disease.

Genetic Resistance. The degree of infection and discoloration in barley kernels seems to be influenced by different factors including the pathogen, plant genotype and environment (Miles *et al.*, 1987). Genetic resistance is considered the most practical tool for control of kernel blight and breeding studies for kernel discoloration caused by *C. sativus* have been in progress for some time. (Banttary *et al.*, 1975; Wilcoxson *et al.*, 1980; Miles *et al.*, 1987; Miles *et al.*, 1989; Gebhardt *et al.*, 1992). The standard screening process involves enhancing development of the disease by overhead irrigation with concomitant inoculation with *C. sativus* (Miles *et al.*, 1987). In one of these studies it was found that irrigation significantly increased black stain severity. On the other hand, even though inoculation may have increased black stain severity, the effect was not statistically significant (Miles *et al.*, 1987). Draper (1985), looking for genetic resistance to bacterial kernel spot, reported differences among ten barley cultivars suggesting the existence of genes for resistance. Differences were reported between two cultivars ("Liberty"

and "Moore") in the number of isolatable bacteria per gram of grain (Follstad, 1961). These differences seemed consistent and large enough to lend experimental evidence that there actually are differences among cultivars in susceptibility to invasion by bacteria (Follstad, 1961). A common limitation in some screening programs is the lack of reliability of the screening method used, a factor that has been one of the goals of this project. Symptomatological and etiological studies of kernel blight allow a better understanding of this disease and the results can be utilized in the development of a reliable method of screening for genetic resistance.

CHAPTER 2

SYMPTOMATOLOGY AND STUDY OF THE CAUSAL AGENTS OF KERNEL BLIGHT OF BARLEY IN MONTANA

Introduction

'Kernel blight' and 'kernel discoloration' have been used to describe a barley disease of importance to the malting and brewing industries, referred to as kernel blight in this study. Microorganisms present in infected kernels can detrimentally affect different steps of the malting and brewing processes and thereby the quality of the final product. This disease is commonly present in the upper Midwestern region of the United States (Miles *et al.*, 1987, Gebhardt *et al.*, 1992). In the past several years it has occurred with some severity in Northcentral Montana, where it has caused concern to local growers and users of malting barley.

Two symptom types of kernel blight have been observed in Montana. The most common type consists of dark brown discoloration of the embryo end of the kernel, hereafter referred to as basal kernel blight (Fig. 1a), and generally attributed to fungal infection. This symptom has previously been called 'black point' or 'kernel discoloration' and has been observed on wheat, barley, and rye (Machaceck and Greaney, 1938; Anderson and Banttary, 1976, Mathre, 1982).

Several fungi have been reported to cause this symptom including *Alternaria* spp., *Cochliobolus sativus* (Ito and Korib), and *Fusarium* spp. (Machaceck and Greaney, 1938; Anderson and Banttari, 1976; Banttari *et al.*, 1975; Mathre, 1982). Until now, no one has proposed bacteria as the causal agent of this symptom. The second type of blight, reportedly caused by the bacterium *Pseudomonas syringae* pv. *syringae* (Peters *et al.*, 1983), occurs more sporadically and is characterized by tan to dark brown necrotic spots with distinct margins on the lemma of the kernel, hereafter referred to as spot kernel blight (Fig. 1b). Results of this research indicated that species of *P. syringae* were associated with both types of blight. Preliminary evidence suggested that these strains were phylogenetically different.

For assessment of possible phylogenetic relationships of phytopathogenic bacteria such as *P. syringae*, more than one criterium is necessary. The use of pathogenicity and biochemical tests have traditionally been important in the systematics of phytopathogenic bacteria (Hildebrand *et al.*, 1988). These tests are not always adequate to define pathovars, nor do they appear useful in distinguishing strain differences within a pathovar (Legard *et al.*, 1993). However by combining biochemical and pathogenicity tests with other tools, such as analysis of restriction fragment length polymorphism (RFLP) and serological techniques, it

should be possible to ascertain and assess phylogenetic relationships among phytopathogenic bacteria (Hartung and Civerolo, 1991; Gottwald *et al.*, 1991).

In the case of *P. syringae*, several studies have utilized RFLP analysis in the characterization of strains between and within pathovars, (Hendson *et al.*, 1992; Legard *et al.*, 1993; Denny *et al.*, 1988; Quigley and Gross, 1994). Toxin synthesis and toxin secretion are common characteristics among pathovars of *Pseudomonas syringae*, and it appears that peculiarities of toxigenesis may reflect overall genetic differences (Gross, 1991; Hildebrand *et al.*, 1982). Thus, whole genes or fragments of genes involved in toxin production have proven useful as probes for characterizing toxigenic strains of *P. syringae* (Cuppels *et al.*, 1990; Shaad *et al.*, 1989). Sections of DNA of the tabtoxin biosynthetic region have been used as specific probes for tabtoxin-producing strains of different pathovars of *P. syringae* including *tabaci*, *coronafaciens*, and *syringae* (Kinscherf *et al.*, 1991). Quigley and Gross (1994) demonstrated that *syrB* and *syrD* genes are conserved among a broad spectrum of *P. s.* pv. *syringae* strains that produce syringomycin or one of its analogs, syringotoxin or syringostatin. Thus, these probes proved to be very useful in studies of genetic relationships among syringomycin-producing strains (Quigley and Gross, 1994). Other genes that also appear useful in RFLP analysis are those in the

hrp (hypersensitive reaction and pathogenicity) region. By using these genes as probes, Legard *et al.* (1993) constructed a dendrogram showing that *P. s.* pv. *syringae* strains that were pathogenic to beans belonged to a taxonomic group distinct from the strains of *P. syringae* pv. *syringae* pathogenic in other hosts.

The present study was initiated to determine the causal agent of kernel blight of barley in Montana. When *P. syringae* was found to be the primary causal agent, two types of this bacterium were identified, each appearing to cause a distinct symptom and the molecular methodologies described above were applied to these bacteria to determine their relationships to each other and to other phytopathogens.

Materials and Methods

Samples.

Field samples showing kernel blight symptoms were collected from different barley cultivars grown in Northcentral Montana and from the Midwestern states of North Dakota, Minnesota, and South Dakota. A total of 91 samples were obtained from the 1991, 1992, and 1993 growing seasons. A description of these samples is presented in Table 1. Each sample consisted of approximately 500 g of kernels and was obtained from a single field. Symptomatic kernels showing basal or spot kernel blight were analyzed for the presence of bacteria and fungi.

Fungal Isolation.

Fifty symptomatic kernels were chosen from each sample in 1991 and 10 each from the 1992 and 1993 samples. Each kernel was considered a subsample and the type of symptom was recorded. Symptomatic kernels were surface sterilized in a solution of 0.5% (v/v) sodium hypochlorite (household bleach) and 10% (v/v) ethyl alcohol for 5 minutes followed by three rinses in sterile distilled water. Kernels were placed on potato dextrose agar (PDA) containing 10 ug/ml of streptomycin dissolved in 70 percent ethanol and added after autoclaving. All laboratory chemicals were obtained from Sigma Chemical Company, St. Louis MO., unless otherwise noted. After 4 to 6 days, fungal colonies were purified by the obtention of monosporic cultures and when possible identified under the microscope. Frequency of fungi present in symptomatic kernels was determined.

Bacterial Isolation.

Subsamples were obtained as described for fungal isolation. Symptomatic kernels were soaked individually in sterile phosphate buffer solution (PBS) (0.5M potassium phosphate, 0.75% sodium chloride) for two hours at 4 C, and then transferred to a rotary shaker at 250 rpm at room temperature for an additional 30 minutes. A ten-fold dilution series was prepared in PBS and spread onto the semi-selective medium KBBC, consisting of King's B medium

Table 1. Malting barley samples obtained from Northcentral Montana and North Dakota, South Dakota, and Minnesota during the growing seasons 1991, 1992, and 1993.

Locality	Year	Cultivar	Blight type	# samples
Montana				
	1991	B1202	Spot	9
		B1202	Basal	2
		B2601	Basal	11
		Klages	Spot	2
	1992	B5133	Spot	1
		B1202	Spot	2
		B1202	Basal	4
		B2601	Basal	6
	1993	B1202	Mixture[a]	10
		Harrington	Mixture	2
		Harrington	Mixture	2
		Morex	Mixture	4
		B2601	Mixture	3
North Dakota				
	1991	Robust	Basal	2
	1992	Robust	Basal	2
		Morex	Basal	3
	1993	Robust	Basal	7
		B2912	Basal	1
		Morex	Basal	2
South Dakota				
	1991	Morex	Basal	1
	1992	Morex	Basal	1
Minnesota				
	1991	Campbell	Basal	1
		Crookstan	Basal	1
	1992	Robust	Basal	2
	1993	Robust	Basal	7
		Excel	Basal	3

[a] Mixture = Samples that had kernels with spot and kernels with basal kernel blight.

(KB) (King *et al.*, 1954) amended with 1.5 ug/ml boric acid (autoclaved separately), and 80 ug/ml of cephalexin (Mohan and Shaad, 1987) and 20 ug/ml of cycloheximide. Antibiotics were dissolved in 70 percent ethanol and added after autoclaving. Plates were incubated in the dark at 28 C for 48 hours and Pseudomonas-like colonies were selected visually by pigment production and/or fluorescence under UV (Model UVL-21, BLAK-RAY, Ultra-Violet Products, Inc., San Gabriel, California) for further analysis. Isolates were tested for oxidase activity (Kovacs, 1965); hypersensitivity (HR) in tobacco leaves (*Nicotiana tabacum* cv. *Xanthi*) by infiltration of ca. 10^9 cfu/ml bacterial suspensions in sterile distilled water (Huang *et al.*, 1988); and for arginine dihydrolase activity (Hildebrand *et al.*, 1988). Other non-Pseudomonas-like bacteria were also purified and tested for their hypersensitive reaction in tobacco plants to detect potential pathogens.

Bacterial Inoculation in the Greenhouse.

Pathogenicity tests were performed under greenhouse conditions at temperatures of 22 to 24 C during the day and 12 C during the night with 14 hr light periods. In all greenhouse experiments, each replication consisted of three plants in a 19 cm pot. Each experiment was conducted in a completely randomized design with three replications per treatment. Data were analyzed by Analysis of Variance using

the MSU statistical program (Montana State University, Bozeman MT.). Each experiment was repeated twice.

Two barley cultivars, B2601 (six-row) and B1202 (two-row) from Bush Ag Resources were planted in the greenhouse. The heads of two sets of plants were inoculated during kernel development. One set was inoculated with *P. s.* pv. *syringae*, strain 552 isolated from kernels with basal kernel blight and the other set was inoculated with *P. syringae* strain 554 obtained from kernels with spot kernel blight. For inoculum, the bacteria were grown for 24 hrs on KB and a 1:10 dilution was made of a suspension yielding a turbidity of 80 Klett units on a Klett-Summerson spectrophotometer with a green filter (ca. 10^7 cfu/ml) was prepared in sterile distilled water with 0.025% Tween 20 (polyoxyethylenesorbitan monolaurate). Plants were inoculated by spraying barley heads at the milk stage with the bacterial suspension using a hand air brush (Model Paaschi D500 1/10 H.P.) until runoff. Control plants were sprayed with a water solution of 0.025% Tween 20. After inoculation, plants were incubated for 48 hrs in a mist chamber in the greenhouse providing continuous wetness and then transferred to the greenhouse bench. At maturity, inoculated heads were harvested and kernel blight was visually evaluated and percentage of blighted kernels by weight was obtained by comparison to the weight of the total amount of kernels.

Syringomycin Assay.

The putative *Pseudomonas syringae* isolates were tested for production of syringomycin *in vitro* by measuring antifungal activity against *Geotrichum candidum* Link following the technique of Gross (1985). Bacteria were transferred with a sterile toothpick onto plates of either potato dextrose agar (PDA) supplemented with 1% glucose or the defined toxin production medium SRM and incubated at 25 C for 5 days (Gross, 1985). Toxin production was determined by spraying the plates with a suspension of the toxin-sensitive fungus *G. candidum* strain F-260 (provided by D.C. Gross, Washington State University). After 24 to 48 hours of incubation, a zone of inhibition of *G. candidum* around a bacterial colony was considered indicative of syringomycin production. The zone of inhibition was measured from edge of colony to edge of zone to give a semi-quantitative evaluation of the amount of toxin produced.

Biochemical Tests on Bacterial Strains.

A sample of 60 oxidase negative, HR (+) strains isolated from kernels showing the two different types of blight symptoms were selected from the large collection of bacterial strains obtained during initial isolations. Two known *P. s.* pv. *syringae*, strains B301D (pear pathogen) and SD202 (wheat pathogen), supplied by D.C. Gross, were included for comparison. The strains were tested for

utilization of carbon sources including glucose, lactic acid, trigonelline and quinate (Hildebrand *et al.*, 1988). A subsample of these strains was also tested by using the Biolog MicroStation™ (Biolog, Inc. Hayward, CA, 94545), which is based on the utilization of 95 carbon sources, and by fatty acid profiles which combines cellular fatty acid analysis with computerized high resolution gas chromatography performed by the Microbial Identification System (MIDI, Newark, DE).

Reaction to a Polyclonal Antiserum.

The same 60 strains described above were also tested for the reaction to a polyclonal antiserum. The antiserum was produced against strain 384 which belongs to the *P. syringae* 554 group. Reaction to the antiserum was perfomed by indirect ELISA test employing goat anti-rabbit antibodies conjugated to horseradish peroxidase and detected with azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and hydrogen peroxide (Harlow and Lane, 1988).

RFLP Analysis.

Strains. The same 60 strains were also utilized for genetic characterization studies. *P. syringae* and *E. coli* strains were grown in Luria-Bertani (LB) broth (Sambrook *et al.* 1989) and stored in 15% glycerol at -80 C for long term storage.

Preparation of Total Genomic and Plasmid DNA. *P. syringae* strains were grown overnight in LB broth on a rotary shaker at 28 C. Three ml of bacterial suspension were used for preparation of total DNA by obtaining a pellet by centrifugation. Pelleted cells were washed twice in sterile deionized water and genomic DNA was prepared using the S&S Elu-Quik DNA purification Kit (Schleicher & Schuell, Kleene, NH). The protocol followed was as suggested by the manufacturer with the only modification being that lysed cells were treated with 2 ng of RNase at 37 C for 15 min. All the plasmids used were maintained in *E. coli* strain DH5 and multiplied by growing the bacteria overnight at 37 C in LB broth with the appropriate antibiotics. Plasmid DNA was prepared by the standard boiling method (Sambrook *et al.* 1989).

Digestion of DNA, Separation of Restriction Fragments, and Alkaline DNA Transfer. Total genomic DNA was digested to completion with the restriction enzyme *Pst*I as recommended by the supplier (New England BioLabs). Approximately 0.5 to 1.0 ug of digested DNA was separated by gel electrophoresis on 0.8 % agarose gel in Tris-acetate buffer at 50 to 60 volts for 3-4 hours. Molecular size standards of lambda DNA digested with *Hind*III were run concurrently on each gel to allow size comparisons. DNA fragments were transferred to Hybond-N+ nylon membranes (Amersham International plc, Amersham UK) under alkaline

conditions (0.4 M KOH) overnight and baked at 80 C for 30 minutes, following the manufacturer's instructions.

DNA Probes. Four DNA probes were utilized in this study. Probes consisted of excision fragments obtained from four different plasmids. One probe was excised from p91 and consisted of the 1.1-Kbp *Sal*I internal fragment of the *SyrB* gene, reported to be present in all *P. syringae* pv. *syringae* strains that produce syringomycin or its amino acid analogs. This plasmid was provided by D. C. Gross (Quigley and Gross, 1994). Two probes were individual fragments from the 30 Kbp insert containing the *hrp* region from *P. s.* pv. *syringae* (Huang *et al.*, 1988). These fragments were 10-Kbp and 14-Kbp *Eco*RI inserts excised from pLY9 and pLY14, respectively, provided by S. W. Hutcheson of the University of Maryland (Heu and Hutcheson, 1993). DNA regions included in these fragments are known to be essential for the production of HR activity in *P. s.* pv. *syringae* (Huang *et al* 1991). The fourth probe was a 5.3 Kbp *Pvu*II fragment excised from pQZ4100, a plasmid provided by D. K. Willis, University of Wisconsin (Kinscherf *et al.*, 1991). After plasmid extraction, each vector was digested with the appropriate enzyme and separated by electrophoresis in low melting temperature agarose gels containing ethidium bromide using standard procedures (Sambrook *et al.*, 1989). Inserts were excised from the gel with a razor blade and the DNA was eluted by using the Magic™ PCR Preps DNA purification

system for rapid purification of DNA fragments (Promega, Madison, WI).

DNA Hybridization. DNA probes were labelled with the non-radioactive ECL (enhance chemiluminescence) direct nucleic acid labelling and detection system of Amersham International plc (Amersham, UK). The hybridization buffer was adjusted to 0.25 M NaCl and after one hour pre-hybridization, the labelled probe was added to the solution and hybridization was carried overnight at 42 C. Hybridized membranes were washed in two changes of primary wash buffer (6M urea, 0.4% SDS and 0.1x SSC) at 42 C for 20 min each followed by two five minute washes in 2x SSC at room temperature. Procedures for signal generation and detection were done as described by the manufacturer. Membranes were then exposed to autoradiography film (Hyperfilm-ECL, Amersham) for 1 to 20 min at room temperature. Each probe was individually hybridized to the membranes, scored, and the membrane stripped as recommended by the membrane manufacturer before reprobing with the next probe.

Genetic Relationships of *P. syringae* Strains Based on RFLP Profiles. The size of restriction fragments hybridizing with each probe was determined by comparison with the lambda DNA size standards. Strains were evaluated for the presence or absence of fragments of specific sizes. Data from separate hybridizations of *Pst*I digests with the

three of the DNA probes were combined for analysis as reported by Legard *et al.* (1993). Pairwise comparisons were made by the NTSYS-pc program version 1.8 (Exeter Publishing Ltd.,Setauket, N.Y.) using the following equation: $F = 2n_{xy}/(n_x + n_y)$, where n_{xy} is the number of hybridizing fragments shared by the two strains and n_x and n_y are the total number of fragments in strain x and y, respectively (Legard *et al.*, 1993). A similarity matrix of F values was constructed using the NTSYS program and cluster analysis of distances resulted in a dendrogram using the unweighted pair group method with averages, UPGMA.

Results

Symptoms.

Both basal and spot kernel blight symptoms (Fig. 1a and 1b) occurred in Montana samples in 1991 and 1992, yet all symptomatic kernels from a single sample or field had only one type of blight. In 1993, a year with more rainfall, every Montana sample had kernels with basal and kernels with spot kernel blight. Only basal kernel blight was observed in all symptomatic kernels from the upper midwest samples for the same three years.