ASCOCHYTA RABIEI IN NORTH DAKOTA:

CHARACTERIZATION OFTHE SECRETED PROTEOME AND

POPULATION GENETICS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

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ABSTRACT

Mittal, Nitin, M.S., Genomics and Bioinformatics Program, College of Graduate and Interdisciplinary Studies, North Dakota State University, October 2011. ***Ascochyta rabiei* in North Dakota: Characterization of the Secreted Proteome and Population Genetics**. Major Professor: Dr. Steven W. Meinhardt.

Chickpea is one of the most important leguminous crops grown in regions of southern Europe, Asia, the Middle East, and the United States. Ascochyta blight, caused by *Ascochyta rabiei*, is the most important foliar disease of chickpea. In favorable conditions, this disease can destroy the entire chickpea field within a few days. In this project the secreted proteins of *Ascochyta rabiei* have been characterized through one and two-dimensional polyacrylamide gel electrophoresis. This is the first proteomic study of the *A. rabiei* secretome, and a standardized technique to study the secreted proteome has been developed. A common set of proteins secreted by this pathogen and two isolates that exhibit the maximum and minimum number of secreted proteins when grown in modified Fries and Czapek Dox media have been identified. Population genetic studies of *Ascochyta rabiei* populations in North Dakota have been conducted using microsatellites and AFLP markers. Population genetic studies have shown that the ascochyta population in North Dakota has not changed genetically in the years 2005, 2006 and 2007, but the North Dakota population is different from the baseline population from the Pacific Northwest. The ascochyta population in North Dakota is a randomly mating population, as shown by the mating type ratio.

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LITERATURE REVIEW

Chickpea (*Cicer arietinum*) Production

Chickpea is one of the most important leguminous crops grown in regions of southern Europe, Asia, the Middle East and the northwestern United States (Wilson and Kaiser, 1995). Chickpeas can be grown in semiarid regions with relatively poor soil and enhance the soil by fixing nitrogen. As a food chickpeas are 21-24% protein and can provide all of the amino acids except for tryptophan, methionine and cysteine (Iqbal et al, 2006). Chickpeas also contain the minerals sodium, potassium, calcium, zinc, copper, manganese and magnesium. It has 5% fat and 52% carbohydrates of which 20% is insoluble dietary fiber (de Almeida-Costa et al, 2006). These properties make it a candidate for a primary nutritional source for many developing third world countries.

According to the Food and Agricultural Organization of the United Nations (FAOSTAT, 2007), India is the largest producer of chickpeas followed by Pakistan, Iran and Turkey. India is also the largest consumer of chickpeas. In 2007 a total of 11.5 million hectares were harvested producing approximately one million tonnes of chickpeas worldwide (FAOSTAT). The last two decades have seen a major expansion of chickpea production in Canada, South America, and the United States (USDA NASS). According to the National Agricultural Statistics Service (USDA NASS), in 2007 a total of 96,600 acres were planted with chickpea in the United States. Washington and Idaho led in chickpea

production while North Dakota ranked third. In 2008 the total chickpea production in North Dakota was valued at $5 Million (USDA NASS).The acreage in North Dakota increased from 6,584 acres in 2008 to 14,400 acres in 2009 but decreased in 2010 to 9,676 acres (US Dry Pea and Lentil Council, 2010). The chickpea production has been limited due to its most important fungal disease, ascochyta blight.

Ascochyta rabiei

Müller and von Arx (Trapero-Casas and Kaiser, 1992) classified the causal agent of ascochyta blight as the following:

Kingdom.....Fungi

Division.....Ascomycota

Class.....Loculoascomycetes

Order.....Dothideales

Family.....Dothideaceae

Genus.....Didymella

Species.....D. rabiei (Kovacheski) v. Arx.

Ascochyta Blight

Ascochyta blight is present in all chickpea growing regions of the world (Nene, 1982). International and intranational movement of seed has been

responsible for transmitting this disease worldwide. This process may have also lead to the introduction of more aggressive isolates and compatible mating types (Kaiser, 1997). The first report of ascochyta blight in North America was in Canada in 1974. In the United States it was first reported in the Pacific Northwest in 1983 and in California eleven years later (Peever et al, 2004). It is assumed that this pathogen entered North America through infected chickpea lines imported into Canada from Turkey, India, Bulgaria, Syria and other countries in Europe and Asia (Wilson and Kaiser, 1995).

Cool wet conditions are favorable for ascochyta blight development. In the presence of these conditions, entire fields can be infected and destroyed within a few days (Chongo et al, 2003). Ascochyta exists as both anamorph (asexual or imperfect stage) and teleomorph (sexual or perfect stage). The teleomorph of *Ascochyta rabiei* is termed as *Didymella rabiei* (Trapero-Casas and Kaiser, 1992).

The infection frequency and disease severity are affected by plant age, cultivar, and their interaction (Chongo and Gossen, 2001). For resistant cultivars, the resistance has been found in seedlings and vegetative plants which gradually decreases as the plant matures (Chongo and Gossen, 2001). Susceptible lines are equally susceptible to ascochyta blight at all growth stages (Chongo and Gossen, 2001). Resistance genes are thought to be expressed to a greater extent during early stages of crop development (Chongo and Gossen, 2001). Based on these observations, it has been suggested that flowering is the best time for the first fungicide application because the decline in resistance begins at this stage. Further

applications are dependent upon the disease severity, weather conditions and crop development stage.

Symptoms

All above ground parts, including leaves, pods, stem and branches, of chickpeas can be infected by *A. rabiei*. Leaflets and pods show similar symptoms that are brown and circular but can also be elongated. Within these lesions are concentric circles of pycnidia. Chlorosis can also be observed surrounding the brown lesions. On petioles and stems, during the initial stages of infection, the lesions usually appear to be water-soaked. When the lesions become necrotic they are oval, approximately 3 cm in length, with concentric rings similar to those seen on the leaves. When lesions progress to girdle the stems or petioles they will break and the portion beyond that point will die. If the girdling occurs at the base of the stem the whole plant will die.

On the cellular level a complete breakdown of the inner leaf structure can occur. Cells collapse due to plasmolysis in areas near the fungal hyphae. Cells distant from the hyphae often show symptoms indicating the presence of exotoxins in the disease process (Höhl et al, 1991). It is probable that the toxins released from the fungus disrupt the selective permeability of the plasma membrane resulting in plasmolysis which appears as a water soaked area. This

collapse of the cells would then result in the stems being unable to support the plant (Hamid and Strange, 2000).

Patches of diseased plants become prominent in the field with advancement of the disease. Eventually these patches coalesce to cover the entire field (Nene, 1982). The incubation period between inoculation of plants and appearance of symptoms, varies between 5 and 7 days (Nene, 1982).

Life Cycle

Ascochyta rabiei's life cycle incorporates both sexual and asexual reproduction. A single sexual generation per season develops on infected plant debris during the fall and winter which is then followed by several asexual generations during the growing season (Trapero-Casas and Kaiser, 1992). Ascospores provide for the survival of the fungus from one growing season to the next (Kaiser, 1997), and were first observed in Bulgaria in 1936 on overwintering pods in the field (Kaiser, 1997). The initial infection of a field comes from airborne ascospores and conidia derived from the pathogen overwintering on crop residue. Splash dispersal of conidia contributes to short distance spread (Traperso-Casas and Kaiser, 1992), while airborne ascospores are responsible for long distance dispersal.

Trapero-Casas and Kaiser (1992) showed that specific environmental conditions are required for the formation of the teleomorph of *A. rabiei.*

Development of the sexual stage, *D. rabiei,* has not been observed on actively growing plants or on artificial media, while the sexual stage develops extensively on overwintered chickpea crop residue (Trapero-Casas and Kaiser, 1992).

High moisture is required for saprophytic growth and pseudothecial development. The development of *D. rabiei* fruiting bodies on chickpea debris is not affected by the temperature when the moisture level is appropriate for growth. Although temperature has little effect on pseudothecial formation, an optimum cool temperature between 5-10°C is necessary for their maturation (Trapero-Casas and Kaiser, 1992). With the advancement in pseudothecial development any temperature effect present decreases (Navas-Cortés et al, 1998). The pseudothecia produce large numbers of ascospores that are released during the spring and provide, along with pycnidia, the primary inoculum of the disease (Trapero-Casas and Kaiser, 1992; Navas-Cortés et al, 1998). The peak time for ascospore maturation is in the beginning of the spring and discharge of ascospores stops by the beginning of summer. Ascospores and conidia are usually multinucleated (Wilson and Kaiser, 1995). There are 8 ascospores per ascus and ascospores are divided into two unequal cells that are strongly constricted at the septum (Nene, 1982).

Production of sporulating fruiting bodies, known as pycnidia, is a characteristic feature of the asexual stage of the fungus. Asexual spores produced by pycnidia are known as pycnidiaspores or conidia. Pycnidia can be easily seen as minute dots in the lesions produced by the disease (Nene, 1982).

Pycnidiaspores are transparent, may be slightly bent at the ends, and vary in size (65 μm to 245 μm). The size of these spores varies when they are developed on a host or artificial media. Pycnidiaspores produced on an artificial media are usually smaller than those produced on the host (Nene, 1982).

Pycnidiaspores are the source for secondary infections. Asexual cycles result in repeated infections during the growing season. The rate and severity of disease development, and its spread is usually determined by the number of infection cycles (Wilson and Kaiser, 1995). Gossen and Miller (2004) found that *A. rabiei* can survive in infected crop residue for more than 4 years, although the production and viability of spores declined with time, and survival was not affected by the depth of burial (Gossen and Miller, 2004).

Pathogenic Variation

Significant pathogenic variation has been found in *A. rabiei* but no completely avirulent isolates have been identified (Geistlinger et al, 1997b). Terms such as pathogenic groups, races, virulence forms and pathotypes have been used to classify its pathogenicity. Vir and Grewal (1974) described 13 pathogenic groups in India, 6 races were reported in Syria and Lebanon by Reddy and Kabbabeh (1985) and 11 virulence forms were reported in the Palouse region of the US by Jan and Wiese (1991). Pathogenic variation has also been found in other parts of world including Pakistan, Turkey, Syria, and Canada.

Udupa et al (1998) divided *A. rabiei* isolates into three different pathotypes depending upon their aggressiveness. The pathotypes are distinguished by their ability to infect and kill three differential chickpea lines. Isolates belonging to pathotype I are the least aggressive and will infect line ILC1929 but will not infect lines ILC482 and ILC3279. Pathotype II isolates are moderately aggressive and will infect and kill both ILC1929 and ILC482 but not ILC3279, while pathotype III isolates are the most aggressive and will kill all three lines. These pathotypes are polyphyletic and do not represent evolutionarily coherent groups or lineages (Udupa et al, 1998) indicating that the pathogenic specialization traits are controlled by multiple genes. The pathotypes cannot be distinguished by molecular methods, but they are stable and can be distinguished by a standardized pathogenicity assay. Pathotype III is thought to have evolved in response to the use of partially resistant varieties (Udupa et al, 1998). Migration can be another factor in the evolution of more aggressive strains (Udupa et al, 1998). The third pathotype has not been reported in the US while pathotypes I and II have been reported in the Pacific Northwest (Chen et al, 2004). At present only pathotype II has been found in North Dakota (Kiersten Wise personal communication). The designation proposed by Udupa et al (1998) is the most accepted pathogenic classification system in the US.

Recently a fourth pathotype has been described. A new chickpea line, ICC12004, was added to the previous three lines. The line ICC12004 was resistant to all pathotypes except the new pathotype IV (Imtiaz et al, 2011).

The variability in classification schemes is most likely due to the absence of a standard method for scoring disease severity or host susceptibility. Therefore, comparison of results from different studies is difficult (Chen et al, 2004). The mini-dome bioassay technique, developed by Chen et al (2005), suggested that only two pathotypes of *A. rabiei* are present in the United States.

Mating Types

D. rabiei is heterothallic and therefore requires two mating types (Trapero-Casas and Kaiser, 1992; Wilson and Kaiser, 1995). Both mating types, MAT1-1 and MAT1-2, have been found in most countries (Kaiser, 1997) while only mating type I has been reported in Australia (Khan et al, 1999). Both mating types were introduced into the US in 1983 and were widely distributed in commercial plantings by 1984. Only mating type MAT1-2 was present in California when the disease was first imported in 1994, but the MAT1-1 mating type has been subsequently introduced (Kaiser, 1997; Peever et al, 2004). Sexual reproduction, which is responsible for gene recombination (Kaiser, 1997; Wilson and Kaiser, 1995), is responsible for major genetic and population variation (Peever et al, 2004), and defines the genetic make-up of a population (Phan et al, 2003). Peever et al (2004) found that contemporary subpopulations in the Pacific Northwest of the US are recombining regularly, indicating that the sexual stage of *A. rabiei* is important in creating the population's genetic diversity. Since mating

of *A. rabiei* is dependent on environmental conditions, the presence of both mating types in a population does not confirm sexual reproduction (Geistlinger et al, 1997a). It is not known if the teleomorphic stage is important for changes in aggressiveness and pathogenicity (Kaiser, 1997).

Before 2003, the mating type of an *A. rabiei* isolate was determined through conventional laboratory crosses with mating type tester strains. In 2003, Barve et al cloned and characterized the *MAT* locus from *A. rabiei.* They developed mating type specific primers (TAIL5, Com1 and SP21) for use in a multiplex PCR assay. This PCR assay provided an accurate, easy and rapid determination of the mating type (Barve et al, 2003).

Genetic Structure of *A. rabiei*

The first steps towards developing a genetic linkage map of *A. rabiei* was performed by Geistlinger et al (1997a) by producing a highly polymorphic mapping population using mating types, microsatellite fingerprinting and aggressiveness data. They used ten ^{32}P-end labeled simple repetitive oligonucleotide probes (for microsatellite fingerprinting) in combination with two restriction enzymes and found 230 polymorphic bands. They determined that the genomes of both mating types consisted of similar motifs. Their arrangement and abundance was found to differ between isolates and pathogenic species. This

difference was also seen between US isolates and Old World isolates (Geistlinger et al, 1997a).

Lichtenzveig et al (2002) also constructed a linkage map of *Didymella rabiei* by testing 232 DNA Amplification Fingerprint (DAF) markers and 37 Sequence Tagged Microsatellite Site (STMS) markers. The linkage map consisted of 25 DAF markers and six STMS markers spanning a total of 261.4 cM. This map was organized into 10 linkage groups. Nineteen of the markers remained unlinked (Lichtenzveig et al, 2002).

Phan et al (2003) developed a detailed linkage map of *A. rabiei* using Random Amplified Polymorphic DNA (RAPD), Sequence Characterized Amplified Region (SCAR), Amplified Fragment Length Polymorphic DNA (AFLP) and STMS markers. Sixty three RAPD, thirty seven AFLP, nine STMS, and one SCAR marker were mapped in 28 linkage groups covering a total of 1271 cM with an average distance of 15.1 cM between markers. This map was not highly saturated as shown by the large number of linkage groups and unlinked markers, and the large average distance between markers. This map could not be integrated with the map developed by Lichtenzveig et al (2002) because only two STMS markers were common in both maps. A SCAR primer specific for MAT-2, the mating type 2 loci, was also identified.

DNA markers and morphological characteristics have shown a high level of genetic variation in *A. rabiei* (Santra et al, 2001). Santra et al (2001) compared genetic variability between *A. rabiei* isolates from India, the US, Pakistan and

Syria. Isolates from the US were found to be significantly different from Indian isolates. They divided isolates from India into two classes, A and B according to their cluster position in the dendrogram (Santra et al, 2001). Rhaiem et al (2008) used six SSR (Simple Sequence Repeats) markers to determine the population structure of *A. rabiei* in Tunisia. In 2001 they made the first report of the sexual stage in Tunisia and suggested that the MAT1-2 mating type was introduced that year (Rhaiem et al, 2008).

Chickpea Genetics and Ascochyta Blight Resistance

Chickpeas are a diploid organism with 8 chromosomes. While a considerable number of linkage maps have been generated for chickpea, they have been limited by the lack of common markers. Early on Winter et al (2000) used 354 markers consisting of inter simple sequence repeat (ISSR), STMS, DAF, AFLP, RAPD, SCAR, and isozyme markers and Fusarium wilt resistant loci to create 16 linkage groups covering 2077.9 cM. Cho et al (2002) used 76 recombinant inbred lines and 78 markers to develop a chickpea molecular map consisting of 16 linkage groups. They developed markers associated with genes for several important morphological traits. In 2003 several linkage maps were published. Flandez-Galvez et al (2003) used 66 markers (51 STMS markers, 12 resistance gene analogue markers and three ISSR markers) to assemble 8 linkage groups. Collard et al (2003) produced a map with 83 markers where six of the

eight linkage groups corresponded to the Cicer consensus map. Millán et al (2003) produced a limited map with one linkage group but the markers used did not correspond to those used by other groups. In 2005 Cobos et al used 160 markers and 159 recombinant inbred lines to produce a linkage map with 10 linkage groups and related their linkage groups to those published by Winter et al (2000). In 2009 Anbessa et al used four populations and 144 SSR markers to create a map with 8 linkage groups. Most recently Millán et al (2010) attempted to produce a consensus map with 10 populations and 555 markers/loci to produce 8 linkage groups that allowed the combination of many of the previous maps. The authors were also able to correlate some of the chickpea chromosomes with those of *Medicago truncatula.*

In many of these mapping studies the researchers looked for resistance genes to *Ascochyta rabiei.* Singh and Reddy (1983) were the first researchers to identify two sources of resistance in chickpea against ascochyta blight. They found that two alleles are involved in ascochyta blight resistance, a recessive allele termed as *rar1* and a dominant allele they called *Rar*2 (Singh and Reddy, 1983). Tekeoglu et al (2000) studied the segregation in three recombinant inbred lines and reported that three major recessive genes control resistance to ascochyta blight in chickpeas. Many other minor genes appeared to be involved in modifying the action of these genes suggesting that minor genes are important in the variation in the degree of resistance (Tekeoglu et al, 2000). Udupa and Baum (2003) used recombinant inbred lines and 140 microsatellite markers to develop a

linkage map with eight linkage groups (LG). Their study found that resistance to pathotype I is controlled by a major locus, *ar1*, on LG 2 while resistance to pathotype II is controlled by two recessive genes, one on LG 2, *ar2a*, and the other on LG 4, *ar2b*, exhibiting complementary gene action. Collard et al (2003) identified two resistance QTLs on linkage group 4 that were associated with seedling resistance. They also reported five markers associated with stem resistance and four additional markers associated with seedling resistance located on LG 2, 3, 4, 5, and 6. Iruela et al (2006, 2007) identified two QTLs associated with resistance on LG 2 and 4. They linked this QTL with 3 SCAR markers, 4 RAPD markers and 2 STMS markers. Bian et al (2007) identified six QTLs with one in LG 1, three in LG 4, and two in LG 8. Most recently Anbessa et al (2009) used 144 SSR markers to identify five QTLs one each on LG 2, 3, 4, 6 and 8.

Although it is difficult to correlate these studies, it is clear that linkage group 4 contains at least one and as many as 3-5 different QTLs for ascochyta resistance. Two groups identified a resistance QTL on linkage group 2 and these may be the same since they map to the same general region. Linkage group 8 was identified by two groups to contain one or two resistance QTLs. Two groups identified one QTL on linkage group 3. The markers associated with these QTLs are close enough on the consensus map that it is not clear if they represent two different loci or a single locus. Only Anbessa et al (2009) have found a resistance QTL on linkage group 6 and so this resistance locus may be unique to the parents used for generating the populations used.

Coram and Pang (2006) identified a number of resistance predictive genes by examining their expression. Their results suggested that overall resistance is controlled by more than one gene in agreement with other studies. They suggested that the expression level of defense related genes may be less in susceptible genotypes as compared to resistant genotypes. They also suggested that susceptible genotypes may possess an allele producing ineffective protein products or that the resistant genotype may possess a gene which can regulate a wide range of defense responses (Coram and Pang, 2006). Coram et al (2007) investigated gene expression changes (using a microarray) in response to three defense signaling compounds: salicylic acid, methyl jasmonate and aminocyclopropane carboxylic acid. They found that these compounds independently regulated the gene expression levels and that the change in expression level depended upon the genotype of the isolates (Coram and Pang, 2007).

Grafting and reciprocal grafting in varying combinations of scion and rootstock genotypes have shown that local resistance to *A. rabiei* is controlled by the scion genotype (Chen et al, 2005).

Several cultivars exhibit moderate resistance in the seedling stage but the resistance declines considerably as plants mature and flower (Gan et al, 2006). Partial blight resistance is not enough to stop ascochyta blight from infecting (Cho et al, 2004) but disease severity is reduced in resistant lines (Chongo and Gossen,

2001). Epidemics develop more slowly on resistant varieties when compared to susceptible lines.

Solanapyrones

Solanapyrones were first isolated by Ichihara et al in 1983 from *Alternaria solani*, the fungal pathogen responsible for causing early blight of potato. Alam et al (1989) were the first to find that solanapyrones A and C are produced by *A. rabiei* when the pathogen was grown in Czapek Dox nutrients supplemented with hot water extracts of chickpea seed. Solanapyrones A, B, and C have been found in culture filtrates of almost all isolates (Alam et al, 1989; Höhl et al, 1991; Latif et al, 1993). Later it was found that cytochalasin D was produced by one of the isolates which caused epinastic symptoms when cuttings were placed in a cytochalasin D solution (Latif et al, 1993).

Höhl et al (1991) studied the production of solanapyrones during the *A. rabiei* growth cycle. They confirmed the production of solanapyrones A, B and C when *A. rabiei* was grown on chickpea seed extract media (Höhl et al, 1991). They found that on day 6 cultures started producing solanapyrone A while the other two solanapyrones were produced in low quantities. The production of all the solanapyrones peaked at 16-18 days. Overall, solanapyrone C is produced in the lowest amount. The toxin concentration present during hyphal development was quite low but increased extensively with fungal aggregation and spore

production. Toxin production has also been linked with pycnidia formation (Höhl et al, 1991). In cultures grown in plant sap media (PSM), solanapyrones were not detected in 4 day old cultures but were detected in low concentrations on days 7 and 12 (Höhl et al, 1991).

Chen and Strange (1991) developed a defined media for solanapyrone production. They found that addition of Zn, Mn, Ca, Co and Cu cations to Czapek Dox media induced solanapyrone production. These cations also increased fungal growth by 21-71%. Zn was found to be the most essential cation for production of solanapyrones in Czapek Dox media. These cations are known to influence both primary and secondary metabolism of *A. rabiei* (Chen and Strange, 1991). When compared to cultures grown in Czapek Dox media supplemented with cations, solanapyrone production in plant sap media was found to be very low (Chen and Strange, 1994). *A. rabiei* produces the highest concentrations of solanapyrone A after 14-18 days in Czapak Dox media (Bahti and Strange, 2004; Höhl et al, 1991) but the amount of solanapyrone produced is also dependent on the isolate (Latif et al, 1993).

Only solanapyrone C has been found in infected plants while all three solanapyrones, A, B and C, are produced in culture (Chen and Strange, 1994; Höhl et al, 1991; Latif et al, 1993). None of the solanapyrones have been purified from infected plants. The inability to purify the solanapyrones from plants may be due to chemical modification by metabolism, or binding to some other

components of the plant. Interestingly, the production of these toxins is inhibited on leaves of resistant plants (Höhl et al, 1991).

Höhl et al (1991) found that the symptoms caused by ascochyta blight in chickpea are similar to those caused by application of solanapyrones to healthy chickpea leaves. This observation suggests that solanapyrones could account for most of the toxic effects in ascochyta bight disease. They also identified solanapyrones B and C in the germinating fluids of fungal spores suggesting that solanapyrones might play an important role in early disease development, especially in suppressing the general resistance mechanism of the host. A positive correlation has been found between severity of disease symptoms with sensitivity to solanapyrones A and B, which again indicates their role in pathogenesis (Hamid and Strange, 2000).

Solanapyrone A has been found to be 2.62-12.64 times more toxic than solanapyrone B depending upon the conditions. It has been proposed that solanapyrones have an important role in the disease since they are always produced in culture under appropriate conditions independent of other differences between isolates (Hamid and Strange, 2000). It has also been suggested that solanapyrones are nonspecific since they affect different organisms such as potato and chickpea (Hamid and Strange, 2000).

Solanapyrones are produced by a polyketide synthase and are secreted along with hydrolytic or cell wall degrading enzymes (Höhl et al, 1991; White and Chen, 2007; Alam et al, 1989). Kasahara et al (2007) cloned the

solanapyrone biosynthesis gene cluster from *Alternaria solani*. A novel Diels-Alderase solanapyrone synthase, which is required for production of solanapyrone through Diels-Alder reaction in the fungus, has also been identified and purified from *Alternaria solani* (Katayama et al, 2008).

Solanapyrone A, isolated from *A. solani*, has been found to inhibit the activities of mammalian DNA polymerases, pol β and λ (Mizushina et al, 2002). However, there is no evidence that these enzymes are affected in chickpea. There have been unsuccessful attempts to identify an enzyme that can reduce the highly virulent solanapyrone A to the less virulent form, solanapyrone B (Bahti and Strange, 2004).

Confirmation of the role of solanapyrones in the disease process may prove to be helpful in studying resistance to ascochyta blight in chickpea (Hamid and Strange, 2000).

QoI Fungicides and Resistance

Strobilurins were first isolated by Anke et al (1977) from the basidiomycete fungus *Strobilurus tenacellus*. Two types of strobilurins were isolated, strobilurins A and B, and mass spectroscopy yielded the formulas $C_{16}H_{18}O_8$ and $C_{17}H_{19}ClO_4$ (Anke et al, 1977). They were found to be highly active against filamentous fungi and yeasts, while bacteria were not affected by concentrations up to 20 μg/ml (Anke et al, 1977). Strobilurins, along with several

other compounds such as oudemasin and myxothiazol, inhibited at the same site and have the methoxyacrylate structure in common. The comparison of these compounds led to the identification of the inhibitor structure and the development of the QoI type fungicides.

Strobilurin fungicides or QoI (quinone outside inhibitor) fungicides inhibit mitochondrial respiration by binding to the ubiquinone oxidation site (Qo) located in the cytochrome *b* subunit of complex III. Aerobic respiration is blocked resulting in an energy deficiency due to the lack of ATP production. Cytochrome *b* is encoded by the mitochondrial *cyt b* gene (Grasso et al, 2006). Since cells contain numerous mitochondria, each containing its own genome, cells can contain multiple variants of the genes encoded by the mitochondria without harm to the organism. This heteroplasmy can lead to rapid mutations in mitochondrial genes. Other factors that affect the speed and extent of fungicide resistance development are the chemical nature of the fungicide, mode of action, application frequency and the nature of the pathogen including its genetic makeup, life cycle and fitness level (Brent and Hollomon, 2007).

At present over 22 different plant pathogens have been found to be resistant to QoI fungicides (FRAC, 2011). QoI fungicides were introduced to the cereal market in 1996 and two years later, in 1998, a resistant strain of *Blumeria graminis* was isolated in Northern Europe. Four years later a strobin resistant *Mycosphaerella graminicola* strain was isolated (Sierotzki et al, 2007). The rapid

appearance of resistant pathogens has continued and has led to recommendations for maintaining susceptibility to this group of fungicides.

Grasso et al (2006) studied the *cyt b* gene and identified the point mutations responsible for resistance to QoI fungicides in 14 different plant pathogens. They found two important point mutations, G143A and F129L. The G143A point mutation changes the glycine codon GGT to alanine GAT. The F129L mutation occurs through changes in two bases of the codon and can result in four different codons. A third point mutation at G137 was identified by Sierotzki et al in 2007. The G137R mutation provides the same level of resistance as the F129L mutation in *P. tritici-repentis*. Both of these provide less resistance than the G143A mutation in *P. tritici-repentis* (Sierotzki et al, 2007). All mutations other than the G143A point mutation have a limited effect on resistance (Hollomon, 2007). Grasso et al (2006) proposed that the presence of an intron that splits the codon for G143 or located just after the codon would prevent the formation of the G142A point mutation and they suggested that the cytochrome *b* gene should be analyzed to determine whether the pathogen will remain susceptible to QoI fungicides before any application of the QoI fungicides (Hollomon, 2007).

Several different methods, including real time PCR (Kianianmomeni et al, 2007; Fraaije et al, 2002) and PCR followed by restriction digestion (Fontaine et al, 2009; Bäumler et al, 2003), have been developed to determine the presence of the various point mutations that provide resistance to QoI fungicides. Each assay

must be tailored to the chosen organism and multiple reactions are necessary to identify all the possible mutations.

Disease Management

Outbreaks of *A. rabiei* can be devastating to a crop and are difficult to manage. The best management plan for controlling ascochyta blight is to adopt an integrated approach that combines strategic agronomic management practices including cultivar selection, appropriate fungicide application, and cultural practices. This approach has proven to be very helpful in minimizing the damage caused by ascochyta blight (Gan et al, 2006).

Cultivars resistant to ascochyta blight are the most economical way to control this disease. As discussed above partially resistant cultivars are available but the resistance breaks down as the plants mature. Fungicides can be used to reduce disease severity but many of the fungicides currently in use are single mode of action fungicides and so are susceptible to resistance development. The QoI fungicides initially provided effective control of ascochyta blight but resistance to this group of fungicides was evident in 2005 just two years after their widespread use began. The use of QoI fungicides is currently discouraged in North Dakota. The recommended regimen for the control of ascochyta blight consists of a preventative spray with multimode fungicides, chlorothalonil (FRAC group M5) or mancozeb (FRAC group M3), before flowering followed by a spray

of either boscalid (FRAC group 7) or prothioconazole (FRAC group 3) at flowering or when the disease is observed (Wise et al, 2007). Cultural practices currently used to control the disease include use of ascochyta free seed, seed dressing with appropriate fungicides, deep burying and burning of chickpea stubble, crop rotation, sowing chickpea at a distance from previous chickpea crops, optimum sowing date and deep sowing (Gan et al, 2006). Two recent findings may affect the cultural practices currently used. The first is the finding of Dugan et al (2009) that treatment of the chickpea debris with the biocontrol fungus *Aureobasidium pullulans* results in a 38% reduction in the number of ascochyta blight leasions the following year. Secondly, Trapero-Casas and Kaiser (2009) found that *A. rabiei* can survive on 16 weed and crop species that are commonly found in the Pacific Northwest. These isolates remained virulent on chickpea, although they did not appear to be virulent on the alternative hosts.

Thesis Project

This project addresses two aspects of the population diversity of *Ascochyta rabiei*. One project is the characterization of secreted proteome of *A. rabiei* over a large number of isolates, while the other project is a population genetics study of *A. rabiei* population in North Dakota.

CHAPTER I

CHARACTERIZATION OF THE SECRETED PROTEOME OF THE *ASCOCHYTA RABIEI* POPULATION IN NORTH DAKOTA

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INTRODUCTION

Most pathogenic fungi develop a germ tube and produce an appresoria which then penetrates the plants epidermis independent of whether the plant is a host or not. In the case of a non-host the pathogenic process ceases at this point due to the lack of a compatible interaction between the host and the pathogen. In a compatible reaction, the pathogen interacts with the host through secreted proteins, cell surface proteins and small molecules and in turn senses molecules produced by the host as well as the products produced by the secreted proteins. This interaction leads to a continued pathogenic process and colonization of the host. An understanding of the secreted proteins and their function can therefore provide information on the initial infection process and provide information about how the pathogen interacts with its host to signal continued growth of the pathogen.

Several proteomic studies have been performed on the secreted proteomes of plant pathogens. The most completely studied secretome is from the plant pathogen *Botrytis cinerea* with three complete studies. In each case several different growth media were used to look for similarities and differences in the secretome. Shah et al (2009a) grew this pathogen on cellophane membranes initially set on NY agar without supplement or supplemented with extracts from a ripe tomato, a ripe strawberry, and an Arabidopsis leaf. After 5-8 days the

cellophane was set on top of acetate buffer and left for 10 days. In this study samples were taken from a one dimensional gel and analyzed by LC-MS/MS. They identified 89 different proteins under the four growth conditions with only seven being present in all systems. The majority of the proteins could be grouped into carbohydrate metabolism (28), 8 peptidases, 12 hypothetical proteins, one pathogenicity factor (ceratoplatanin), and miscellaneous proteins. The second study by Shah et al (2009b) investigated the secreted proteins produced when the fungus was grown on a defined media supplemented with citrus pectin, a 30% esterified pectin, and sucrose. Again using slices from one dimensional gels they identified only 67 different proteins. As expected the majority of the enzymes were for pectin digestion and carbohydrate metabolism (30). Ten hypothetical proteins were observed with only two being identified in the previous study. Only two proteases were observed, the pathogenicity protein ceratoplatanin, two lactases and three phytases. In the last study by Fernández-Acero et al (2010) defined media was supplemented with five different carbon sources (glucose, carboxymethyl cellulose, starch, pectin, and tomato cell walls). In two-dimensional gels the largest number of spots was observed in the cell wall extract and was used for the protein analysis. Seventy eight spots were analyzed identifying 56 unique proteins. As in the other two studies most proteins (39) were involved in cellulose and carbohydrate metabolism. Nine hypothetical proteins were identified with one being identified in all papers and one being found by Shah et al (2009a). Four peptidases were observed with the aspartate

protease being observed in all three papers while the tripeptidase was also observed in the first study. In all three studies 28 different hypothetical proteins were identified, 11 unique peptidases and approximately 30 proteins involved in carbohydrate metabolism.

Two studies using one dimensional gel electrophoresis were performed by the same group looking at the rutin induced proteins in *Aspergillus flavus* (Medina et al, 2004, 2005). In the earlier study, 15 proteins induced by the addition of rutin (3 peptidases, 4 catalases, 7 carbohydrate metabolism enzymes, and one other) were identified (Medina et al, 2004). In the non-induced cultures, seven proteins were identified of which only two were not identified in the rutin-induced culture. In a second study 51 unique proteins were identified, 11 proteases, 13 for carbohydrate metabolism, three involved in reactive oxygen degradation, and 8 miscellaneous proteins (Medina et al, 2005). Only 16 were found under all three growth conditions used, with 10 being unique to glucose as the carbon source, 14 unique to rutin and only two being unique to potato dextrose broth.

Two other studies looked at the secreted proteome of *Fusarium graminearum* (Phalip et al, 2005) and *Sclerotinia sclerotiorum* (Yajima and Kav, 2006). In *S. sclerotiorum* 52 different protein spots were reproducibly observed but only 14 unique proteins were identified by MS/MS (two proteases, eight carbohydrate metabolism, three hypothetical and one miscellaneous). In *F. graminearum* 84 unique proteins were identified when gown on hop cell wall preparation or glucose. Of these only four were found under both growth

conditions. Of the 84 proteins 45% were involved in cell wall/carbohydrate metabolism, 8% in cell wall biogenesis, 9% in protein digestion, and 33% were hypothetical or doubtful.

One unique study was performed by Vincent et al (2009) on *Leptosphaeria maculans* where they tested various protein extraction and precipitation methods and used solution isoelectric focusing to concentrate/ purify the proteins prior to 2D gel electrophoresis. In their studies they found that lyophilization reduced the number of proteins resolved due to increased horizontal streaking. Concentrating the samples by ultrafiltration increased the protein concentration but did not increase the protein diversity. Trichloroacetic acid/acetone precipitation increased the concentration of high molecular weight proteins but resulted in a relative decrease in the low molecular weight proteins. The process appeared to enhance the acidic proteins and decrease the relative concentration of basic proteins. Solution isoelectric focusing resulted in the depletion of highly abundant proteins, due to precipitation, and the subsequent increase the in lower abundance proteins. In addition this method appeared to remove many of the compounds that interfered with two-dimensional gel electrophoresis, giving better resolution in the subsequent gels. In this study over 2000 protein spots were observed indicating that the secretome is much more complex than previously observed. As with the other methods, it appears that the basic proteins were not concentrated and that the majority of proteins observed

were acidic proteins (pI <7). The authors state that other researchers using the same technique reported difficulty in resolving basic proteins after fractionation.

There are several unique aspects of this present study of the *Ascochyta rabiei* secretome. The first is that multiple isolates were investigated looking for differences in the secretome produced by an isolate under the same growth conditions. The second is we concentrated the proteins through repetitive dialysis and vacuum distillation which resulted in the protein spots being more evenly distributed between acid and basic pH ranges. Lastly we investigated the secretome under conditions where the solanapyrone toxins would or would not be induced. Currently it is thought that the solanapyrones will modify the amino acids of the proteins and so should induce a considerable change in the apparent proteome.

MATERIALS AND METHODS

Collection of *A. rabiei* Isolates

Isolates from North Dakota were collected by Dr. Kiersten Wise between 2005 and 2007. Table A1 in Appendix A lists the isolates, year collected, location of collection and other pertinent data.

Growth of *A. rabiei* Cultures

A. rabiei isolates were grown by placing filter papers containing the isolate on petri dishes with Potato Dextrose Agar (PDA). Cultures were incubated at 20°C with diurnal light (12 hrs light – 12 hrs dark) for 7-10 days. Three 1.0 cm circular agar plugs covered with sporulating mycelia from the growth edge of each culture were added to either a 125 or 250 ml flask containing 50 ml of modified Fries media or 30 ml of Czapek Dox media (Hamid and Strange, 2000). Each isolate was grown in 2-3 flasks for 7-14 days without agitation. Modified Fries media cultures were incubated at room temperature in the dark. Cultures in Czapek Dox media were grown at 20°C in continuous light (500 lux). After growth, the mycelia were harvested by filtering the culture through Whatman No. 2 filter paper. The mycelial mass was then used for DNA extractions for population genetics studies and the filtered culture media was used for analysis of the secretome.

Representative isolates were grown three times in both modified Fries and Czapek Dox media. Each batch of culture media was started from a different petri dish. All cultures were grown for 10 days.

Protein Sample Preparation

Crude culture filtrates for each isolate were stored at -20°C in 50 ml tubes. A 40-45 ml sample was dialyzed overnight against 4 liters of deionized water in dialysis tubing with a molecular weight cut off of 3500 Da. The dialyzed sample was then concentrated to 4-4.5 ml, 10X concentrate, using a roto-evaporator. The roto-evaporator water bath was set to 30°C and a water aspirator was used to create a vacuum. The protein concentration was measured in 96 well plates with the Bradford assay using the Bio-Rad Protein Assay dye concentrate (catalogue #500-0006). The 10X concentrated samples were then used for SDS PAGE. For 2D gel electrophoresis, the 10X concentrate was dialyzed again and then concentrated another 5 fold. The protein concentration was again measured using the Bradford assay as stated above.

SDS-PAGE

The secretome was first investigated using one dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein, 8-10 μg, was precipitated by adding four volumes of acetone with 12% TCA

(trichloroacetic acid) to one volume of the 10X concentrated sample containing the secreted proteins. This mixture was incubated overnight at –20°C. After incubation the samples were centrifuged at 3000g for 10 minutes. The supernatant was discarded without disturbing the protein pellet and the pellet was washed twice with 1 ml cold acetone and stored at -20°C.

The SDS sample buffer was prepared by adding 10.8 gm of urea, 2.5 ml 1.5M Tris-HCl pH 8.8, 6 ml 50% glycerol, 12 ml 10% SDS, 0.3 ml 0.25% bromphenol blue and distilled water up to 30 ml final volume. This buffer was stored in -20°C as 630 µl aliquots. Seventy microliters of 2-mercaptoethanol was added to each aliquot prior to use. The protein pellet was resuspended in 35 µl of the SDS sample buffer by vortexing the solution and storing the mixture overnight at 4°C. The next day, the sample was transferred to a 1.5 ml centrifuge tube and centrifuged at 16000g to remove undissolved particles before loading the sample onto the SDS-PAGE gel.

A tris-tricine buffer system was used to separate the proteins in a 12% total acrylamide gel, 3% crosslinker, which was 0.75 mm thick, with a stacking gel of 4% total acrylamide, 3% crosslinker, as described by Schägger and Von Jagow (1987). Into each well 20 µl of sample was loaded. Kaleidoscope prestained standards (Bio-Rad #161-0324), 3 µl, was loaded in one well. The Bio-Rad Miniprotean II gel system was used to run the SDS-PAGE. Gels were run at 100-125 V for 60-70 minutes. The gels were then stained overnight with colloidal

coomassie blue (GE Healthcare 2-D Electrophoresis Principles and Methods, Appendix III). The stained gels were washed 2-3 times with deionized water and the image photographed using the KODAK Gel Logic 2200 Digital Imaging System (Carestream Health, Inc, Rochester, NY).

Two-Dimensional Gel Electrophoresis

Sixty micrograms of protein was precipitated from a 50X concentrated double dialyzed sample, using the 2D Clean Up Kit (GE Healthcare). The precipitate was rehydrated in 135 µl of destreak rehydration buffer (GE Healthcare) for 2-3 hours at room temperature. Before loading the samples, 0.68 µl of IPG buffer (pH 3-11) was added to the samples. Immobiline dry strips, 7 cm in length, with a non-linear pH range of 3-11 were used to run the isoelectric focusing (IEF) gels in the GE Healthcare IPGphor III system. The sample was quickly spun at top speed in a bench top microcentrifuge to sediment the undissolved particles. One hundred and twenty five microliters of sample was loaded into the sample holder and 125µl of mineral oil was loaded at the top of the strip to avoid evaporation of the sample. Gels were run for 26 hours with maximum voltage reaching 5000V, for a total of 36750 volt-hours. The strips were stored at -80^{o}C until they were subjected to the second dimension SDS-PAGE (1mm thick, 12% acrylamide, 3% bis acrylamide gel) which was poured without a stacking gel. The Schägger and Von Jagow (1987) tris-tricine buffer

system was used. The strips were equilibrated in 3 ml equilibration buffer (GE Healthcare 2-D Electrophoresis Principles and Methods) twice for 20 minutes. Dithiothreitol (DTT) was added to the equilibration buffer, 1% final concentration, just prior to use. The equilibrated strips were slid into the large well of the second dimension gel (one strip per gel) with the negative end towards the standards. The strips were pressed against the gel surface to ensure they were in contact. Two percent agarose, prepared in equilibration buffer, was used to seal the strips in the well. Kaleidoscope prestained standards, 0.5 μl, were loaded into the standards well. The gels were run for 70-80 minutes at 100-115V. After running, the gels were silver stained using the Bio-Rad Silver Stain Plus Kit (catalogue #161-0499).

RESULTS AND DISCUSSION

An important aspect of this research was to identify unique individuals within the North Dakota population of *Ascochyta rabiei* that could be useful in studying the host-pathogen interactions in this pathosystem. This was accomplished by looking at the secretome through one and two-dimensional polyacrylamide gel electrophoresis. A total of 167 different isolates obtained in North Dakota between 2005 and 2007 were examined by one dimensional gel electrophoresis.

SDS-PAGE of the *A. rabiei* Secretome

Characterization of the proteome of *A. rabiei* began with the collection of culture filtrates from isolates grown in modified Fries media which were then separated by one dimensional SDS-PAGE gels using the Schägger (2006) buffer system which resolves down to 3 kDa. To determine if any bands might be obtained from the media, modified Fries media without inoculation was treated in the same manner as a cultured sample and run on SDS-PAGE. No bands were observed demonstrating that all protein bands observed in the gels were due to secreted proteins (data not shown). The isolates, point of collection and the figures where the images of the one dimensional gel for each isolate can be found, are listed in Table A1 in Appendix A.

The basic banding pattern of proteins in all gels was very similar (Figure 1, Appendix A, Figures A1-A26). There were 13-15 bands clearly evident in these gels, representing the secreted proteins, which ranged in relative mass from 111 kDa – 6 kDa. The protein band at approximately 30 kDa was always present in high abundance when compared to other proteins, while the other bands changed in relative intensity. Small variations were seen in the number of protein bands and these were most often expressed at low levels. Some of these bands are indicated by the arrows in Figure 1.

As the samples were processed it became evident that at some point the banding pattern had consistently changed for all the samples run after a certain

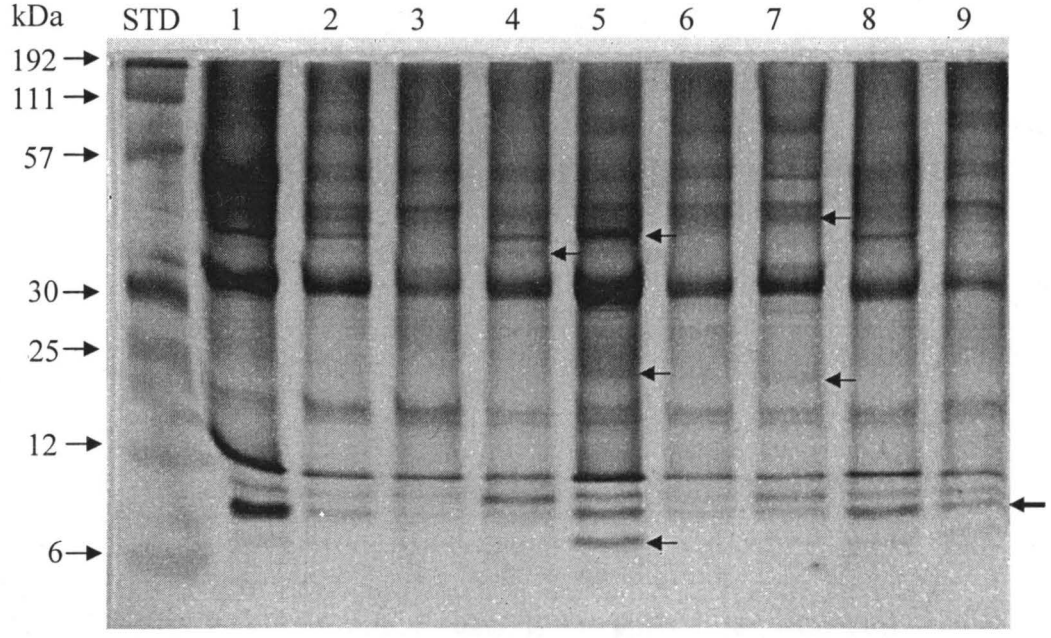

Figure 1: SDS-PAGE of the secreted proteome from nine different *A. rabiei* isolates grown in modified Fries media; Lane 1: 06BWEF2-10, lane 2: 05110M4, lane 3: 05105C8, lane 4: 06KND2A-18, lane 5: 06MB10, lane 6: 05105M9, lane 7: 06101M2, lane 8: 06KND2A-10, lane 9: 06MSTS2. Figure A4 in Appendix A.

date. The most obvious difference occurred between 25 and 12 kDa where additional bands were resolved in the second buffer and indicated by the arrows on the side of Figures 1 and 2. This was most likely due to changes in the buffers used to make and run the gel electrophoresis and so the buffers used in the gel electrophoresis were remade. This gave a third banding pattern that was slightly different from the first buffer set used, Figure 3.

To demonstrate that the change was due to the buffers and not due to a change in the proteins secreted by the isolates, four isolates from both the first and second buffer set were run using the last buffer set. Figure 4 is a composite from several gels showing samples run in buffer stock I and II. These same samples

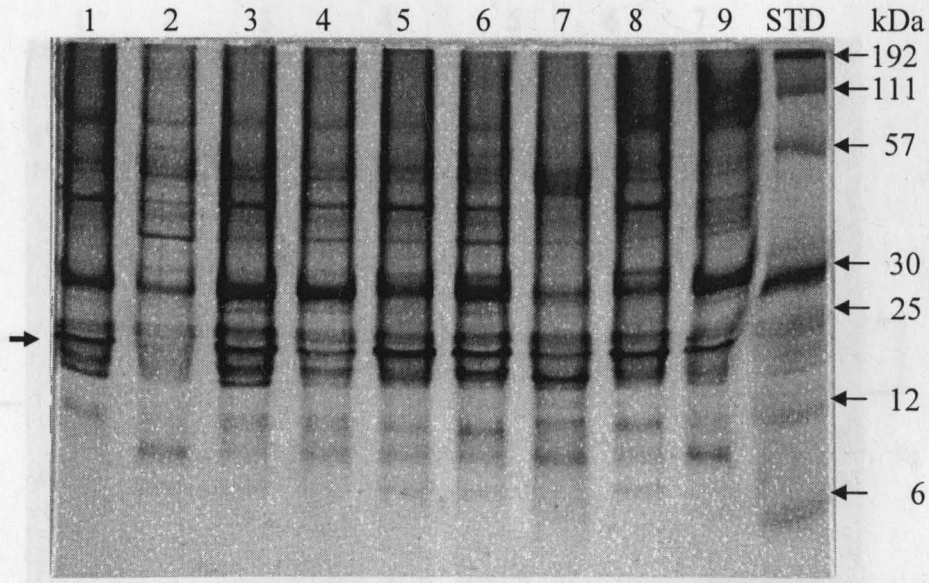

Figure 2. SDS-PAGE of nine *A. rabiei* isolates separated with buffer stock II. Samples were run according to the text with the reagents made after 6-16-08. Lane 1: 07HRF26, lane 2: 07GF-F2-10, lane 3: 07GF-F2-1, lane 4: 07GF-F1-18, lane 5: 07TSF2-46, lane 6: 07KMB9F2-17, lane 7: 05101CA1, lane 8: 05109M9, lane 9: 06TLND57. Figure A21 in Appendix A.

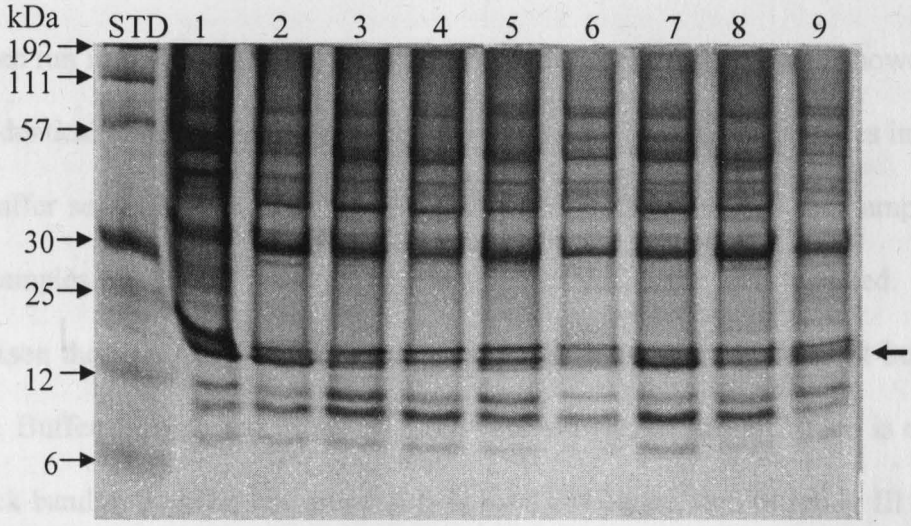

Figure 3. SDS-PAGE of nine *A. rabiei* isolates separated with buffer stock III. Samples were run according to the text with the reagents made after 9-16-08. Lane 1: 07CWF3-20, lane 2: 07CWF2-15, lane 3: 07GF-F1-27, lane 4: 07GF-F2-9, lane 5: 07GF-F1-28, lane 6: 07BMXP-13, lane 7: 07M204-7, lane 8: 07H406-6, lane 9: 07CWF2-13. Figure A8 in Appendix A.

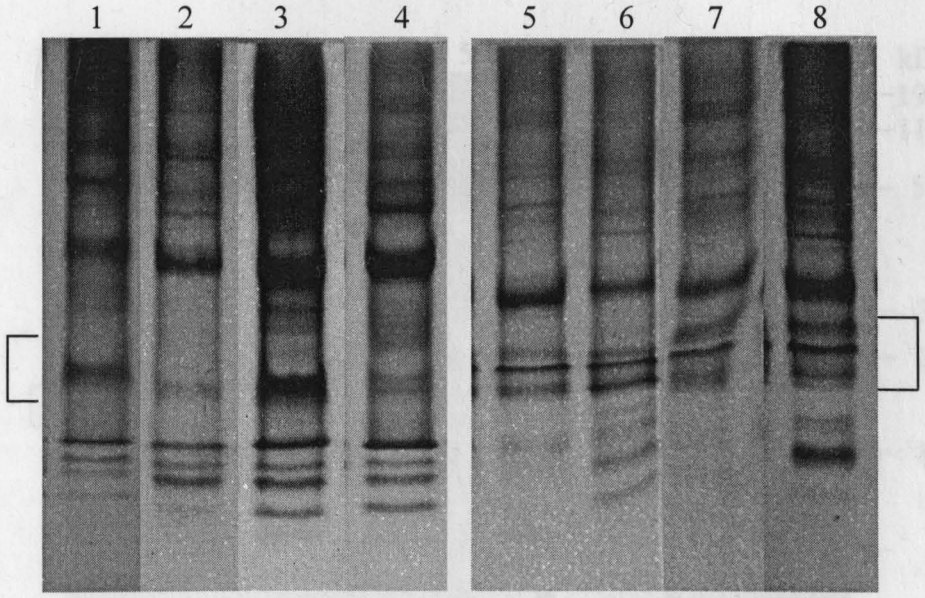

Figure 4. Selected lanes of *A. rabiei* isolates separated with buffer stocks I and II. Lane 1: 05108M1 (A15), lane 2: 05111M1 (A5), lane 3: 05211C1 (A17), lane 4: 06MB10 (A4). Lane 5: 06BND2 (A6), lane 6: 06KND1A-2 (A6), lane 7: 06MSTD2 (A19), lane 8: 05202STM2 (A3), Lanes 1-4 are buffer stock I and lanes 5-8 are buffer stock II.

were then run in buffer stock III, Figure 5. In buffer stock III all isolates showed a nearly identical banding pattern even though there were obvious differences in the other buffer sets. Since the change was due to the buffers and not the samples, those samples separated prior to the last buffer set were not repeated. For comparison the gels were divided into three groups based on the different buffer batches. Buffer group I and group III were very similar. In group I there is only one thick band at 20 kDa, but group II gels have 3-4 bands, and in group III this thick band is resolved into two bands (brackets, Figures 4 and 5). The three bands between 12 kDa and 6 kDa are more intense and compact in group I and III gels than in group II gels.

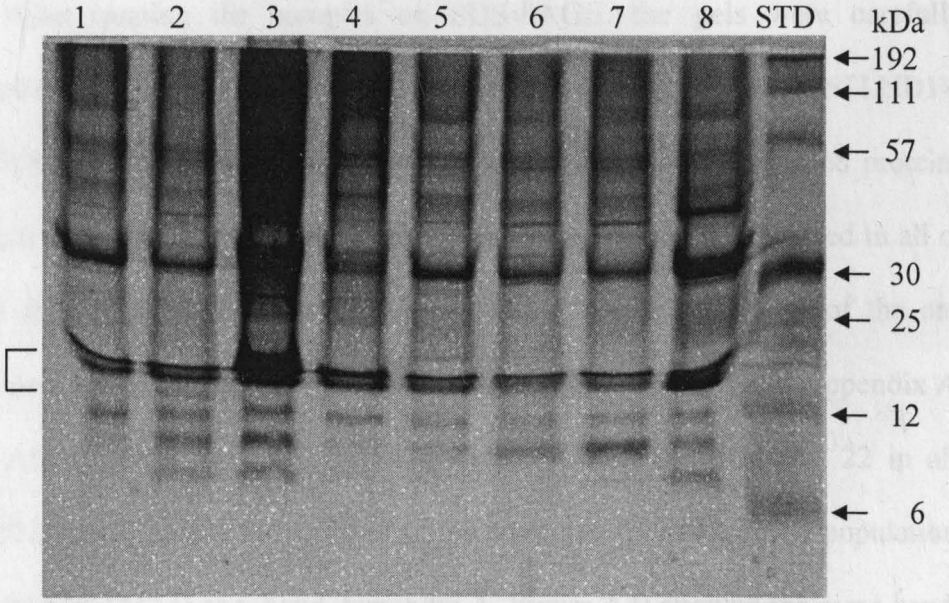

Figure 5. SDS-PAGE of samples from group I and II with buffer stock III. Lane 1: 06BND2, lane 2: 06KND1A-2, lane 3: 05211C1, lane 4: 05108M1, lane 5: 06MSTD2, lane 6: 05111M1, lane 7: 05202STM2, lane 8: 06MB10

The differences observed between the gels when different buffer stocks are used, indicates the sensitivity of the separation to the experimental conditions. The most apparent differences occurred in the low molecular weight regions, less than 25 k Da, although some changes were seen in the middle molecular weight ranges, 25-60 kDa. We were not able to resolve what the differences were between the buffer stocks. To ensure that no more changes would occur, the last buffer stocks were prepared in a large quantity and frozen in aliquots. The results did not change after this was done. We have not observed the same sensitivity to changes in the buffers when using the Laemmli (1970) buffer system. On the other hand the Laemmli buffer system does not resolve well below 14 kDa and is more sensitive to the presence of salts.

After running the samples on SDS-PAGE, the gels were carefully analyzed for similarities and differences. Four isolates, 05105M2, 06TLND14, 05202STM2 and 05APNS3, showed the greatest variation in expressed proteins from each other and represented all the unique protein patterns expressed in all of the one dimensional gels from the population. A composite image of the one dimensional gels is shown in Figure 6. Isolate 05105M2 (Lane 1 and Appendix A, Figure A17) showed the maximum number of well resolved bands, 22 in all. These 22 bands appeared to represent all the proteins expressed by the population. Isolate 05202STM2 (Lane 2 and Appendix A, Figure A3) showed the same bands as 05105M2 except it exhibited one thick band at 10 kDa while there were 2 less intense bands present in 05105M2. Isolate 06TLND14 (Lane 3 and Appendix A,

Figure A2) was exceptionally different from other isolates. In the figure its lane was pinched at the center giving it a non-uniform run most likely due to a high salt concentration in spite of efforts to remove salts. The proteins below 17 kDa were expressed in very low amounts resulting in very light bands. The smallest

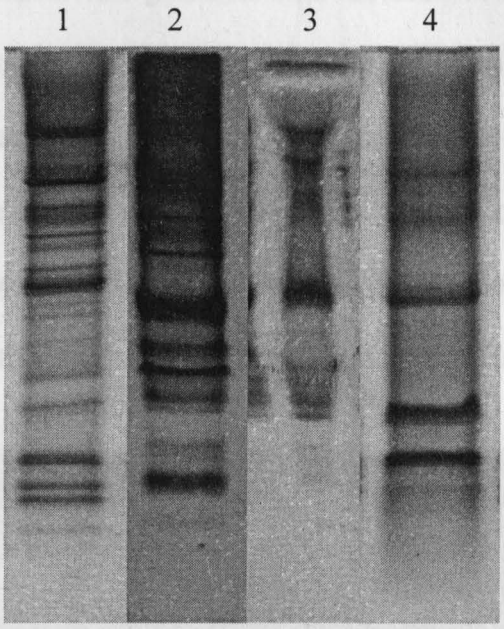

Figure 6. Isolates selected for further study. Lane 1: 05105M2, lane 2: 05202STM2, lane 3: 06TLND14, lane 4: 05APNS3

number of bands observed came from culture filtrates from isolate 05APNS3, (Lane 4 and Appendix A, Figure A17) with only 12 bands of which 2 bands, 12 kDa and 15 kDa, were exceptionally thick. These isolates were chosen as representative isolates and were studied in greater detail.

The representative isolates were grown three separate times as described in the materials and methods section, to check the reproducibility of the results

shown by each isolate. There was no difference in the bands observed between the three different biological replicates of the same isolate grown in the same media. One dimensional gels of these isolates are shown in Appendix A, Figures A11-14. Shown in Figure 7 (Appendix A, Figure A11) is a one dimensional gel where culture filtrates from each isolate grown in both modified Fries and Czapek Dox media were run. All isolates were very similar with only small differences in the banding pattern and protein intensity. In Figure 7 there are 15-18 proteins present that are secreted by the isolates. Out of these detected proteins, 12 proteins were found to be similar between the different culture media. The major protein near 30 kDa was secreted in both culture media and in high amounts. In Fries media

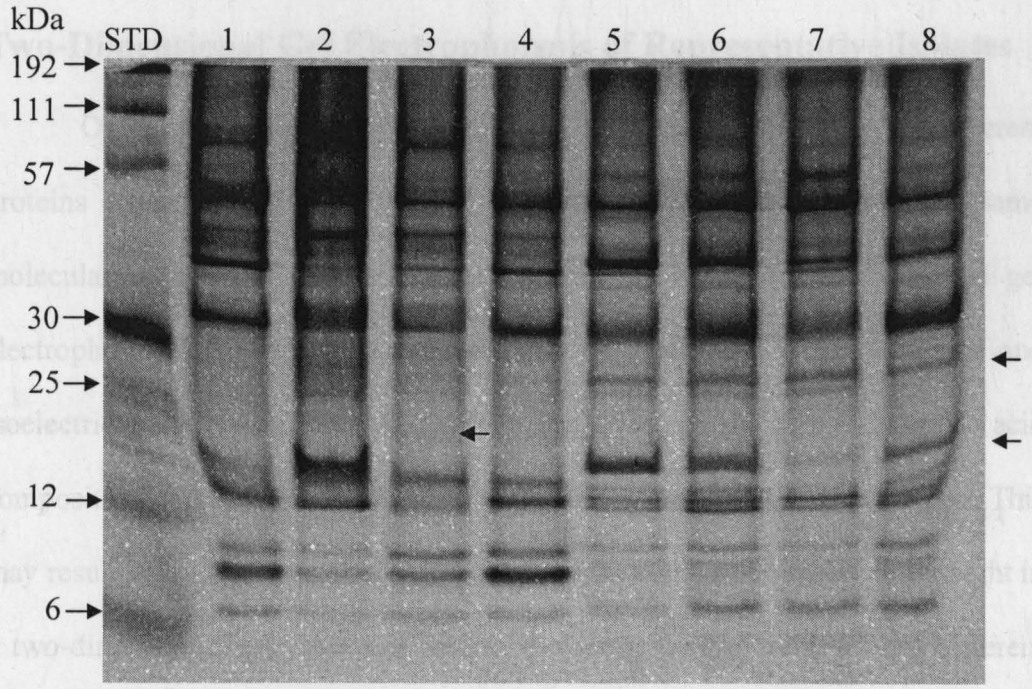

Figure 7. SDS-PAGE of secreted proteome from the representative isolates. Lane 1: 06TLND14, lane 2: 05105M2, lane 3: 05APNS3, lane 4: 05202STM2, lane 5: 06TLND14, lane 6: 05105M2, lane 7: 05APNS3, lane 8: 05202STM2. 1, 2, 3, 4 were grown in modified Fries media while 5, 6, 7, 8 were grown in Czapek Dox media.

cultures, the protein at approximately 18 kDa was secreted by all four isolates but was secreted to a greater extent by 05105M2. In Czapek Dox media cultures, the band near 15 kDa was secreted to a lesser extent by 05APNS3. The 27 kDa protein was observed only in Czapek Dox media. In modified Fries media there was only one band at 12 kDa while in Czapek Dox media the protein was resolved into two to three bands. When isolate 05105M2 was grown in modified Fries media, one thick smeared band was present near 25 kDa while three proteins with very little difference in molecular weight, 26 kDa, 27 kDa and 28 kDa, were secreted by all isolates in Czapek Dox media.

Two-Dimensional Gel Electrophoresis of Representative Isolates

One dimensional gel electrophoresis is limited in resolving the different proteins present in a sample since two proteins can have nearly the same molecular weight and the separation is on a log scale. Two-dimensional gel electrophoresis allows separation based on two physical properties, size and isoelectric point. The isoelectric point is dependent on the protein's amino acid composition and any modification to that protein, such as phosphorylation. This may result in numerous spots running left to right at the same molecular weight in a two-dimensional gel which is due to the same protein with several different degrees of modification. In the two-dimensional gels presented here the first dimension, isoelectric focusing was performed with a non-linear immobilized pH

gradient which gave greater separation in the pH range from approximately 5 to 8 but compressed the pH ranges of 3-5 and 8-10. This allows greater separation of proteins in the region where they are most abundant while still retaining the proteins that would be found at the very high and low pH values, where proteins are usually less abundant. The second dimension, SDS polyacrylamide gel electrophoresis, was performed with the buffer system devised by Herman Schägger (2006). This buffer system allows greater separation of proteins at low molecular weights. A similar separation can be obtained by using a gradient gel but these are difficult to pour reproducibly in the laboratory.

Two-dimensional gel electrophoresis was performed with a fifty fold concentrate of the secreted proteins from each of the representative isolates grown in both Fries and Czapek Dox media. A total of 13 different two-dimensional gels were run of the four representative isolates grown in Fries media and 11 for Czapek Dox media. Images of each of these gels with and without the spots marked can be found in Appendix B. A listing of the gels for each isolate is given in Table B1 of Appendix B. The number of gels made, the number of times a protein spot was observed for each isolate, and the total times a spot was observed in all isolates are listed in Table B2.

When all gels from the isolates grown in Fries media were analyzed a total of 128 non-redundant protein spots were detected in concentrated culture filtrates from *Ascochyta rabiei*. The same isolates grown in Czapek Dox media generated 143 different protein spots of which many were observed in only one isolate. On

average 10 unique protein spots were observed for each isolate in Czapek Dox media. In Figures 8 and 9 are shown two-dimensional gels from isolate 05105M2 grown in modified Fries and Czapek Dox media, respectively. In order to enhance the ability to see the spots in gels run with the Czapek Dox media samples, regions of the gel were adjusted individually to give the best resolution resulting in a mosaic look. Although somewhat similar, there are significant differences between the gels obtained for the different media. Attempts to overlay the protein spots from modified Fries media on those from Czapek Dox were unsuccessful suggesting that it would not be sensible to attempt to correlate the proteins expressed under the different growing conditions. Therefore gels from the two

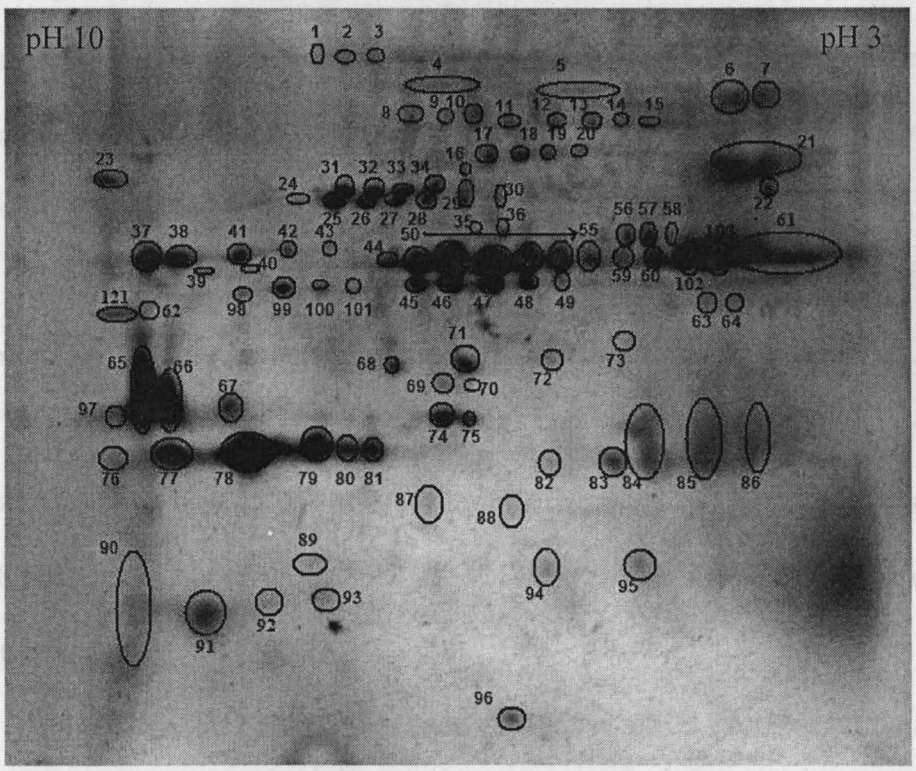

Figure 8. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 5-27-09.

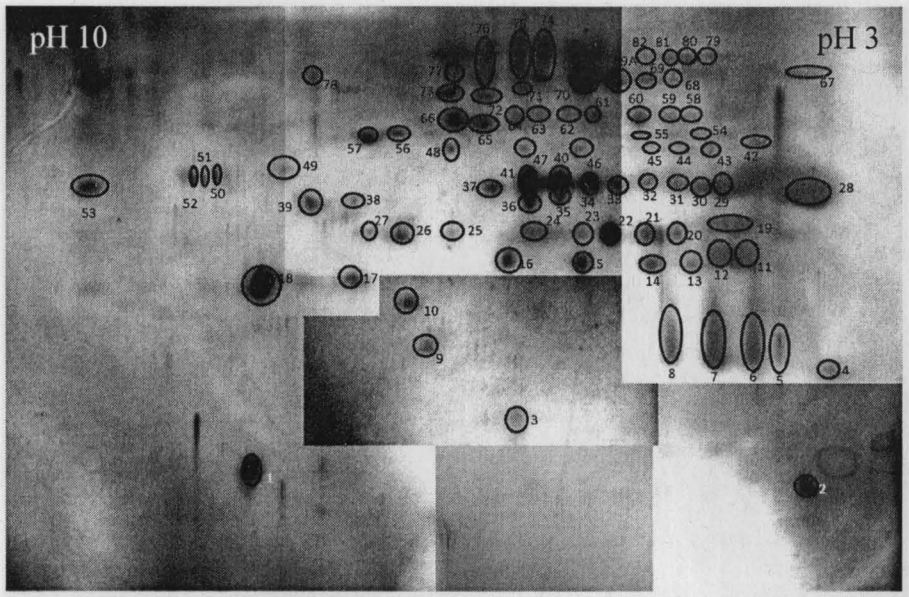

Figure 9. Modified image of the two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 2-05-09.

different growth media were numbered separately. Since in Czapek Dox media several isolates showed unique proteins, proteins that appeared to be in common between the isolates were given numbers below 83 and most unique proteins were numbered from 84 to 143.

In modified Fries media isolate 05105M2 showed the most spots with 127 in one gel. All of the protein spots observed in 05105M2 were observed in the other isolates when grown in modified Fries media except for one protein which was observed one time in 06TLND14. In modified Figures 10 and 11 are shown two-dimensional gels of the isolate 05APNS3 grown in Fries and Czapek Dox media, respectively.

Isolate 05105M2 shows a general movement of all proteins to the acidic range of pH, as indicated by a shift to the right, and an apparent reduction in the

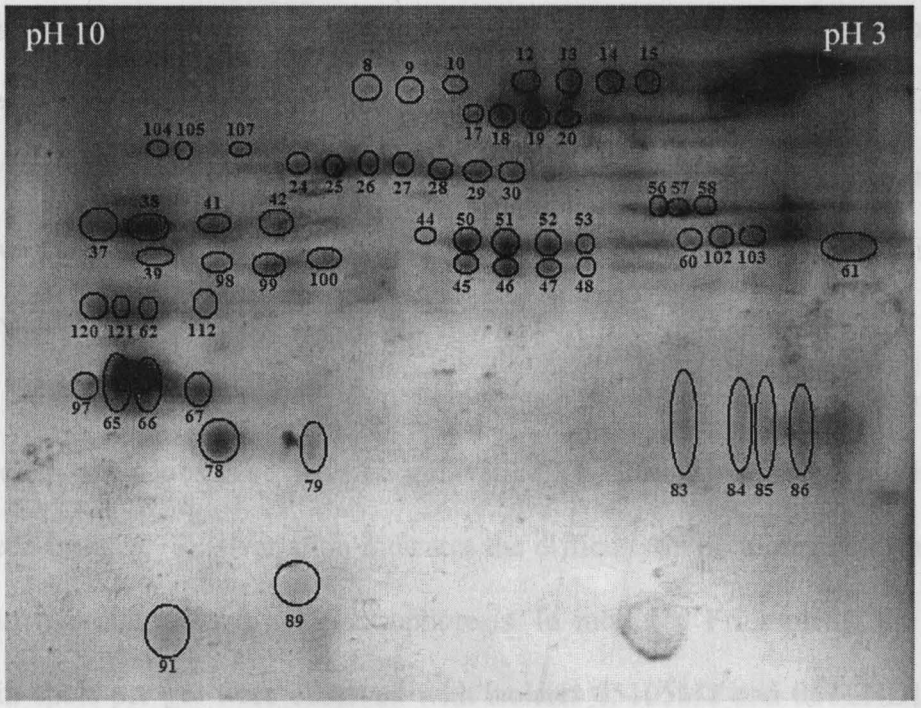

Figure 10. Modified image of the two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 8-4-09.

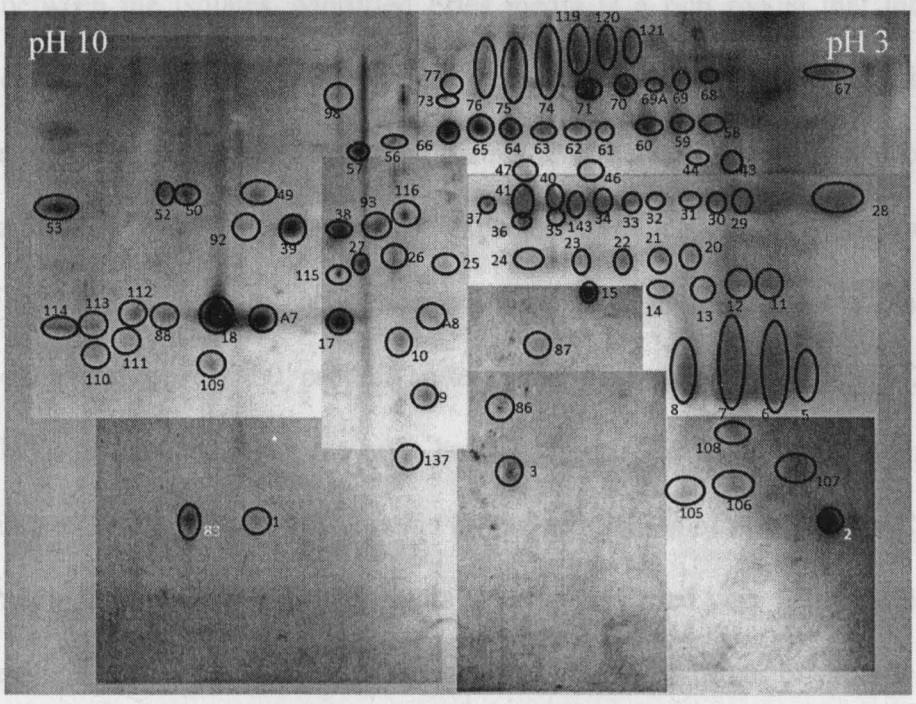

Figure 11. Modified image of the two-dimensional gel of isolate 05APNS3 grown in Czapek Dox media on 8-04-09.

number of low molecular weight proteins when grown in Czapek Dox media. There is also an apparent reduction in the number of protein spots. Isolate 05APNS3 on the other hand shows more protein spots in Czapek Dox media relative to modified Fries media and the shift to more acid pH values is not observed.

Table 1 lists the number of protein spots observed in each gel. The number of protein spots observed in each gel varied significantly within isolates and between isolates. This variation indicates the difficulty in obtaining reproducible gels in two-dimensional gel electrophoresis. In modified Fries media the most protein spots per gel were observed with isolates 05105M2 and 06TLND14, on average 20 more protein spots. In Czapek Dox media the number of spots varied little between the isolates. Modified Fries media is a rich media that includes yeast extract which is a complex nutrient source. Czapek Dox media is a minimal media providing solely the required minerals and a simple nitrogen and carbon source. Modified Fries media would be expected to induce a much more complex set of secreted proteins to accommodate the multiple nutrient sources. The fact that, on average, only 50 protein spots were observed for 05202STM2 and 05APNS3 suggest that they may not be able to use the additional nutrients or contain a more limited array of proteins for the acquisition of nutrients. In Czapek Dox media the number of protein spots on average varied very little. Still there were several gels which showed a significantly reduced number of protein spots. One possibility is that there is significant variability in the number of proteins

Table 1. Total Number of Protein Spots Observed in Each Gel When Grown in Modified Fries and Czapek Dox Media.

Isolate	Media	Gel number					Total spots observed	Average number of spots per gel
		1	2	3	4	5		
05105M2	Fries	104	96	74	85	6	427	85
05202STM2	Fries	73	56	45	----	----	174	58
06TLND14	Fries	77	68	----	----	----	145	72
05APNS3	Fries	61	43	50	----	----	154	51
05105M2	Czapek Dox	83	75	65	----	----	223	74
05202STM2	Czapek Dox	83	53	50	----	----	186	62
06TLND14	Czapek Dox	70	95	57	----	----	222	74
05APNS3	Czapek Dox	91	61		----	----	152	76

secreted when grown under essentially the same conditions and that there are factors that we are unaware of and unable to control that affect the protein expression.

In many cases a protein spot was observed only once in a single gel. For the most part these represent proteins that are not central to the growth of the organism. Table 2 lists the number of protein spots observed only once in each gel. As expected, the greater the number of samples separated by 2D gels, the greater the number of spots identified multiple times and the number of unique spots decreased in each subsequent gel. Interestingly, after 2 gels, the number of unique spots is very limited, relative to the total number of protein spots. In most instances three gels with three replicate samples are considered sufficient to have identified most proteins that are expressed in high quantities.

Table 2. Number of Protein Spots Observed Only Once in Each Gel When Grown in Modified Fries Media.

		Gel number					
Isolate	Media	1	2	3	4	5	Total
05105M2	Fries	14	4	0	1	0	19
05202STM2	Fries	19	4	4	----	----	27
06TLND14	Fries	24	11	----	-----	----	35
05APNS3	Fries	14	12	6	----	----	32
05105M2	Czapek Dox	16	10	2	----	----	28
05202STM2	Czapek Dox	24	8	3	----	----	35
06TLND14	Czapek Dox	10	29	2	----	----	41
05APNS3	Czapek Dox	36	6	----	----	----	42

As stated above, when the isolates were grown in modified Fries media there was only one protein that was unique to one isolate. There were only 17 protein spots that were unique to 05105M2. Of the 17 proteins expressed exclusively by 05105M2, one was expressed in four of the five gels, the majority was observed two to three times and only five were observed only once. Since most of these proteins were expressed more than once, they most likely represent proteins unique to this isolate. Several protein spots were shared between 05105M2 and only one other isolate. Isolates 05105M2 and 05202STM2 shared 17 protein spots that were not shared with the other isolates. Isolates 06TLND14 and 05APNS3 shared 4 and 9 spots exclusively with 05105M2. When the isolates were grown in Czapek Dox media the number of unique protein spots for a specific isolate or pair of isolates increased significantly. Nine protein spots were expressed exclusively in 05105M2, nine in 05202STM2, ten in 6TLND14, and six were expressed exclusively in 05APNS3. Of these unique proteins only two from

05105M2 were expressed two or more times suggesting that these are unique to this isolate. A few protein spots were also shared exclusively between isolates. Only two spots were shared between 05105M2 and 05202STM2, while seven spots were shared only between 05105M2 and 06TLND14. No spots were exclusive to only 05105M2 and 05APNS3 or to 05202STM2 and 06TLND14. Interestingly, eleven protein spots were shared between 06TLND14 and 05APNS3 and not by any of the other isolates.

Within an isolate most of the protein spots were observed two or more times. Table 3 gives the number of spots observed multiple times for each isolate in both growth media. The protein spots that appear only once are usually considered questionable. If we consider only the spots observed two or more times, isolate 05105M2 secreted significantly more proteins than the other isolates

Table 3. Number of Protein Spots Observed in Each Isolate When Grown in Modified Fries and Czapek Dox Media.

		Number of spots observed						
Times observed		1X	2X	3X	4X	5X	Total	Total observed >2X
Isolate	Media							
05105M2	Fries	19	21	23	23	41	127	108
05202STM2	Fries	29	23	33	-----	-----	85	56
06TLND14	Fries	35	61	-----	-----	-----	96	61
05APNS3	Fries	32	28	22	-----	-----	82	50
05105M2	Czapek Dox	29	22	50	-----	-----	101	72
05202STM2	Czapek Dox	36	27	32	-----	-----	95	59
06TLND14	Czapek Dox	35	21	46	-----	-----	102	67
05APNS3	Czapek Dox	42	55	-----	-----	-----	97	55

and only when grown in modified Fries media. In general 60 spots were observed two or more times. From these results we would suggest that typically only fifty proteins are secreted during growth in either media. Only isolate 05105M2 appears to be different and typically expresses 10 to 20 more proteins than the other isolates. Isolate 05APNS3 appears to secrete the minimum number of proteins.

Table 4 lists the number of spots found to be common between the isolates and Tables B2 and B3 in Appendix B list the number of times each spot was observed for each isolate. In Table 4, the columns marked as "1X" indicate that the protein spot was observed at least one time in both isolates and in the columns marked "2X", the spot appeared in least 2 gels for each isolate.

Table 4. Number of Protein Spots Found in Common Between Isolates When Grown in Modified Fries and Czapek Dox Media.

		05105M2		05202STM2		06TLND14		05APNS3	
Isolate	Media	1X	2X	1X	2X	1X	2X	1x	2x
05105M2	Fries	128	108						
05202STM2	Fries	85	55	85	56				
06TLND14	Fries	95	59	76	41	96	61		
05APNS3	Fries	82	50	67	39	72	41	82	50
05105M2	Czapek Dox	101	72						
05202STM2	Czapek Dox	81	47	94	59				
06TLND14	Czapek Dox	84	58	76	52	108	67		
05APNS3	Czapek Dox	76	46	70	36	90	43	97	55

Out of the 127 protein spots observed in modified Fries media, only 12 protein spots were present in all of the gels used to resolve the secreted proteins.

In Czapek Dox media 20 of the protein spots were present in all of the gels. The protein spots found in all gels may represent those proteins that are absolutely required for growth. In modified Fries media only 34 spots were observed two or more times in all four isolates while in Czapek Dox media only 35 proteins were observed two or more times in all isolates. These proteins most likely represent the minimal protein complement used in growth. An additional 16 spots were observed two or more times in three of the four isolates. All 128 spots were observed in the isolate 05105M2 with a subset of these being found in each of the other isolates. As expected 05APNS3 gave the fewest number of spots on average and 05105M2 gave the most. Interestingly isolate 05202STM2 presented almost the same number of spots in the two-dimensional gels as 05APNS3 in spite of appearing to have more bands in the one dimensional gels.

CHAPTER II

POPULATION GENETIC STUDY OF THE *ASCOCHYTA RABIEI* POPULATION IN NORTH DAKOTA

INTRODUCTION

Studies of the genetic diversity of *Ascochyta rabiei* populations have been made in at least six countries: Iran (Nourollahi et al, 2011), Turkey (Bayraktar et al, 2007), India (Varshney et al, 2009), Tunisia (Rhaiem et al, 2008), Canada (Vail and Banniza, 2008), and the United States of America (Peever et al, 2004). All of these studies found a high genetic diversity within populations and multiple populations within each country. In some cases the population appeared to be mating but there were also many cases where the population was not mating across small geographic distances but the populations could be mixed within a region or spread over all of the area studied.

In the United States the population genetic study performed in the Pacific Northwest by Peever et al (2004) revealed that there is a high genetic polymorphism between *Ascochyta rabiei* populations obtained from different cultivars, different years and different geographical regions. That study proved the occurrence of genetic evolution through genetic drift and host selection. The North Dakota ascochyta population was not used in Peever's study. Genetic polymorphism could lead to development of a new pathogen race which could be more virulent, as recently reported (Imtiaz et al, 2011) and resistant to currently available fungicides as was recently found in North Dakota (Wise et al, 2007).

Therefore, a population genetic study of *Ascochyta rabiei* in North Dakota is very important for better management strategies.

In this project we have used Microsatellites and AFLP marker systems, previously used by Peever et al (2004), for population genetic study of the *A. rabiei* population in North Dakota from years 2005, 2006 and 2007. Mating type test has also been performed on this population in this project. Population genetic study in ND will be used to define the genetic variation of the *A. rabiei* population in North Dakota. These populations have also been compared to a small *A. rabiei* population obtained from Pacific Northwest. This study will reflect the general genetic pattern of evolution of *A. rabiei* in North Dakota.

MATERIALS AND METHODS

DNA Extraction

A. rabiei isolates were collected and grown in modified Fries media as explained in the material and methods section chapter I. DNA was extracted from mycelia grown in modified Fries media using a modified CTAB method (Doyle and Doyle, 1990). Mycelia were ground to a fine powder, dispersed in CTAB extraction buffer (2% hexadecyltrimethylammonium bromide, 200mM Tris-HCl pH 7.5, 1.4M NaCl, 20mM EDTA, 0.2% β-mercaptoethanol), and incubated at 65°C for 1 hour. After extraction with an equal volume of chloroform, phases were separated by centrifugation. Nucleic acids were precipitated by the addition of an equal volume of isopropanol and collected by centrifugation. Pellets were washed with ethanol, dissolved in TE (10mM Tris-HCl, 1mM EDTA, pH8.0) containing RNaseA and incubated at room temperature for 30 minutes. Following incubation, 7.5M ammonium acetate was added to volume equal to ½ the volume of the sample and incubated for another 30 minutes at room temperature. The samples were centrifuged and the supernatant was retained. DNA was precipitated with ethanol, resuspended in an appropriate volume of TE buffer, and quantified at 260 nm with the ND 1000 Nanodrop Spectrophotometer (Nanodrop, Wilmington, DE). The extracted DNA was diluted to the appropriate concentration for each experiment with TE buffer.

Amplification of Microsatellites

The polymorphic loci *ArA06T*, *ArH02T*, *ArH05T* and *ArR12D*, three to six alleles per locus (Geistlinger et al, 2000; Peever et al, 2004), were measured in the North Dakota *A. rabiei* population. Four different primer sets specific for each locus were used, Table 5. These primers were the same as used by Geistlinger et al (2000) except that to all of the forward primers the M13 sequence (5' CACGACGTTGTAAAACGAC 3', IDT, Coralville, IA) was added to make them compatible with the IRDye 700 labeled M13 primer used in the LICOR 4300 DNA Analyzer (LI-COR Biosciences, Lincoln, NE).

The reaction mixture consisted of 0.6 µl of labeled M13 primer (5 µM), 1 µl of 10X standard *Taq* reaction buffer (NEB, B9014S), 0.8 µl of dNTP mix (Promega, U1515), 0.15 µl of *Taq* DNA Polymerase (5 units/µl, NEB, M0273X), 0.1 µl of forward primer (10 µM), 0.5 µl of reverse primer (10 µM), 1 µl of DNA (25 ng/µl) and 6.45 µl of sterile dH_2O to make a final volume of 10 µl.

For amplification the following PCR program was used: initial denaturation at 95°C for 5 min; 3 cycles of: denaturation at 95°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 1 min 20 sec, followed by 25 cycles of: denaturation at 96°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 45 sec; and finally one extension cycle at 72°C for 5 min.

Table 5. Primers Used for Microsatellite Amplification.

Primer	Primer sequence
ArA06T	F – 5' CACGACGTTGTAAAACGACCTCGAAACACATTC CTGTGCA 3' R - 5' GGTAGAAACGACGAATAGGGC 3'
ArH02T	F - 5' CACGACGTTGTAAAACGACGTTACTGCGTGTAT AGGCAAG 3' R - 5' TCCATCCGTCTTGACATCCGT 3'
ArR12D	F - 5' CACGACGTTGTAAAACGACATACACCCAAACCG GGTATCC 3' R - 5' GTATGGAATGTGCGATAGGAG 3'
ArH05T	F - 5' CACGACGTTGTAAAACGACCATTGTGGCATCTGA CATCACT 3' R - 5' TGGATGGGAGGTTTTTGGTAG 3'

The LICOR 4300 DNA Analyzer gel imaging system was used to separate and image the bands. The PCR products were diluted 100X by adding 50 µl of dH_2O and 49 µl of formamide loading dye to 1 µl of PCR product. The loading dye consisted of 47.5 ml of formamide, 2 ml of 0.5M EDTA at pH 8.0, 40 mg of bromophenol blue and 0.5 ml of water to make a final volume of 50 ml. Just before loading the samples were heated at 95°C for 5 min and then placed immediately in ice. A gel of 0.25 mm thickness was prepared by adding 15 µl of TEMED and 150 µl of 10% APS to 20 ml of premade RapidGel-XL 6%

acrylamide (USB Corporation, 75861). The gels were prerun for 25 minutes. A 50-700bp standard labeled with IRDye 700 (Li-Cor product no. 4200-60), was loaded along with the samples. Gels were run for 90 minutes according to the manufacturer's instructions.

AFLP Production

AFLP markers developed by Peever et al (2004) were used to study the *A. rabiei* population in North Dakota. The EcoR1 adapter was prepared by adding 0.028 µl (1 µg/µl) EcoR1-AdF primer (5'- CTCGTAGACTGCGTACC - 3'), 0.025 µl (1 µg/µl) EcoR1-AdR primer (5'- AATTGGTACGCAGTCTAC – 3'), 0.947 µl of sterile water for a final volume of 1 µl EcoR1 adapter (5 pmole/µl). The Mse1 adapter was prepared by adding 0.267 µl (1 µg/µl) Mse1-AdF primer (5'-GACGATGAGTCCTGAG – 3'), 0.233 µl (1µg/µl) Mse1-AdR primer (5'-TACTCAGGACTCAT – 3'), and 0.5 µl sterile distilled water to give a final volume of 1 µl Mse1 adapter (50 pmol/µl). These mixtures were heated at 95°C for 5 min and allowed to slowly cool at room temperature. They were stored at -20°C.

EcoR1 and Mse1 restriction enzymes (New England Biolabs) were used to digest 500 ng of genomic DNA. The digestion mixture consisted of: 0.5 µl of Mse1 enzyme (10 units/µl), 0.25 µl of EcoR1 enzyme (20 units/µl), 4.0 µl of 10X EcoR1 buffer (NEB, 1X is 50mM NaCl, 100mM Tris-HCl, 10mM $MgCl_2$,

0.025% Triton X-100, pH 7.5 at 25°C) and 0.4 µl of 100X BSA (New England Biolabs, 10 mg/ml). Genomic DNA and sterile dH_2O were added to a final volume of 40 µl. The DNA was digested at 37°C for 4 hours followed by 15 min at 70°C to inactivate the enzymes.

EcoR1 and Mse1 adapters were ligated to the digested DNA using T4 DNA ligase (Promega, M1801). The ligation mixture consisted of: 1.0 µl EcoR1 adapter (5 pmol/µl), 1.0 µl Mse1 adapter (50 pmol/µl), 1.0 µl 10X ligase buffer (10X is 300mM Tris-HCl, pH 7.8, 100mM $MgCl_2$, 100mM DTT, 10mM ATP), 0.5 µl T4 DNA ligase (3 units/µl), and 6.5 µl sterile dH_2O for a final volume of 10.0 µl. This mix was added to each tube of digested DNA and incubated at room temperature for 3-4 hours.

The primers used in the preamplification reaction had no additional selective nucleotides. Each reaction mixture consisted of: 5.0 µl of ligated product (1:10 dilution), 0.5 µl EcoR1 primer (5'- GACTGCGTACCAATTC – 3', 50 ng/µl), 0.5 µl Mse1 primer (5'- GATGAGTCCTGAGTAA – 3', 50 ng/µl), 0.4 µl dNTP mix (10mM, Promega, U151A), 2.0 µl 10X standard *Taq* buffer (NEB), 0.25 µl *Taq* DNA polymerase (5 units/µl, NEB, M0723X), and 11.35 µl of sterile distilled water to give a final volume of 20 µl. The PCR program for the pre-amplification reaction consisted of the following steps: 94°C for 2 min, 40 cycles of: denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min, and one final extension cycle at 72°C for 5 min. The pre-

amplification product was diluted 10 fold with TE buffer (pH 8.0) and subjected to selective PCR amplification. Each reaction consisted of: 1.0 µl 10X standard *Taq* Buffer (NEB), 0.25 µl dNTP mix (10mM, Promega), 0.25 µl EcoR1 + TA (EcoR1-AdF-TA) IRD700 labeled primer (10ng/µl, Eurofins MGW Operon, Huntsville, AL), 1.0 µl Mse1 + A (Mse1-AdF-A) primer (50 ng/µl, IDT), 0.25 µl *Taq* DNA Polymerase (5u/µl, NEB), 2.5 µl diluted pre-amp DNA, and 4.75 µl of sterile distilled water to give a final volume of 10.0 µl. The PCR program for selective amplification consisted of the following steps: denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, extension at 72°C for 1 min, 13 cycles of: denaturation at 94°C for 30 sec, annealing at 65°C for 30 sec, decreasing by 0.7°C each cycle, extension at 72°C for 1 min, followed by 24 cycles of: denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 1 min, and a final extension cycle of 72°C for 5 min.

The PCR product was diluted 2 fold by adding 10 µl of 1X formamide loading dye to 10 µl of PCR product. The samples were denatured at 95°C for 5 min and then placed in ice. The polyacrylamide gel was prepared as described above for the microsatellite separation. The gels were prerun for 25 minutes. The 50-700 bp size standard labeled with IRDye 700 (Licor, product no. 4200-60) was loaded along with samples. Gels were run for 90 minutes.

Mating Type Determination

The mating type was determined using a multiplexed PCR reaction as described by Barve et al (2003). Three primers, SP21, Tail5, and Com1 were used to amplify MAT-1-1 and MAT-1-2 specific sequences present in a single copy in the genome. The reaction consisted of 5.0 µl of 5X Green GoTaq Reaction Buffer (Promega, M791A), 0.5 µl dNTP mix (Promega, U1515), 1.0 µl of each primer (SP21, Tail5, and Com1 each at 10 µM, Integrated DNA Technologies, Coralville, IA), 1 µl of template DNA (25 µg/ml), 1.25 units GoTaq DNA Polymerase (Promega, M3005), and brought to a final volume of 25 µl by the addition of sterile dH_2O. The PCR program used was: denaturation at 95°C for 2 min; 30 cycles of: annealing at 60°C for 45 sec, extension at 72°C for 45 sec, and denaturation at 95°C for 45 sec; and a final extension at 72°C for 7 min.

The PCR products were visualized by separating 10 µl of the reaction mixture in a 1.3% agarose gel prepared with 1X TAE buffer, along with a 100 bp ladder (New Eengland Bioloabs, N3231F). Gels were run for 1hr at 90 V, stained with ethidium bromide (0.5µg/ml) for 20-30 min, and then destained in nanopure water for 5-10 min. Images were taken with the Kodak Gel Logic 2200 Digital Imaging System (Carestream Health, Inc, Rochester, NY).

Population Genetics Data Analysis

The programs Popgene, version 1.32 (Yeh et al, 1997), and GENALEX 6 (Peakall and Smouse, 2006) were used to analyze the population genetics data. The presence of clones in the experimental population can lead to estimation bias, therefore the samples that represented the same genotype were treated as clones and only one of those samples was used in the analysis. The samples were divided into four different populations, Table 6.

Table 6. Populations Used for Genetic Analysis.

Population	Population size	Description
Population 1	59	Samples collected in North Dakota in year 2005
Population 2	45	Samples collected in North Dakota in year 2006
Population 3	72	Samples collected in North Dakota in year 2007
Population 4	14	Baseline samples from Washington used by Peever et al, 2004

Popgene (Yeh et al, 1997) was used to calculate the heterozygosity (h) and the Shannon's index (I) values within populations and among populations. The hierarchical Analysis of Molecular Variance Analysis (AMOVA) was performed using GENALEX (Peakall and Smouse, 2006). The genetic diversity and distribution of variation among populations (PhiPT), among regions (PhiRT), i.e. between North Dakota and Washington, and among populations within regions

(PhiPR) were examined by this analysis. The statistical P-values were also estimated to test the hypothesis that these populations are different. These probability value estimates were calculated based upon 999 permutations. Pairwise genetic identity and pairwise genetic distance were also calculated by AMOVA analysis using GENALEX.

RESULTS AND DISCUSSION

Microsatellite Analysis

A total of 190 individual samples were analyzed in this population genetics study. Out of these samples, 176 samples were sampled from North Dakota over three years while the other 14 samples were baseline samples obtained from the Pacific Northwest prior to the use of strobin fungicides. These baseline samples were used by Peever et al (2004) in their *Ascochyta rabiei* population genetic studies. The whole population of 190 samples was divided into subpopulations allowing the comparison of the North Dakota *Ascochyta rabiei* populations with the Pacific Northwest population and the comparison of the populations of *Ascochyta rabiei* in North Dakota from different years.

The 176 North Dakota samples were divided into three populations, each comprised of the samples obtained in the years 2005, 2006 and 2007 representing populations one through three, respectively. The 14 baseline samples were grouped as the fourth population. Thus, the whole population was also divided into 2 regions, the North Dakota population having 176 samples and the Pacific Northwest population having only 14 samples.

A total of 34 polymorphic loci were produced by 4 primer sets used for microsatellites. These loci, listed in Table 7, were scored in the four populations as explained in the materials and methods section. The data generated by the

Table 7. PCR Products From Microsatellite Amplification in *A. rabiei.*

Primer	Size of the polymorphic bands generated
ArA06T	150 bp, 160 bp, 175 bp, 180 bp
ArH02T	263 bp, 280 bp, 285 bp, 288 bp, 295 bp, 300 bp, 305 bp, 315 bp, 320 bp, 325 bp, 330 bp, 335 bp, 345 bp, 400 bp
ArR12D	165 bp, 175 bp, 185 bp, 190 bp, 200 bp
ArH05T	202 bp, 204 bp, 208 bp , 217 bp, 221 bp, 225 bp, 235 bp, 240 bp 258bp, 265bp, 325bp

microsatellites was analyzed by two programs, GENALEX and Popgene. The program GENALEX can only calculate the genetic variation between two or more populations but cannot calculate genetic variation within one population while the opposite is true for program Popgene. GENALEX was used to make comparisons between populations 1, 2, 3 and 4 while Popgene was used to calculate genetic variation within each population.

The division of the 190 individual samples into 4 different populations is based upon a hypothesis that each population is different from the other. The null hypothesis for all of the genetic comparisons is that the populations are not genetically different. This null hypothesis was also tested with the program GENALEX using the probability method. A probability value below 5% would result in rejection of the null hypothesis to be tested.

The program GENALEX uses AMOVA (Analysis of Molecular Variance Analysis) to analyze the genetic diversity between the different populations. AMOVA analysis calculates the Phi values which are a statistical representation

of the genetic diversity. PhiRT represents the genetic difference between the regions, thus it has been used to analyze the genetic variation between the North Dakota population and the Pacific Northwest population. In other words, PhiRT represents the genetic difference between populations 1, 2 and 3 from population 4. PhiPR represents the genetic differentiation within the region and has been used to analyze the variation between the North Dakota populations 1, 2 and 3. PhiRT and PhiPR taken together represent the PhiPT which is total differentiation among all 4 populations.

In the AMOVA analysis using GENALEX the total differentiation among populations (PhiPT) is 0.142 with 0.103 being due to differentiation between the regions (PhiRT) and 0.043 due to differentiation between the populations within the region (PhiPR). These results are shown in Table 8. The probability value generated by AMOVA analysis is only 0.1% indicating that the null hypothesis that the populations are different from each other is incorrect.

Pairwise population analysis to compare the populations with each other was calculated using GENALEX. This analysis shows that the maximum genetic distance is between the Pacific Northwest population and the three North Dakota

Table 8: Statistical Values Generated by GENALEX for Microsatellites.

Statistical property	Value	Probability value
PhiRT	0.103	0.001
PhiPR	0.043	0.001
PhiPT	0.142	0.001

populations. There is negligible genetic distance between the North Dakota populations, further supporting the conclusion that the three North Dakota populations are genetically the same with no significant polymorphism between them. The pairwise population matrix of genetic distance is shown in Table 9.

Table 9: Pairwise Population Matrix of Nei Genetic Distance Based on Microsatellite Analysis.

	Pop 1	Pop 2	Pop 3	Pop 4
Pop 1	0.000			
Pop 2	0.022	0.000		
Pop 3	0.010	0.008	0.000	
Pop 4	0.035	0.059	0.043	0.000

Since the GENALEX software can only analyze the molecular variance between populations and cannot analyze the diversity between the individual samples within the population, the program Popgene was used to calculate heterozygosity or variation within the different populations. The analysis of the heterozygosity using Popgene resulted in very low h values (heterozygosity or variation). The heterozygosity within the populations was found to be 0.1832, 0.1498, 0.1656 and 0.1744 for populations 1, 2, 3 and 4 respectively. The heterozygosity among the four populations combined was 0.1788. These very low heterozygosity values clearly indicate that there is very little genetic diversity within the tested populations.

AFLP Analysis

A total of 6 polymorphic loci from a single set of restriction enzymes were scored in the complete set of 190 samples. AMOVA analysis, using GENALEX, determined that the total differentiation among populations (PhiPT) is 0.039 where -0.016 is due to differentiation between the regions (PhiRT) and 0.054 is due to differentiation between the populations within the region (PhiPR). The probability values are shown with the differentiation values in Table 10.

Table 10. Statistical Values Generated by GENALEX Based on AFLP Analysis.

Statistical property	Value	P value
PhiRT	-0.016	0.769
PhiPR	0.054	0.001
PhiPT	0.039	0.048

The high probability value for PhiRT using AMOVA analysis for AFLPs indicates that there is a high probability that the North Dakota population is genetically different from the baseline population from the Pacific Northwest. While the low probability values for PhiPT and PhiPR again indicate that populations 1, 2 and 3 from North Dakota are essentially identical and that there is no significant genetic diversity between them.

Pairwise population analysis for AFLPs using GENALEX, Table 11, shows a maximum genetic distance between the Pacific Northwest population and the North Dakota populations although this genetic distance is not very great.

Table 11: Pairwise Population Matrix of Nei Genetic Distance for AFLPs.

	Pop 1	Pop 2	Pop 3	Pop 4
Pop 1	0.000			
Pop 2	0.031	0.000		
Pop 3	0.037	0.050	0.000	
Pop 4	0.055	0.091	0.094	0.000

Mating Type Distribution

Only the North Dakota population was tested for mating type. Out of 176 isolates, 74 were found to be mating type MAT1-1 while 101 were found to be mating type MAT1-2. The presence of both mating types in nearly 1:1 ratio in North Dakota leads suggests that the *Ascochyta rabiei* population in North Dakota is a randomly mating population.

The population genetic studies define the genetic structure of the population. The genetic structure of the population can be used in assessing the potential of that population to evolve further. This population genetic study has showed that the Ascochyta population in North Dakota across three years 2005, 2006 and 2007 is genetically similar but it is a randomly mating population. Previous studies have shown that natural recombination through the sexual cycle has developed new pathogen races with greater virulence on resistant cultivars. Therefore the North Dakota *Ascochyta rabiei* population possesses a potential to genetically evolve further. Also, the AFLP results have shown that the baseline ascochyta population from the Pacific Northwest is genetically different than the North Dakota population.

The genetic difference in ascochyta populations between different geographical regions poses an interesting problem for the breeder. It suggests that the population can change quickly between different geographical regions given that it has been just over 25 years since the pathogen was introduced into the United States. Although several waves of introduction have occurred, the introduced pathogens may have been different in the two regions resulting in genetically different populations in the two regions. The data suggests that the population in North Dakota is an interbreeding population which would result in the assimilation of the different introductions into one population that could be significantly different from that found in other regions of the country. This then could partially explain, along with environmental factors, the differences in the susceptibility of resistant cultivars to the pathogen found in North Dakota versus the Pacific Northwest.

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APPENDIX A

Listed in Table A1 are the isolates used, the point of collection, and the figures in Appendix A where they were separated by SDS polyacrylamide gel electrophoresis.

Table A1. *Ascochyta rabiei* Isolates Used in This Study.

Isolate	Collection Location	Shown in Figure	Buffer Group
05101CA1	Carrington	A21	2
05101M2	Minot	A5	1
05101STM6	Minot	A15	1
05101STM8	Minot	A3	2
05103M3	Minot	A2	2
05103M6	Minot	A15	1
05103M7	Minot	A18	1
05105C8	Carrington	A4	1
05105M2	Minot	A11- A13, A17	3 - 3, 1
05105M5	Minot	A16	1
05105M9	Minot	A4	1
05106M2	Minot	A6	2
05106M8	Minot	A18	1
05107CA10	Carrington	A16	1
05107C4	Carrington	A15	1
05107M5	Minot	A5	1
05108M1	Minot	A15, 5	1, 3
05109M9	Minot	A21	2
05110M4	Minot	A4	1
05111M1	Minot	A5, 5	1, 3
05112M3	Minot	A16	1
05202STM2	Minot	A3, A11 - A13, 5	2, 3 – 3, 3
05203C1	Carrington	A15	1
05204CA1	Carrington	A17	1
05205C6	Carrington	A16	1
05206CA1	Carrington	A17	1
05206CA4	Carrington	A15	1
05207C2	Carrington	A5	1
05207C5	Carrington	A19	2
05210C6	Carrington	A5	1

Table A1. (continued)

Isolate	Collection Location	Shown in Figure	Buffer Group
05211C1	Carrington	A17, 5	1, 3
05302C1	Carrington	A19	2
05303C1	Carrington	A2	2
05303C6	Carrington	A15	1
05305C6	Carrington	A2	2
05305CA2	Carrington	A18	1
05311CA1	Carrington	A18	1
05412CA3	Carrington	A2	2
05412CA9	Carrington	A3	2
05APNS2	Prosper	A2	2
05APNS3	Prosper	A11 - A13, A17	3 - 3, 1
05APNS4	Prosper	A6	2
05BND1	Fort Berthold	A5	1
05BND2	Fort Berthold	A19	2
05DPNS3	Prosper	A17	1
05DPNS6	Prosper	A18	1
05HSD12	Highmore, SD	A2	2
05MPNS2	Prosper	A17	1
05MPNS4	Prosper	A20	2
05SP2-1	Prosper	A17	1
05SP2-2	Prosper	A3	2
05SUCO2	Sully County, SD	A18	1
06101M2	Minot	A4	1
06212M1	Minot	A20	2
06BND2	Fort Berthold	A6, 5	2, 3
06BND6	Fort Berthold	A20	2
06BND8	Fort Berthold	A19	2
06BND10	Fort Berthold	A5	1
06BND11	Fort Berthold	A6	2
06BRF1-9	Fort Berthold Field 1	A2	2
06BWEF2-8	White Earth Field 2	A17	1
06BWEF2-10	White Earth Field 2	A4	1
06BWEF2-26	White Earth Field 2	A15	1
06BWEF2-31	White Earth Field 2	A19	2
06BWEF2-37	White Earth Field 2	A5	1
06BWEF2-46	White Earth Field 2	A16	1
06FRL1	Fargo	A18	1
06FRL2	Fargo	A20	2

Table A1. (continued)

Isolate	Collection Location	Shown in Figure	Buffer Group
06KND1A-2	Kenmare Field 1	A6, 5	2, 3
06KND1A-10	Kenmare Field 1	A16	1
06KND1A-18	Kenmare Field 1	A19	2
06KND1A-21	Kenmare Field 1	A18	1
06KND2A-1	Kenmare Field 2	A20	2
06KND2A-9	Kenmare Field 2	A20	2
06KND2A-10	Kenmare Field 2	A4	1
06KND2A-18	Kenmare Field 2	A4	1
06MB7	Minot	A5	1
06MB10	Minot	A4, A18, 5	1, 3, 1, 3
06MSTD2	Minot	A19, 5	2, 3
06MSTD13	Minot	A20	2
06MSTS1	Minot	A19	2
06MSTS2	Minot	A4	1
06NDG1	Minot seed increase field	A6	2
06STC2-5	Carrington	A20	2
06STMD13	Minot	A20	2
06TLND14	Turtle Lake	A2, A11 - A13	3, 3 – 3
06TLND57	Turtle Lake	A21	2
06TLND63	Turtle Lake Field 2	A3	2
06TLND65	Turtle Lake	A15	1
06TLNDF1-4	Turtle Lake	A20	2
06TLNDF1-7	Turtle Lake	A16	1
06TNDF1-5	Tioga	A16	1
06TNDF1-8	Tioga	A19	2
06TNDF1-10	Tioga Field 1	A3	2
07BMXP-3	Makoti Field 1	A7	3, 3
07BMXP-13	Makoti Field 1	A8	3, 3
07BMXP-36	Makoti Field 1	A7	3
07BMXP-43	Makoti Field 1	A7	3
07CWF2-11	Williston Field 2	A7	3, 3
07CWF2-13	Williston Field 2	A8	3
07CWF2-15	Williston Field 2	A8	3
07CWF2-16	Williston Field 2	A14	3
07CWF3-15	Divide County Field 3	A7	3
07CWF3-16	Divide County Field 3	A14	3
07CWF3-20	Divide County Field 3	A8	3

Table A1. (continued)

Isolate	Collection Location	Shown in Figure	Buffer Group
07CWF3-22	Divide County Field 3	A14	3
07DF8	Donnybrook	A14	3
07DF9	Donnybrook	A14	3
07DX23	Donnybrook	A14	3
07DX30	Donnybrook	A14	3
07GF-F1-18	Grenora Field 1	A21	2
07GF-F1-2	Grenora Field 1	A10	3
07GF-F1-27	Grenora Field 1	A8	3
07GF-F1-28	Grenora Field 1	A8	3
07GF-F2-1	Grenora Field 2	A21	2
07GF-F2-4	Grenora Field 2	A7	3, 3
07GF-F2-9	Grenora Field 2	A8	3
07GF-F2-10	Grenora Field 2	A21	2
07GF-F3-2	Grenora Field 3	A1	3
07GF-F3-3	Grenora Field 3	A22	3
07GF-F3-4	Grenora Field 3	A1	3
07GF-F3-13	Grenora Field 3	A1	3
07H105-2	Hettinger	A10	3
07H105-6	Hettinger	A10	3
07H201-2	Hettinger	A22	3
07H406-6	Hettinger	A8	3
07HRF11	Ray	A10	3
07HRF21	Ray	A6	2
07HRF26	Ray	A21	2
07HRF37	Ray	A10	3
07KMB9F2-11	Makoti Field 2	A6	2
07KMB9F2-17	Makoti Field 2	A21	2
07KMB9F2-30	Makoti Field 2	A10	3
07KMB9F2-35	Makoti Field 2	A14	3
07KRF-F1-2	Ray Field 1	A1	3
07KRF-F1-5	Ray Field 1	A1	3
07KRF-F1-30	Ray Field 1	A1	3
07KRF-F1-33	Ray Field 1	A1	3
07KRF-F2-2	Ray Field 2	A1	3
07KRF-F2-3	Ray Field 2	A1	3
07KRF-F3-31	Ray Field 3	A9	3
07KRF-F3-32	Ray Field 3	A9	3
07M109-1	Minot	A10	3

Table A1. (continued)

Isolate	Collection Location	Shown in Figure	Buffer Group
07M204-7	Minot	A8	3
07M306-1	Minot	A7	3, 3
07M313-8	Minot	A14	3
07MLMT2	Medicine Lake, MT	A9	3
07MLMT11	Medicine Lake, MT	A22	3
07MLMT13	Medicine Lake, MT	A9	3
07MLMT16	Medicine Lake, MT	A22	3
07TF-F1-10	Tioga	A9	3
07TF-F1-12	Tioga	A22	3
07TF-F1-16	Tioga	A9	3
07TF-F1-17	Tioga	A22	3
07TSF1-21	Tioga Field 1	A9	3
07TSF1-27	Tioga Field 1	A22	3
07TSF1-30	Tioga Field 1	A9	3
07TSF1-37	Tioga Field 1	A9	3
07TSF2-1	Tioga Field 2	A10	3
07TSF2-10	Tioga Field 2	A6	2
07TSF2-46	Tioga Field 2	A21	2
07TSF2-53	Tioga Field 2	A10	3
07W201-3	Williston	A22	3
07W201-16	Williston	A7	3
07W201-18	Williston	A22	3
07W305-10	Williston	A7	3
AR453	Genesse, ID	A2	2

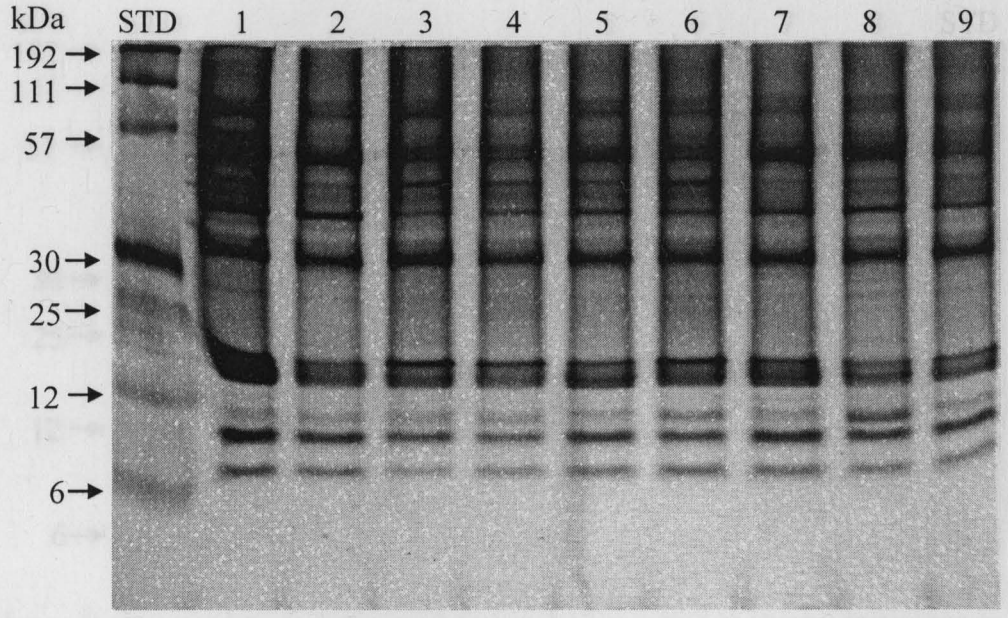

Figure A1. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 1; 1: 07GF-F3-2, 2: 07GF-F3-4, 3: 07GF-F3-13, 4: 07KRF-F1-33, 5: 07KRF-F1-2, 6: 07KRF-F1-5, 7: 07KRF-F1-30, 8: 07KRF-F2-2, 9: 07KRF-F2-3

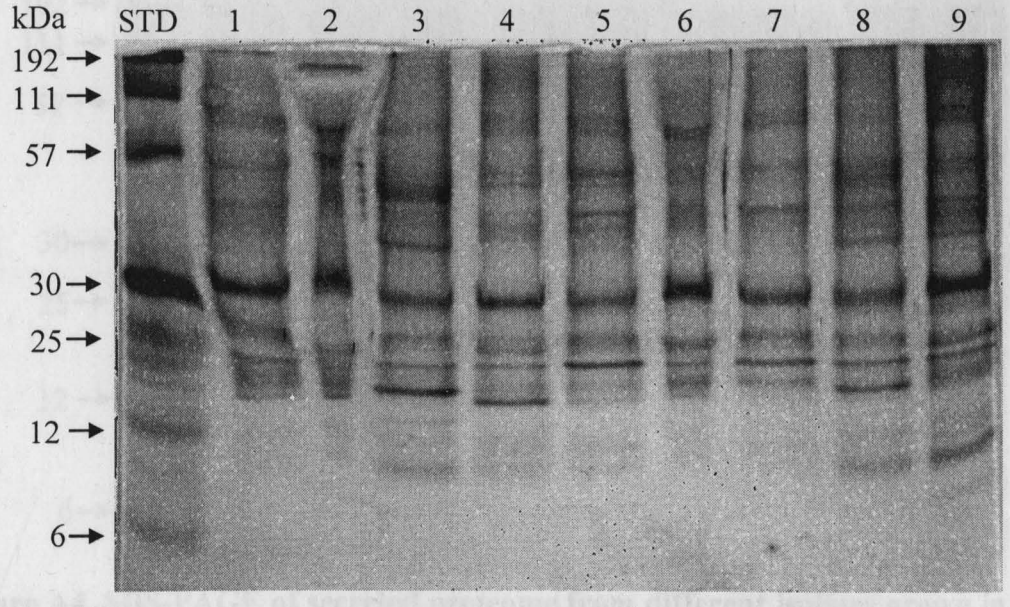

Figure A2. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 2; 1: 05APNS2, 2: 06TLND14, 3: AR453, 4: 05HSD12, 5: 05412CA3, 6: 06BRF1-9, 7: 05103M3, 8: 05305C6, 9: 05303C1

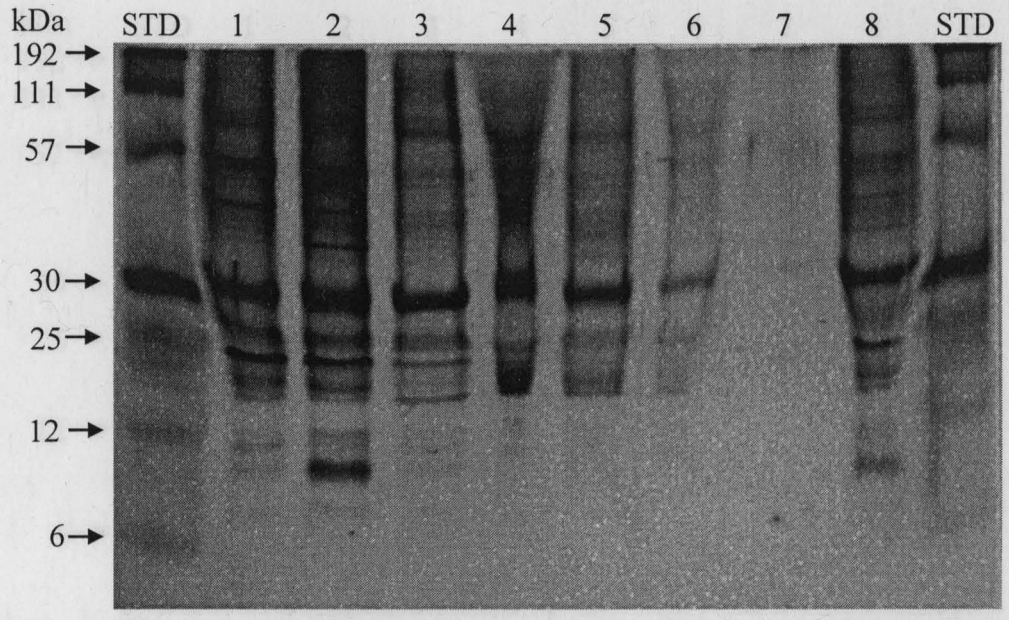

Figure A3. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 3; 1: 05412CA9, 2: 05202STM2, 3: 05SP2-2, 4: 06TLND63, 5: 06TNDF1-10, 6: spill over from lane 5, 7: BLANK, 8: 05101STM8

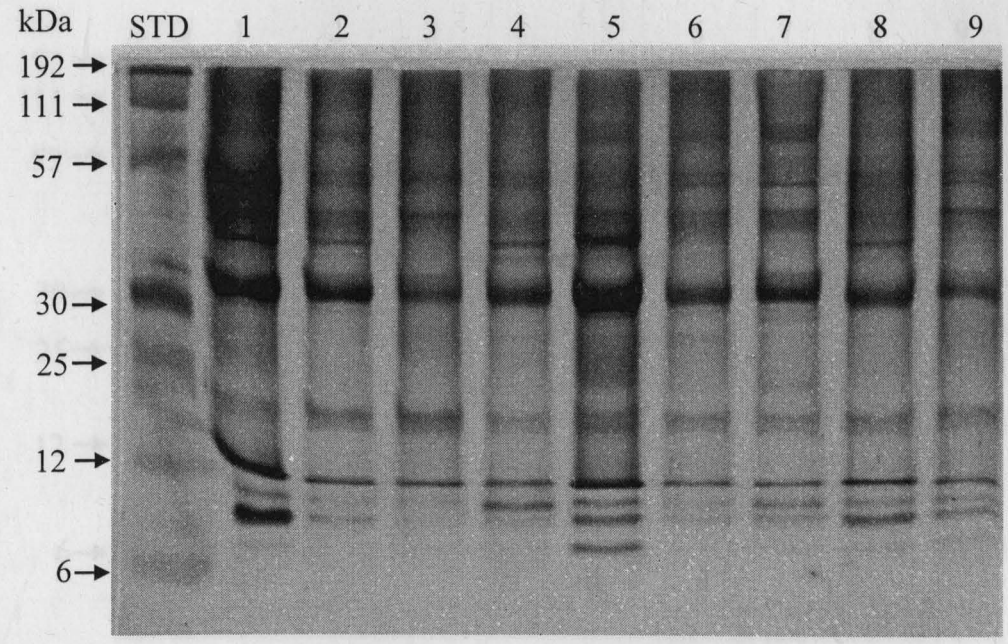

Figure A4. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 4; 1: 06BWEF2-10, 2: 05110M4, 3: 05105C8, 4: 06KND2A-18, 5: 06MB10, 6: 05105M9, 7: 06101M2, 8: 06KND2A-10, 9: 06MSTS2

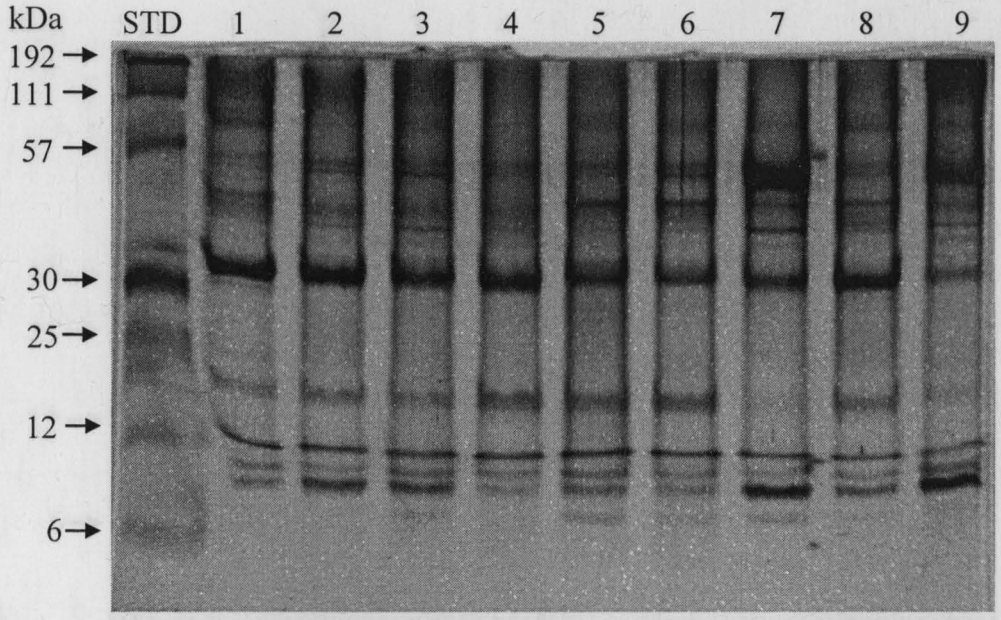

Figure A5. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 5; 1: 06BWEF2-37, 2: 05107M5, 3: 05111M1, 4: 06BND10, 5: 06MB7, 6: 05101M2, 7: 05207C2, 8: 05210C6, 9: 05BND1

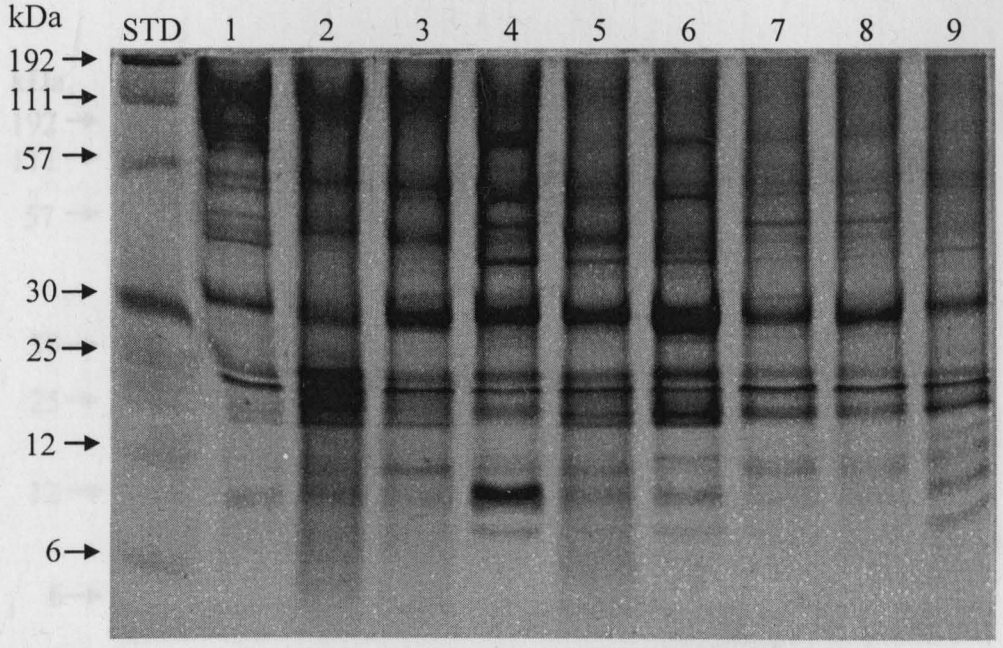

Figure A6. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 6; 1: 05APNS4, 2: 06NDG1, 3: 06BND11, 4: 05106M2, 5: 07KMB9F2-11, 6: 07TSF2-10, 7: 07HRF21, 8: 06BND2, 9: 06KND1A-2

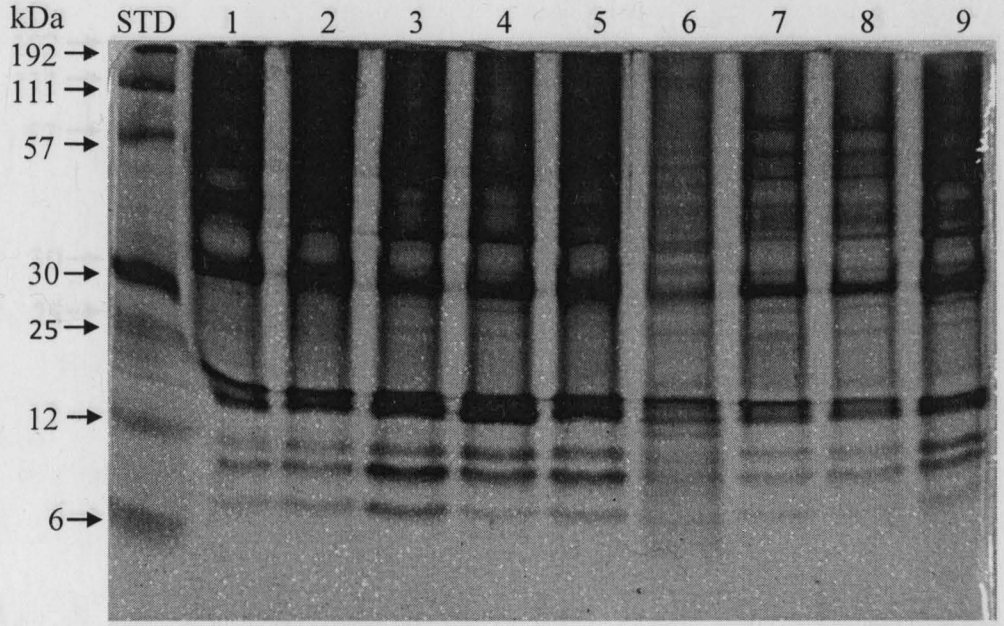

Figure A7. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 7; 1: 07BMXP-3, 2: 07CWF3-15, 3: 07GF-F2-4, 4: 07CWF2-11, 5: 07M306-1, 6: 07W305-10, 7: 07BMXP-43, 8: 07BMXP-36, 9: 07W201-16

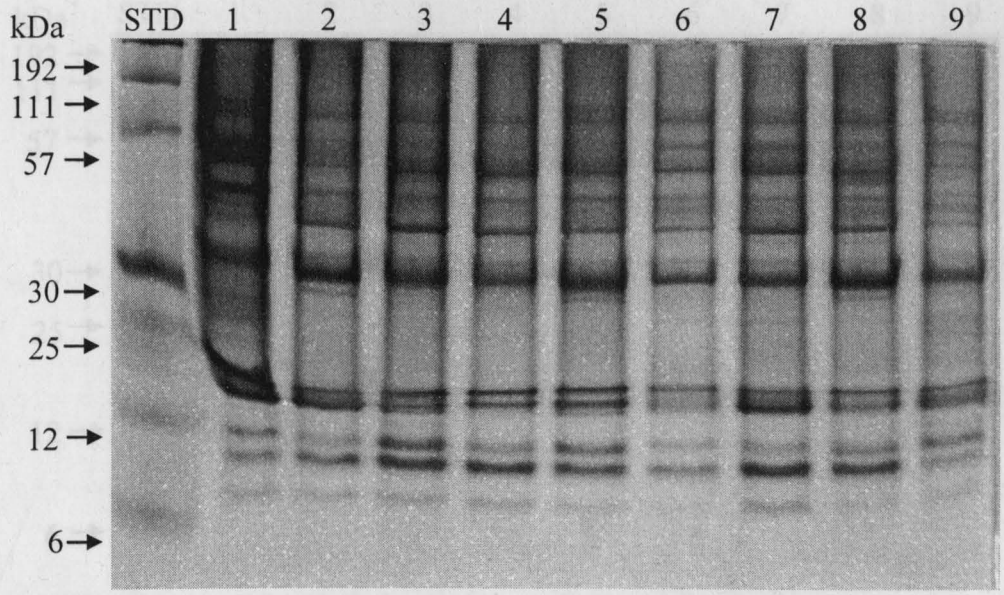

Figure A8. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 8; 1: 07CWF3-20, 2: 07CWF2-15, 3: 07GF-F1-27, 4: 07GF-F2-9, 5: 07GF-F1-28, 6: 07BMXP-13, 7: 07M204-7, 8: 07H406-6, 9: 07CWF2-13

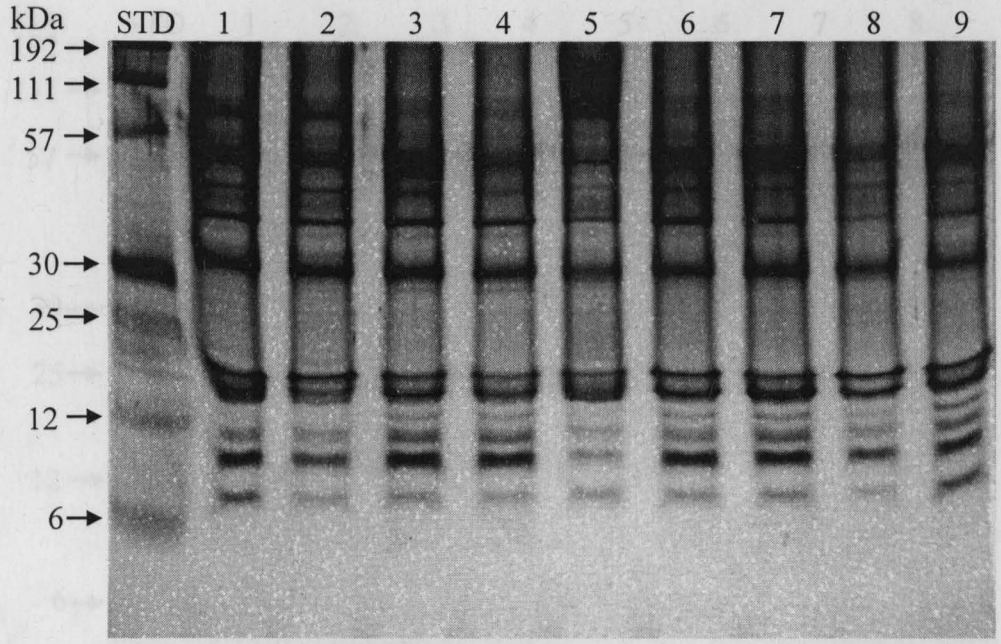

Figure A9. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 9; 1: 07KRF-F3-31, 2: 07KRF-F3-32, 3: 07MLMT2, 4: 07MLMT13, 5: 07TF-F1-10, 6: 07TF-F1-16, 7: 07TSF1-30, 8: 07TSF1-37, 9: 07TSF1-21

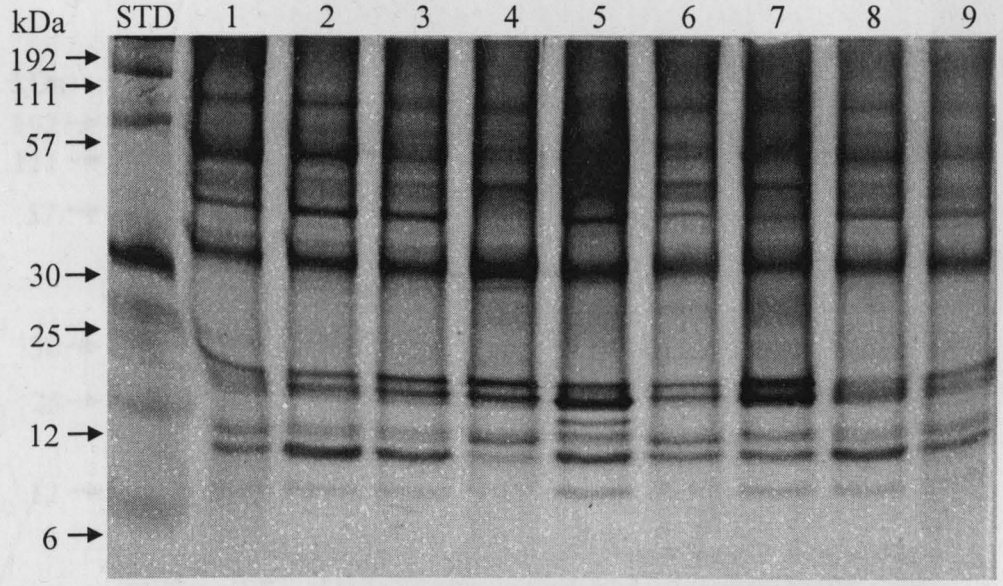

Figure A10. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 10; 1: 07KMB9F2-30, 2: 07TSF2-1, 3: 07TSF2-53, 4: 07M109-1, 5: 07GF-F1-2, 6: 07HRF11, 7: 07HRF37, 8: 07H105-2, 9: 07H105-6

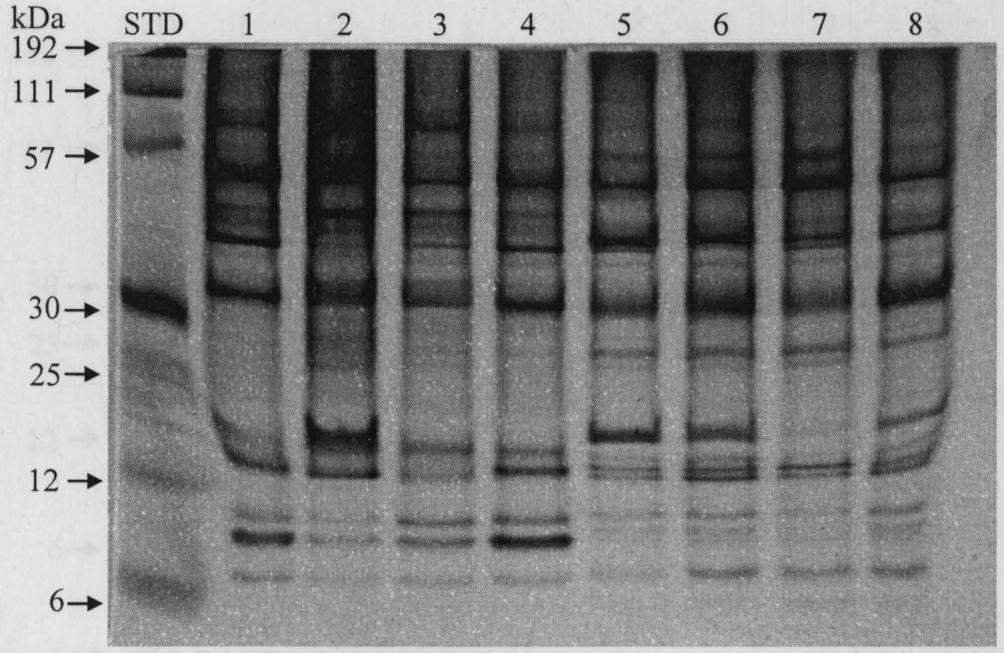

Figure A11. SDS-PAGE of secreted proteome from representative isolates; 1: 06TLND14, 2: 05105M2, 3: 05APNS3, 4: 05202STM2, 5: 06TLND14, 6: 05105M2, 7: 05APNS3, 8: 05202STM2. 1, 2, 3, 4 were grown in modified Fries media while 5, 6, 7, 8 were grown in Czapek Dox media.

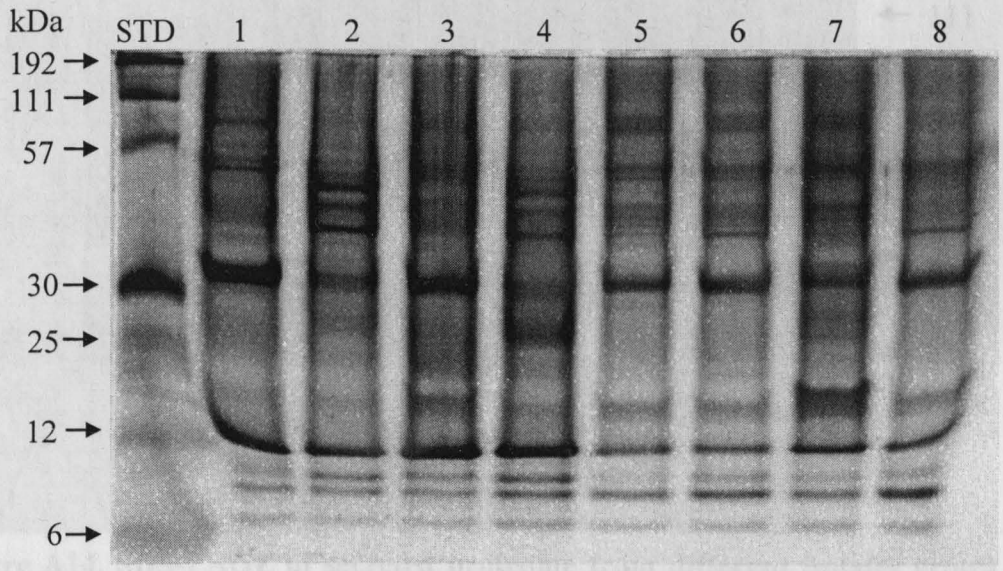

Figure A12. SDS-PAGE of secreted proteome from representative isolates grown in modified Fries media; 1: 05APNS3, 2: 06TLND14, 3: 05105M2, 4: 05202STM2, 5: 05APNS3, 6: 06TLND14, 7: 05105M2, 8: 05202STM2

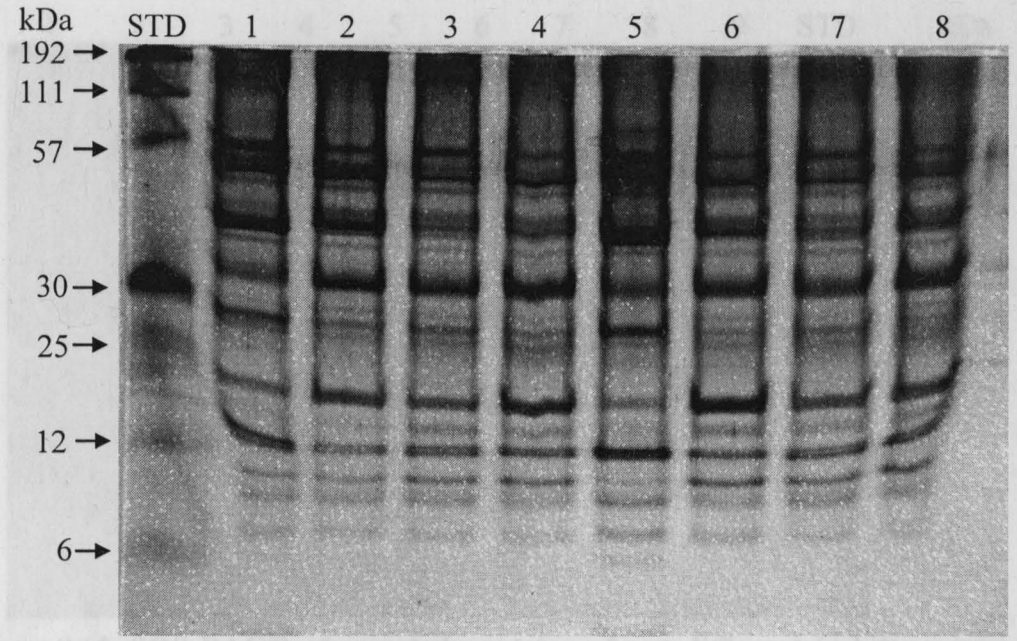

Figure A13. SDS-PAGE of secreted proteome from representative isolates grown in Czapek Dox media; 1: 05APNS3, 2: 06TLND14, 3: 05105M2, 4: 05202STM2, 5: 05APNS3, 6: 06TLND14, 7: 05105M2, 8: 05202STM2

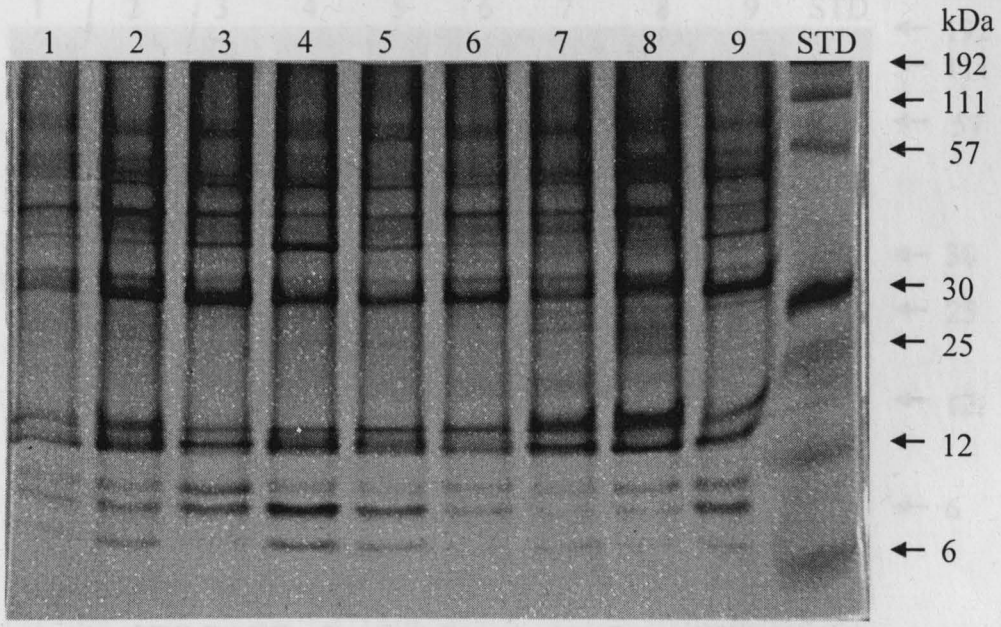

Figure A14. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 14; 1: 07CWF2-16, 2: 07DF8, 3: 07DX23, 4: 07CWF3-16, 5: 07KMB9F2-35, 6: 07M313-8, 7: 07DX30, 8: 07CWF3-22, 9: 07DF9

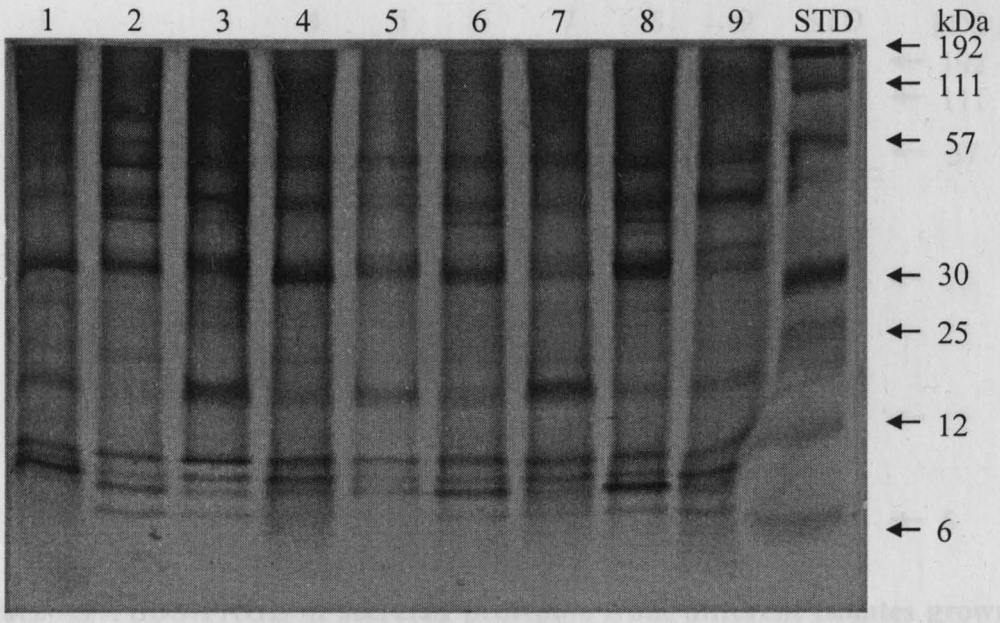

Figure A15. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 15; 1: 06BWEF2-26, 2: 05303C6, 3: 05108M1, 4: 05TLND65, 5: 05206CA4, 6: 05203C1, 7: 05101STM6, 8: 05103M6, 9: 05107C4

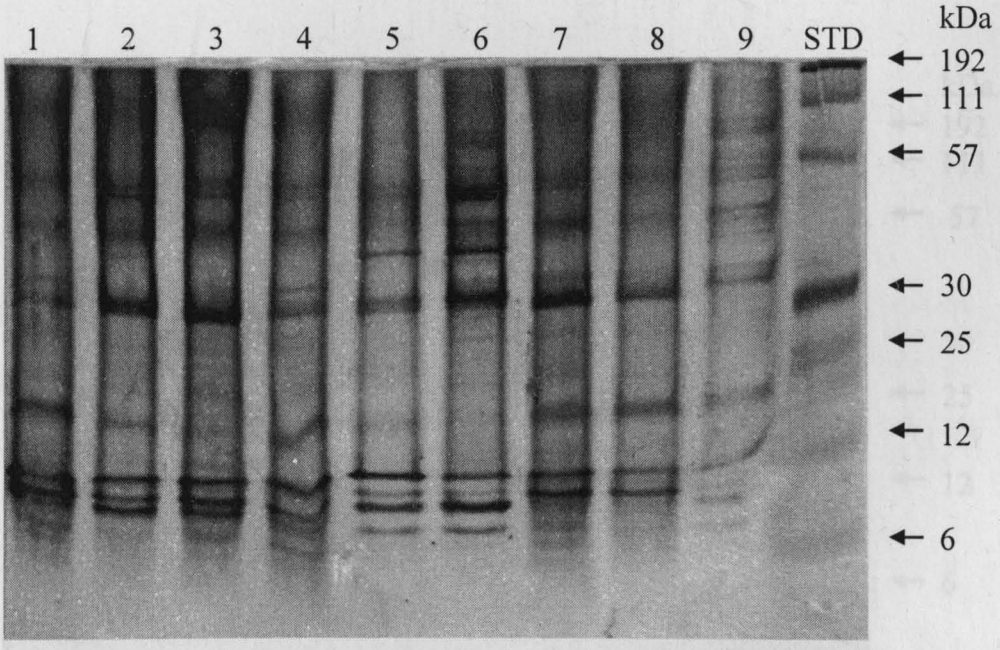

Figure A16. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 16; 1: 06TLNDF1-7, 2: 05107CA10, 3: 06BWEF2-46 (5 day), 4: 06KND1A-10, 5: 05112M3, 6: 05205C6, 7: 06TNDF1-5, 8: 06BWEF2-46 (10 day), 9: 05105M5

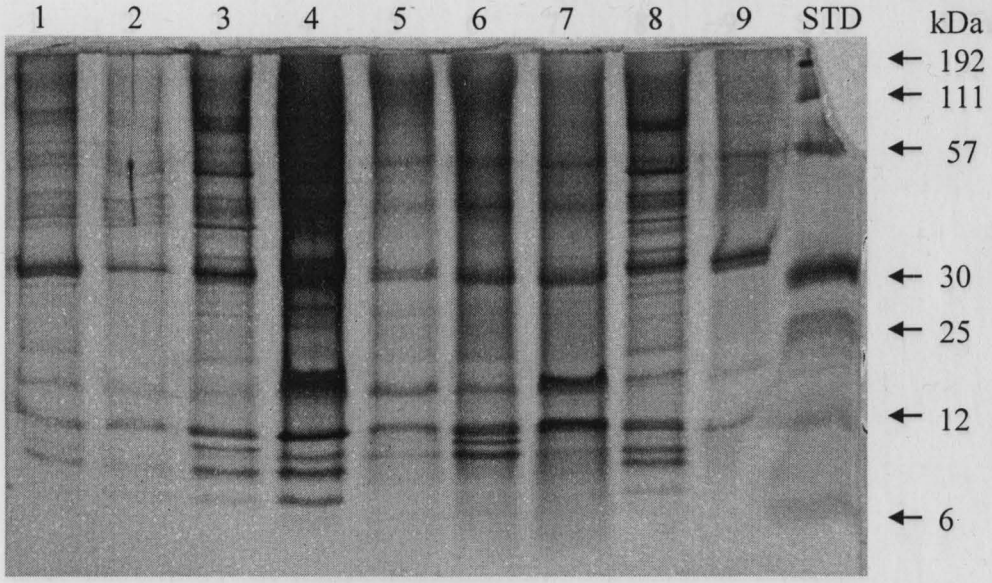

Figure A17. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 17; 1: 05206CA1, 2: 05DPNS3, 3: 05204CA1, 4: 05211C1, 5: 05MPNS2, 6: 05SP2-1, 7: 05APNS3, 8: 05105M2, 9: 06BWEF2-8

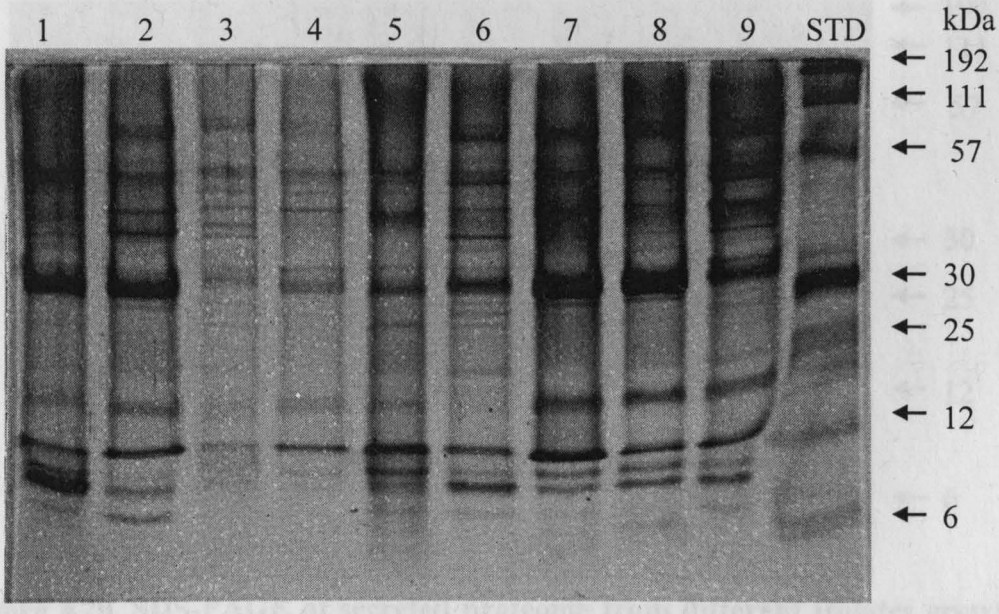

Figure A18. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 18; 1: 06KND1A-21, 2: 06MB10, 3: 05106M8, 4: 05311CA1, 5: 06FRL1, 6: 05305CA2, 7: 05103M7, 8: 05SUCO2, 9: 05DPNS6

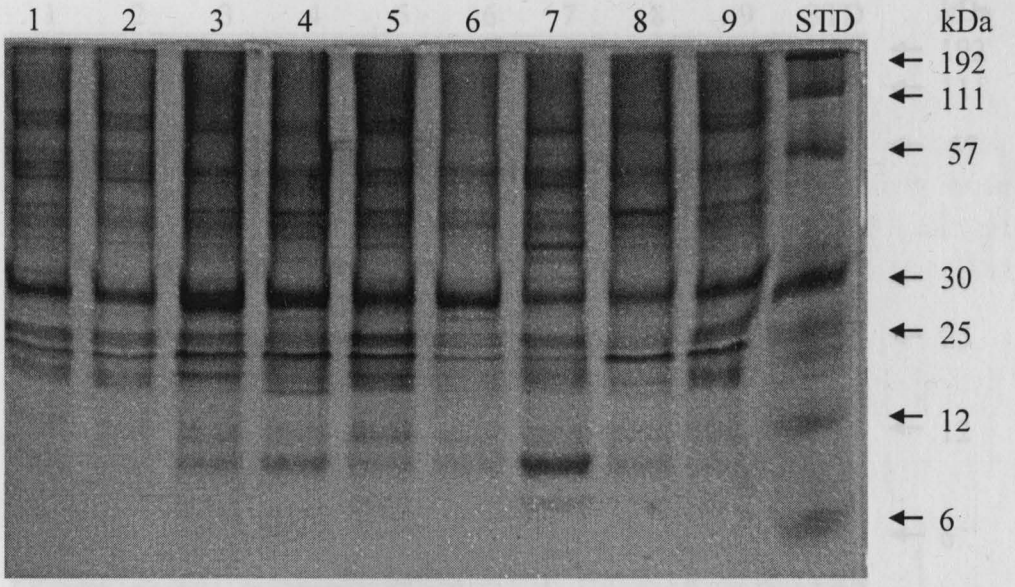

Figure A19. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 19; 1: 06KND1A-18, 2: 06BND8, 3: 06MSTS1, 4: 05302C1, 5: 06TNDF1-8, 6: 06BWEF2-31, 7: 05BND2, 8: 05207C5, 9: 06MSTD2

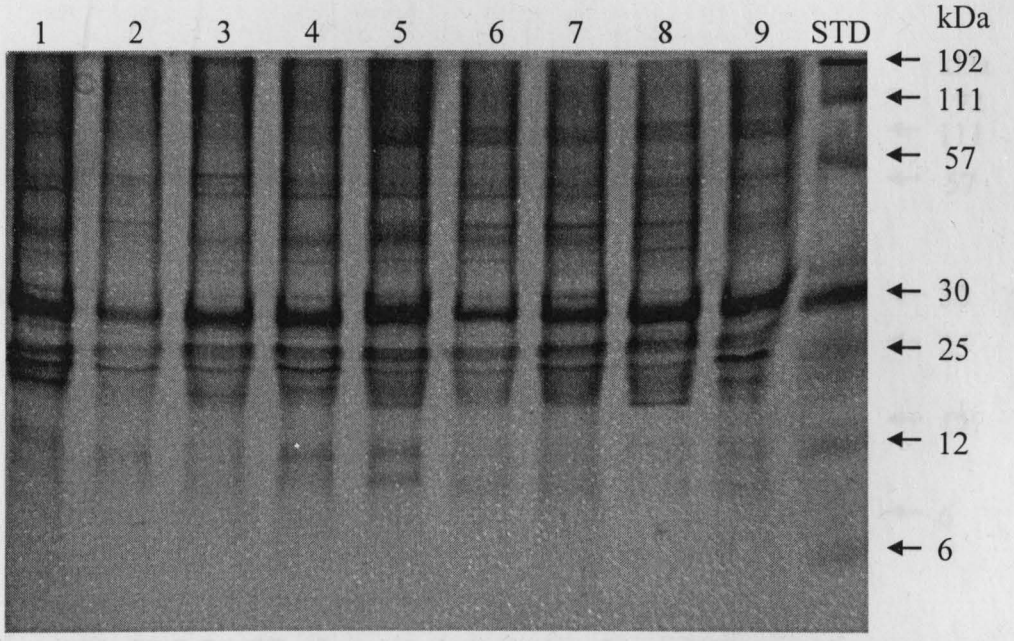

Figure A20. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 20; 1: 06MSTD13, 2: 05MPNS4, 3: 06212M1, 4: 06STC2-5, 5: 06FRL2, 6: 06BND6, 7: 06KND2A-9, 8: 06KND2A-1, 9: 06TLNDF1-4

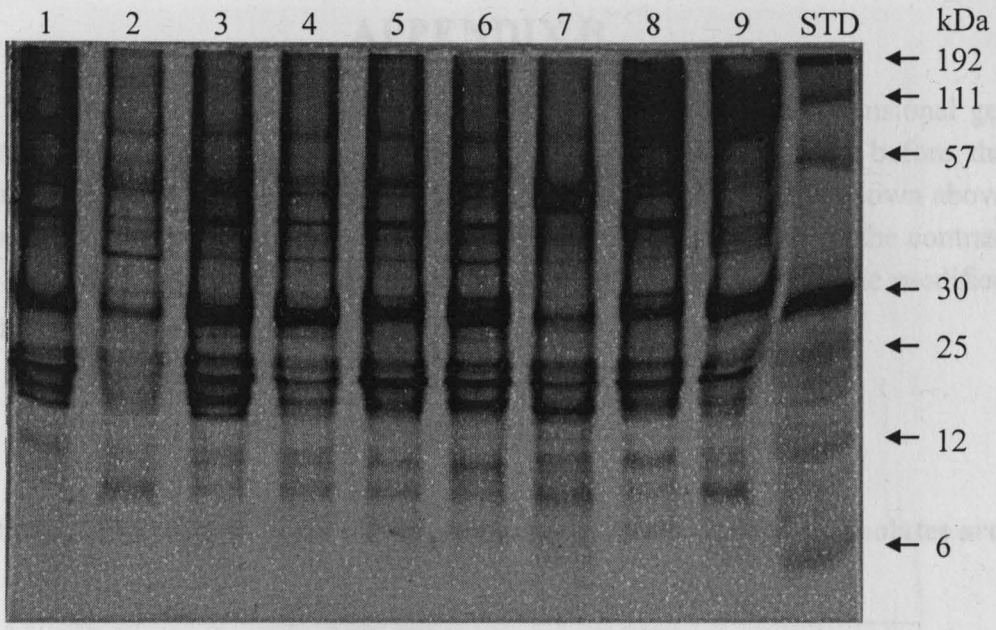

Figure A21. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 21; 1: 07HRF26, 2: 07GF-F2-10, 3: 07GF-F2-1, 4: 07GF-F1-18, 5: 07TSF2-46, 6: 07KMB9F2-17, 7: 05101CA1, 8: 05109M9, 9: 06TLND57

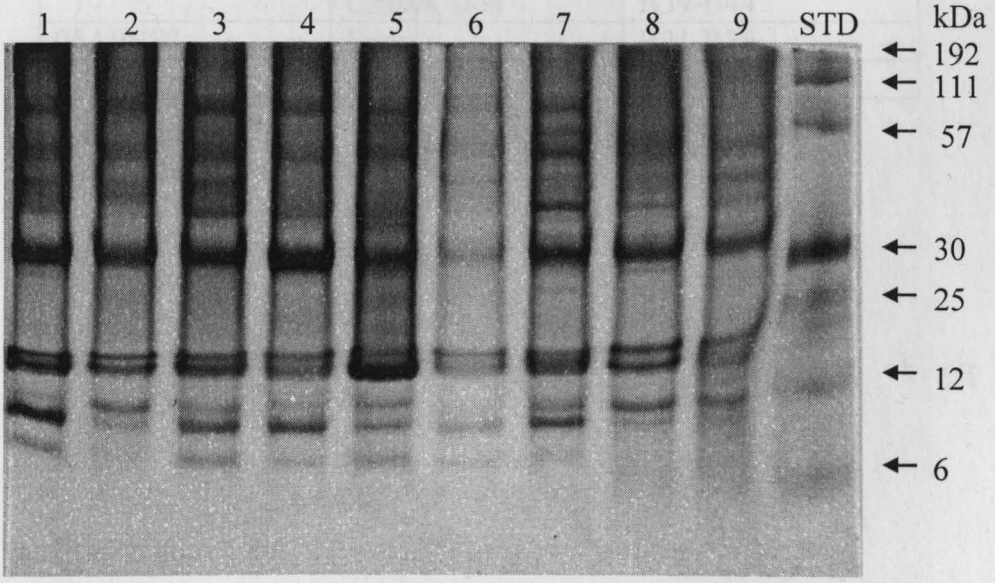

Figure A22. SDS-PAGE of secreted proteome from different isolates grown in modified Fries media, Group 22; 1: 07TSF1-27, 2: 07TF-F1-17, 3: 07TF-F1-12, 4: 07MLMT16, 5: 07MLMT11, 6: 07GF-F3-3, 7: 07W201-3, 8: 07W201-18, 9: 07H201-2

APPENDIX B

Presented in this appendix are the images from the two dimensional gel electrophoresis experiments. Each isolate was grown for 10 days before the culture filtrate was recovered. For each gel an unmodified image is shown above an image where areas of the image were selected for enhancement of the contrast and brightness to better indicate the presence of a protein spot. In the modified images the protein spots are indicated and numbered.

Table B1. Listing of Where Two-Dimensional Gels of Different Isolates are Located.

Isolate	Media	Figure
05105M2	Fries	B1- B10
	Czapek Dox	B27-B32
05202STM2	Fries	B11-B16
	Czapek Dox	B33-B38
06TLND14	Fries	B17-B20
	Czapek Dox	B39-B44
05APNS3	Fries	B21-B26
	Czapek Dox	B45-B48

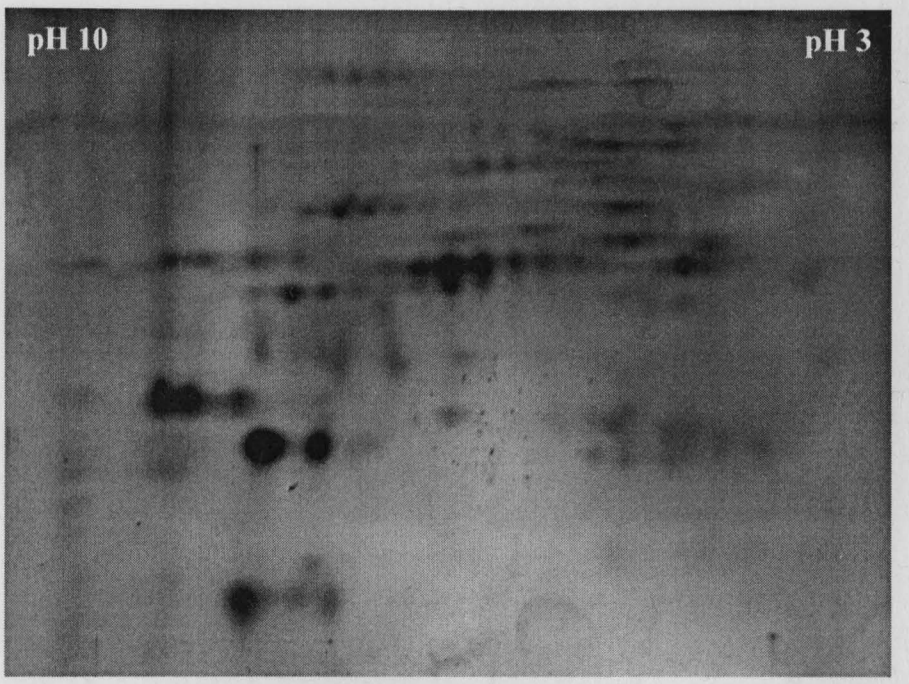

Figure B1. Two-dimensional gel of isolate 05105M2 grown in modified Fries media on 5-22-09.

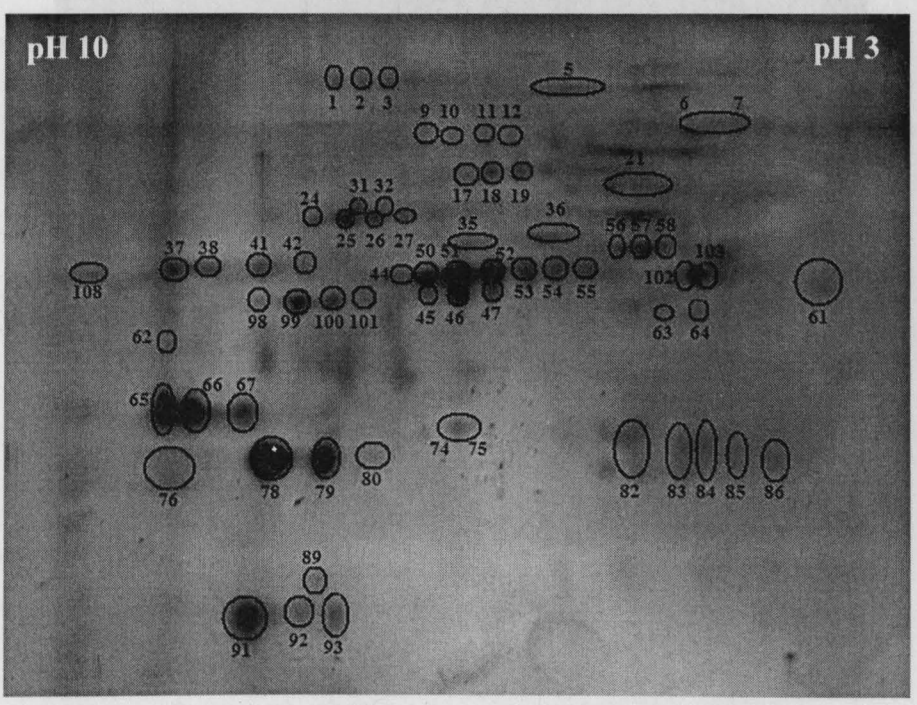

Figure B2. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 5-22-09.

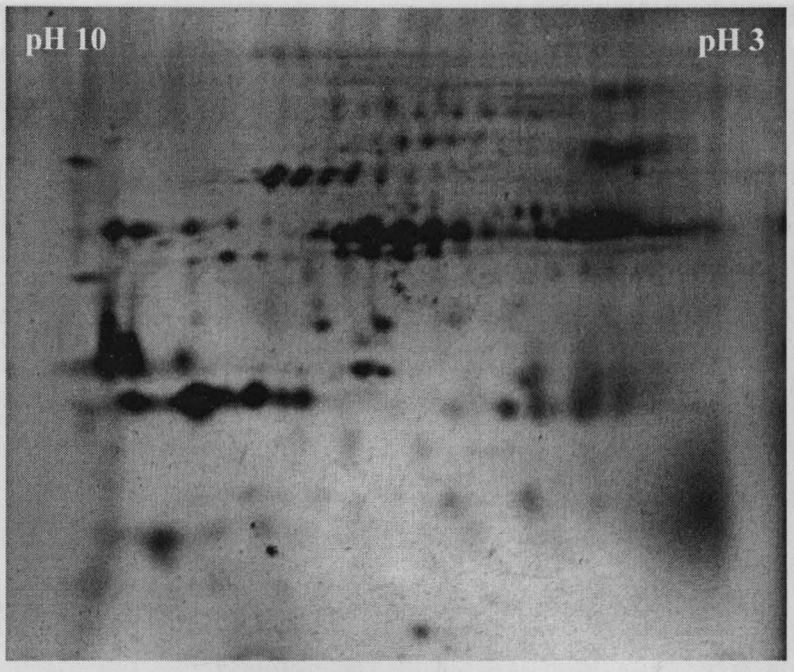

Figure B3. Two-dimensional gel of isolate 05105M2 grown in modified Fries media on 5-27-09.

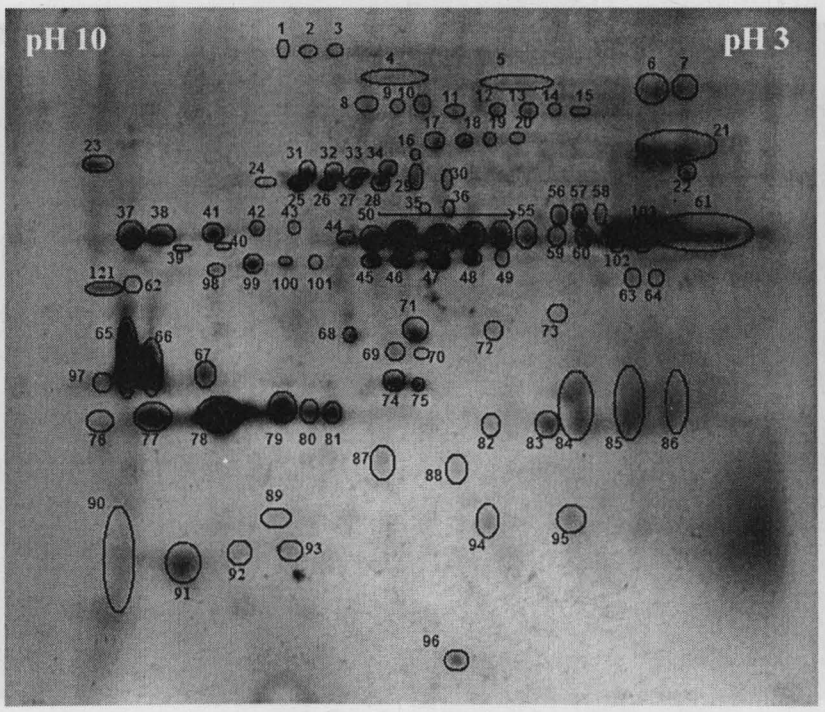

Figure B4. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 5-27-09.

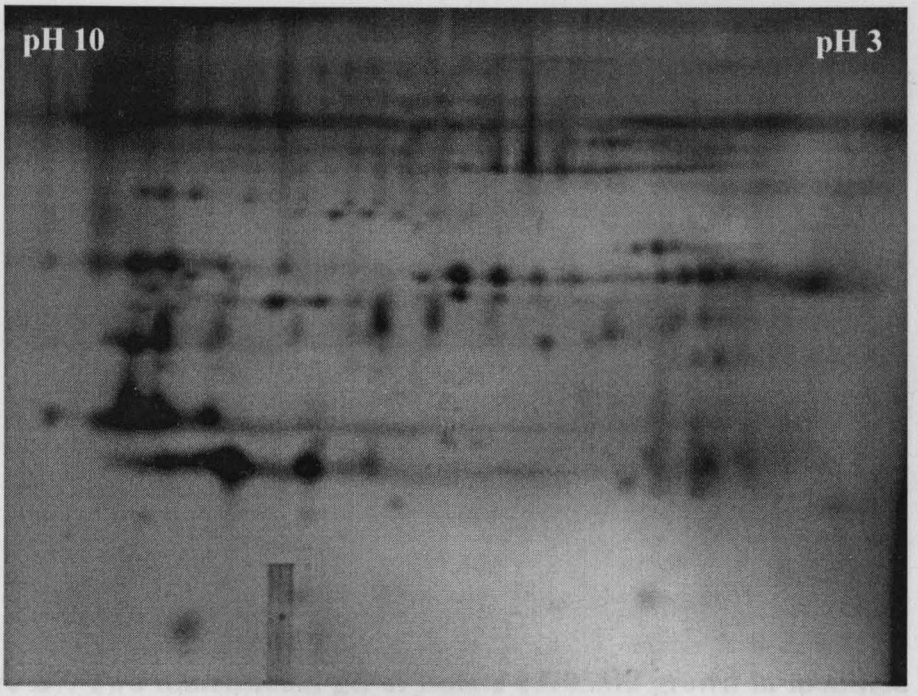

Figure B5. Two-dimensional gel of isolate 05105M2 grown in modified Fries media on 7-15-09.

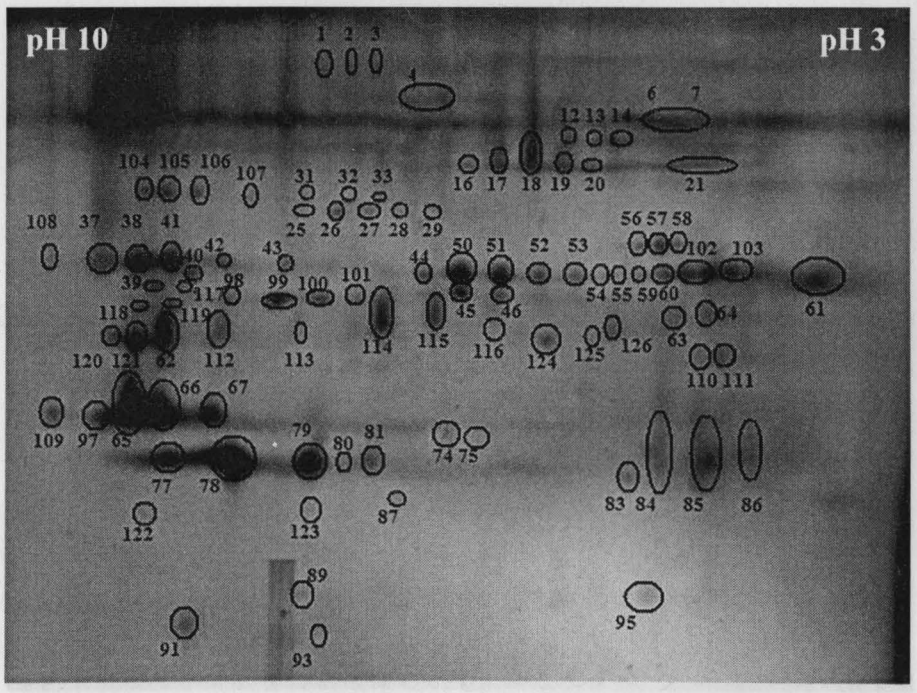

Figure B6. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 7-15-09.

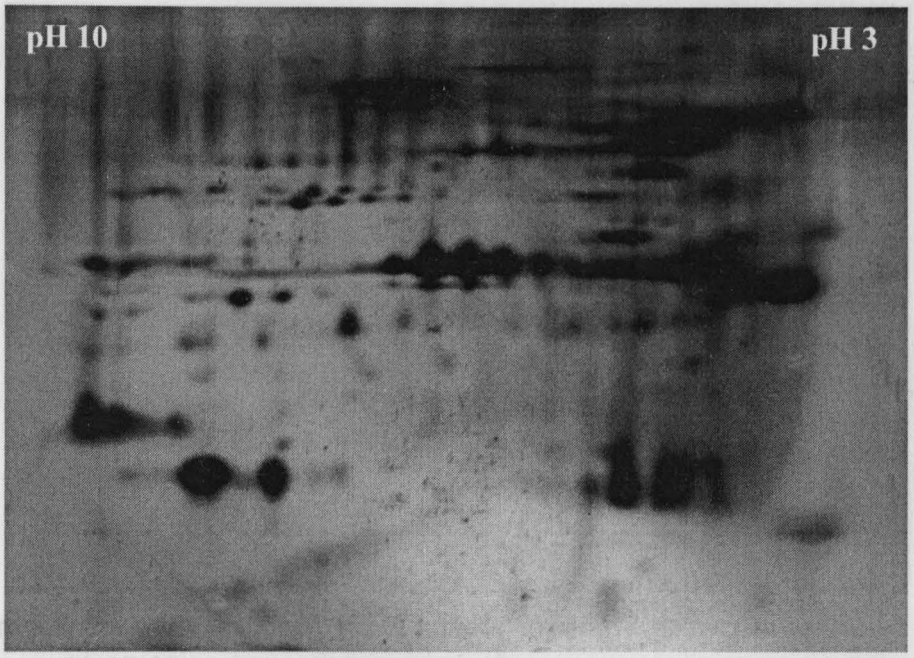

Figure B7. Two-dimensional gel of isolate 05105M2 grown in modified Fries media on 7-16-09.

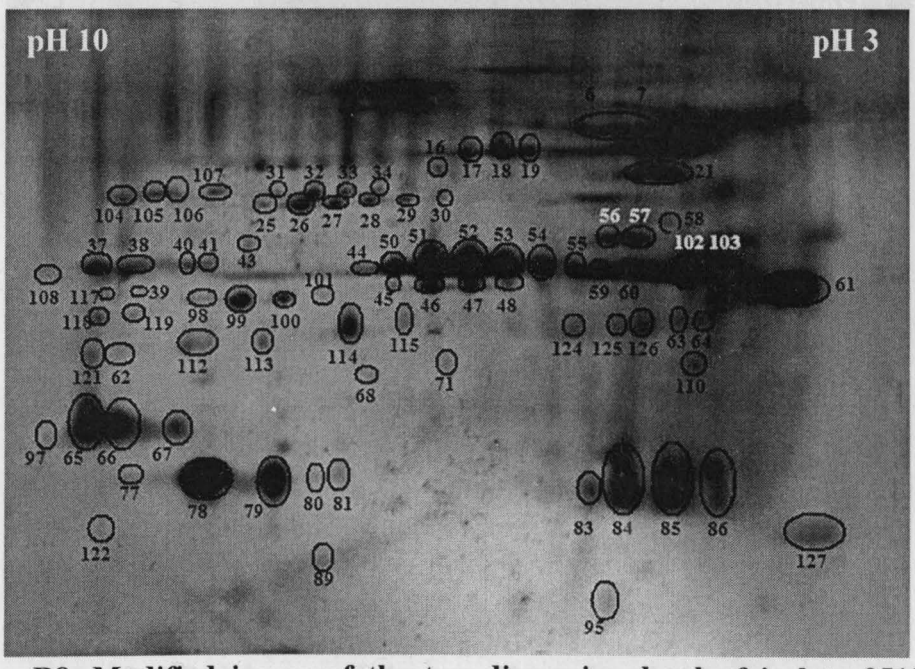

Figure B8. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 7-16-09.

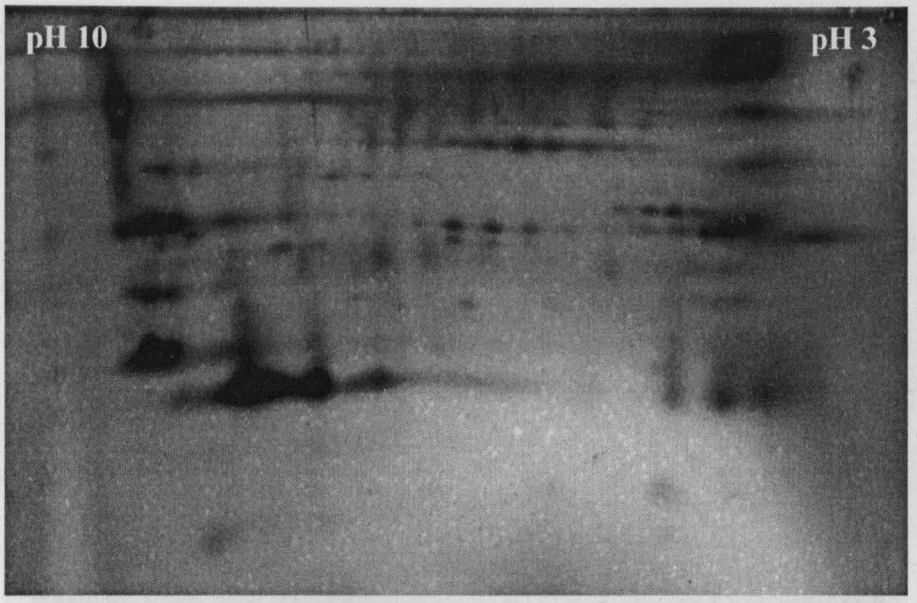

Figure B9. Two-dimensional gel of isolate 05105M2 grown in modified Fries media on 11-05-08.

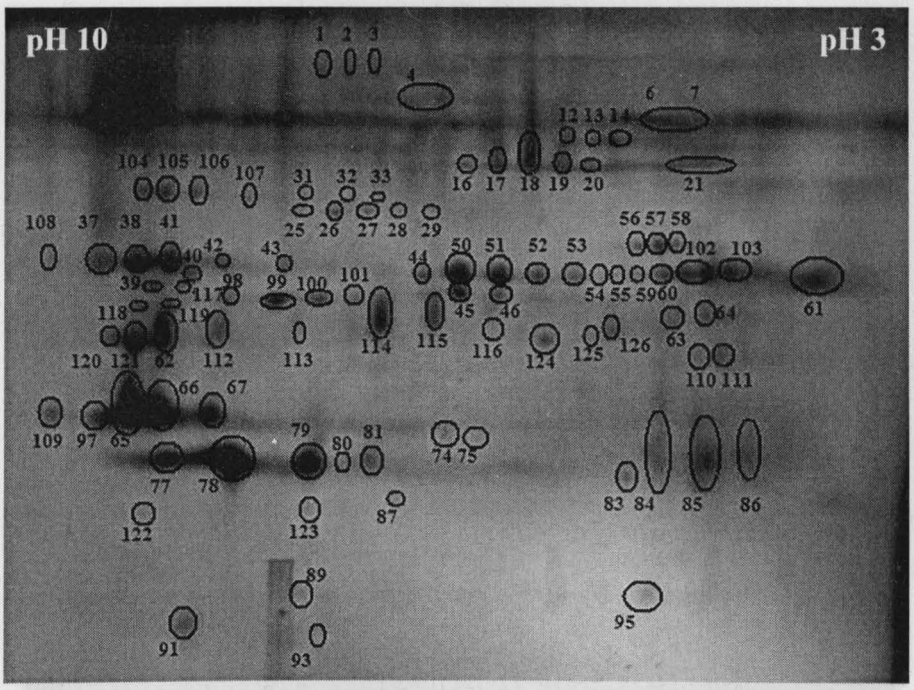

Figure B10. Modified image of the two-dimensional gel of isolate 05105M2 grown in modified Fries media on 11-05-08.

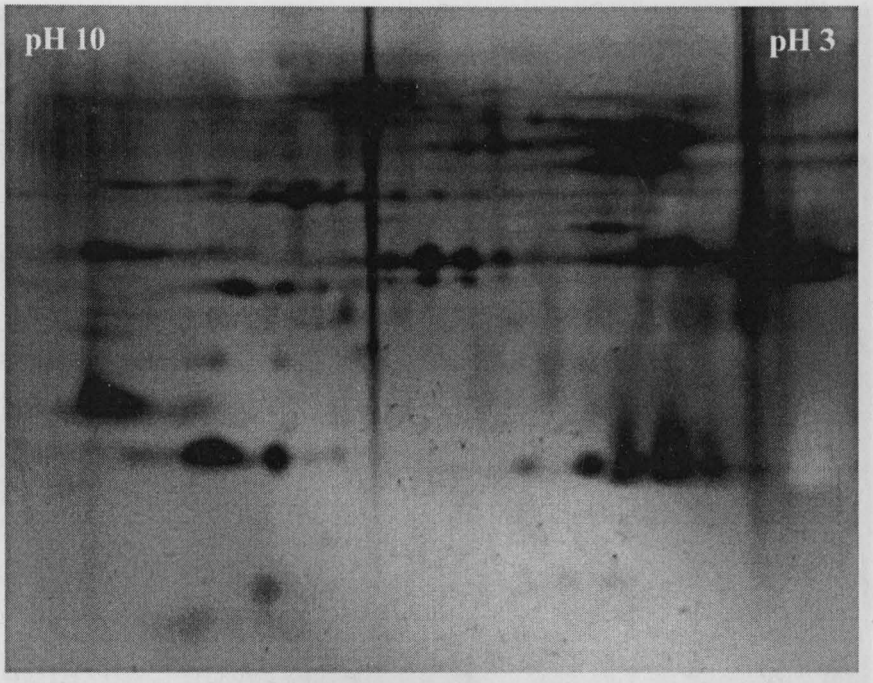

Figure B11. Two-dimensional gel of isolate 05202STM2 grown in modified Fries media on 7-15-09.

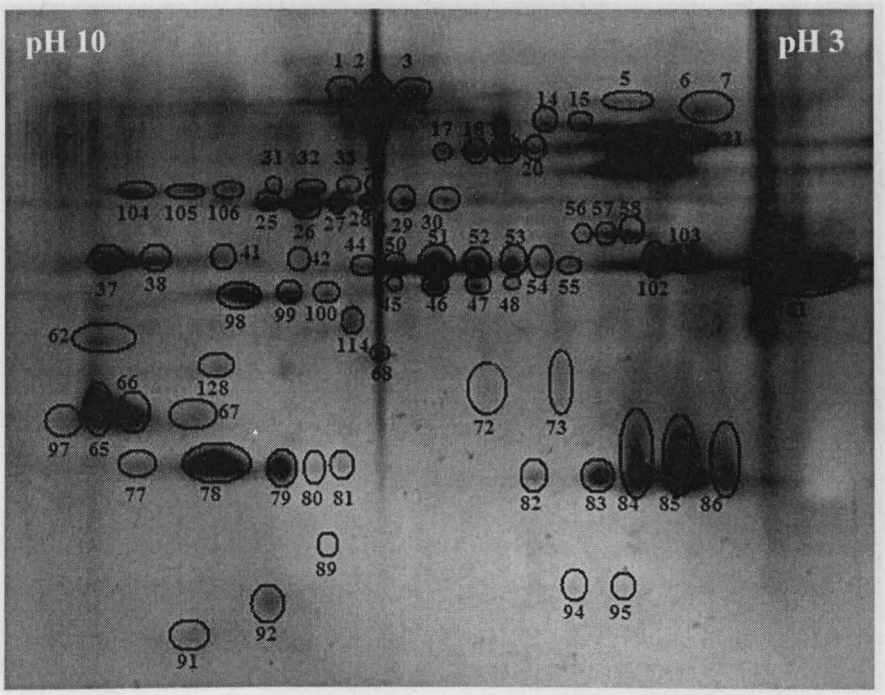

Figure B12. Modified image of the two-dimensional gel of isolate 05202STM2 grown in modified Fries media on 7-15-09.

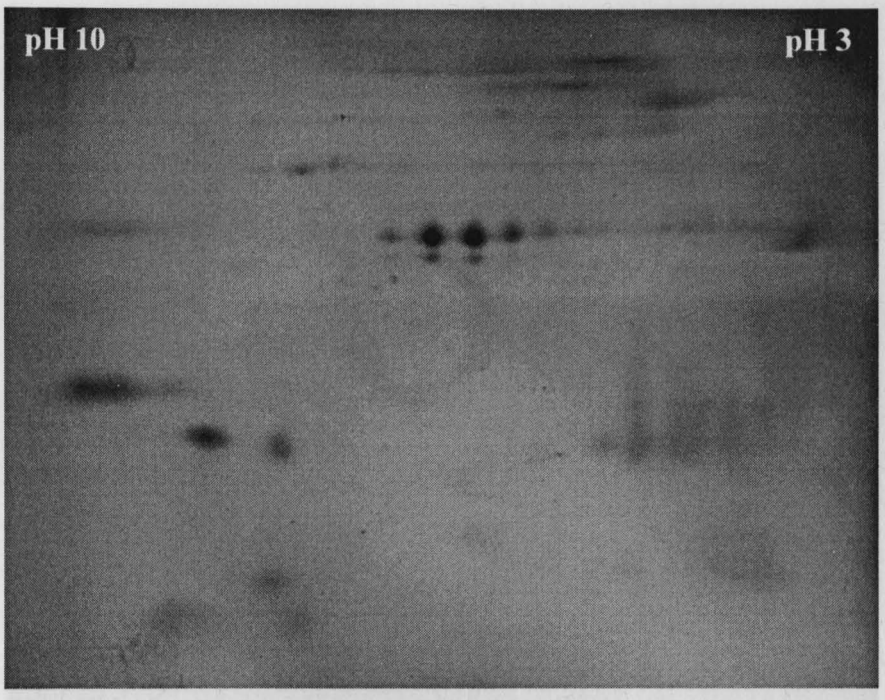

Figure B13. Two-dimensional gel of isolate 05202STM2 grown in modified Fries media on 7-23-09.

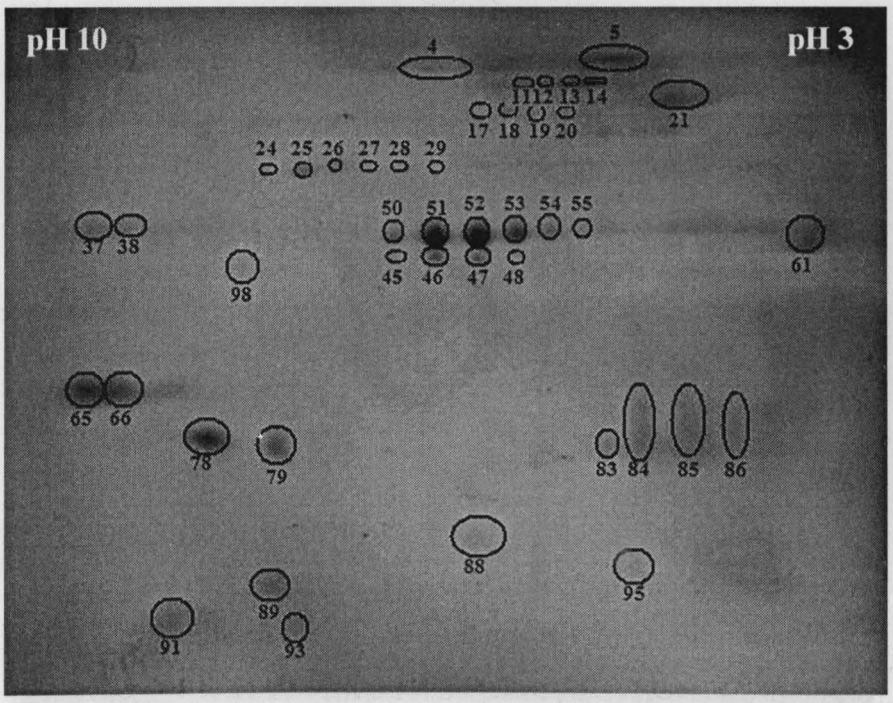

Figure B14. Modified image of the two-dimensional gel of isolate 05202STM2 grown in modified Fries media on 7-23-09.

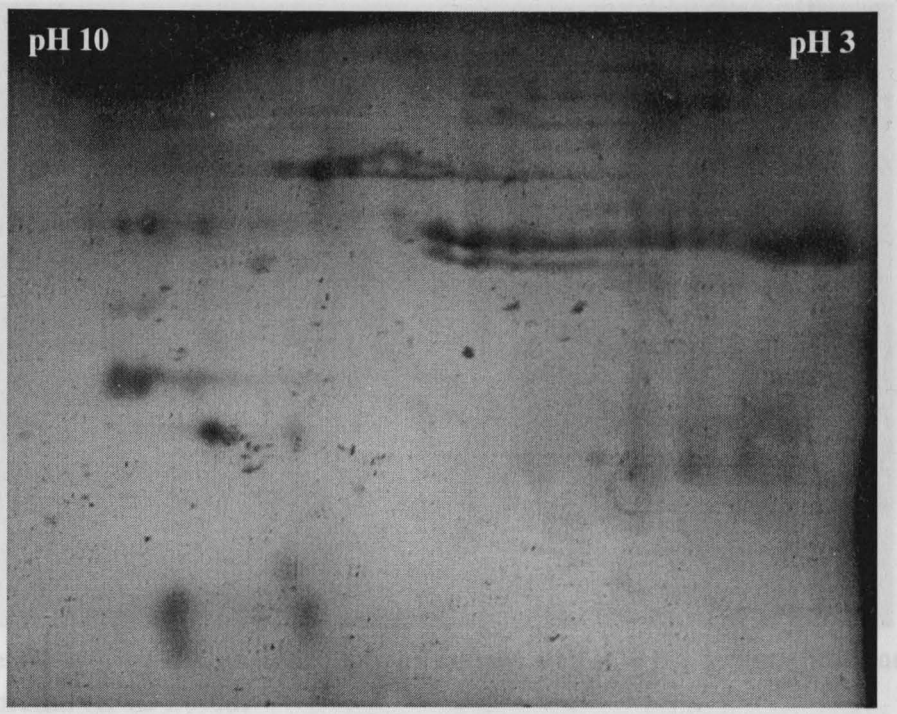

Figure B15. Two-dimensional gel of isolate 05202STM2 grown in modified Fries media on 8-4-09.

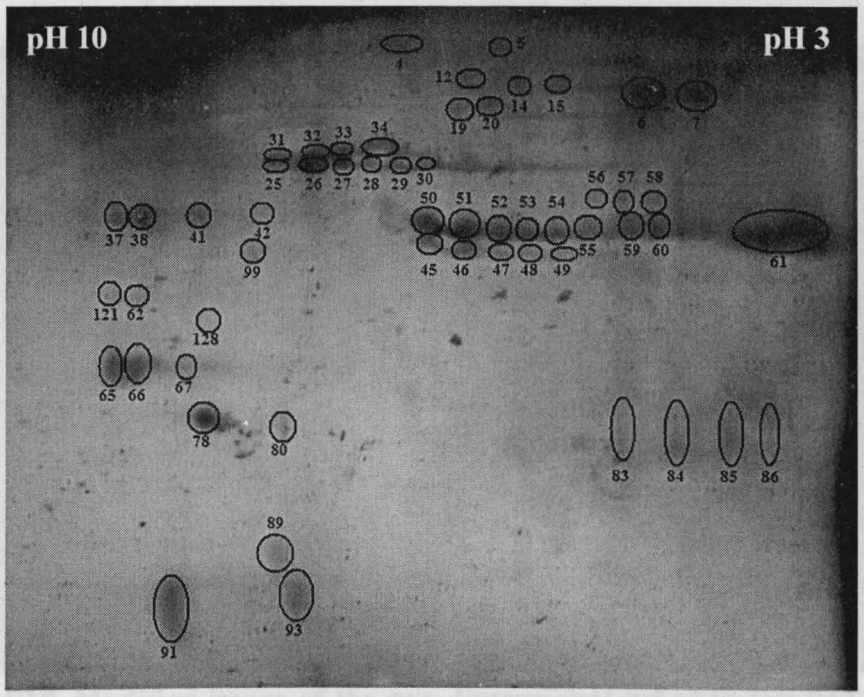

Figure B16. Modified image of the two-dimensional gel of isolate 05202STM2 grown in modified fries media on 8-4-09.

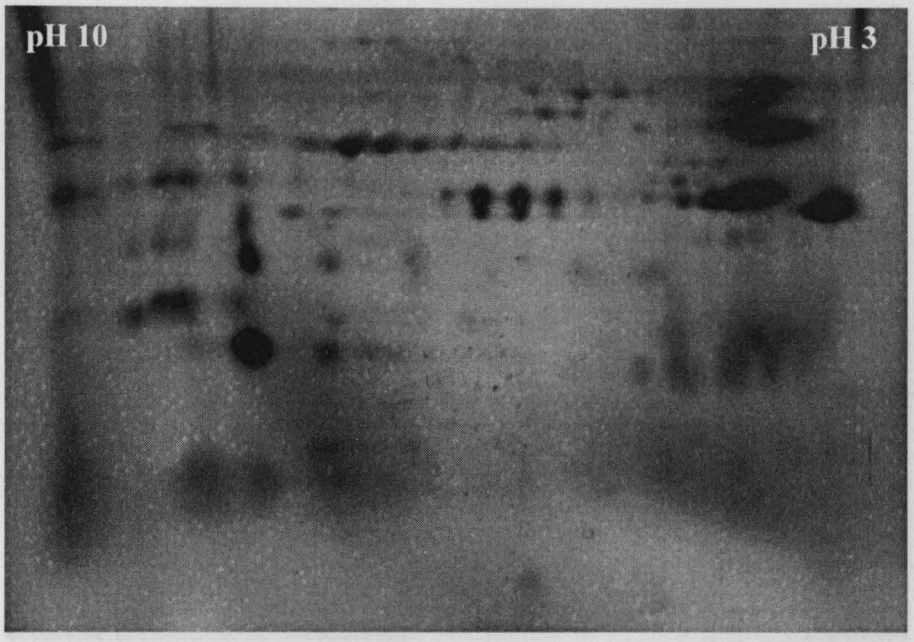

Figure B17. Two-dimensional gel of isolate 06TLND14 grown in modified Fries media on 12-30-08.

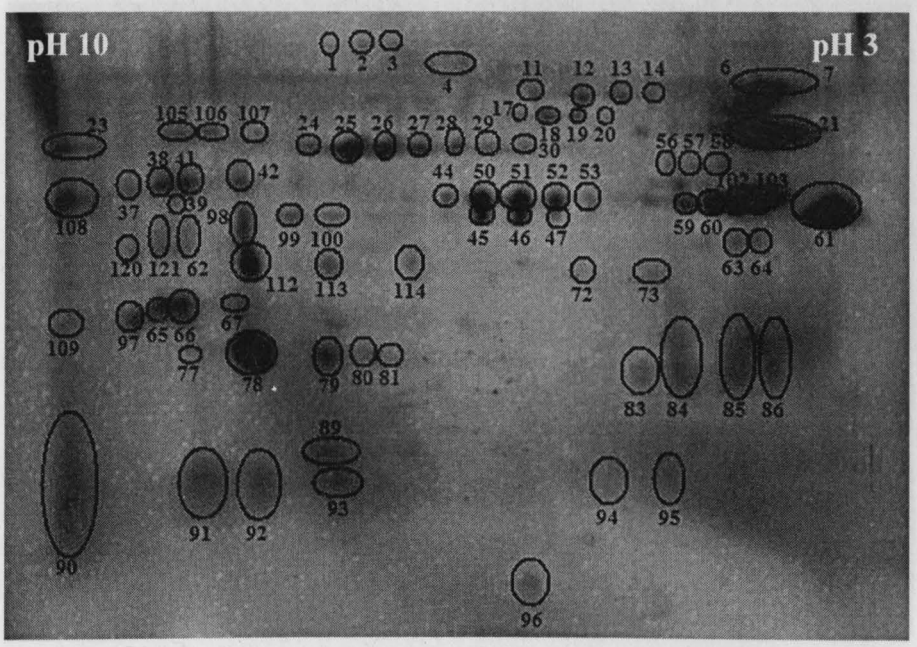

Figure B18. Modified image of the two-dimensional gel of isolate 06TLND14 grown in modified Fries media on 12-30-08.

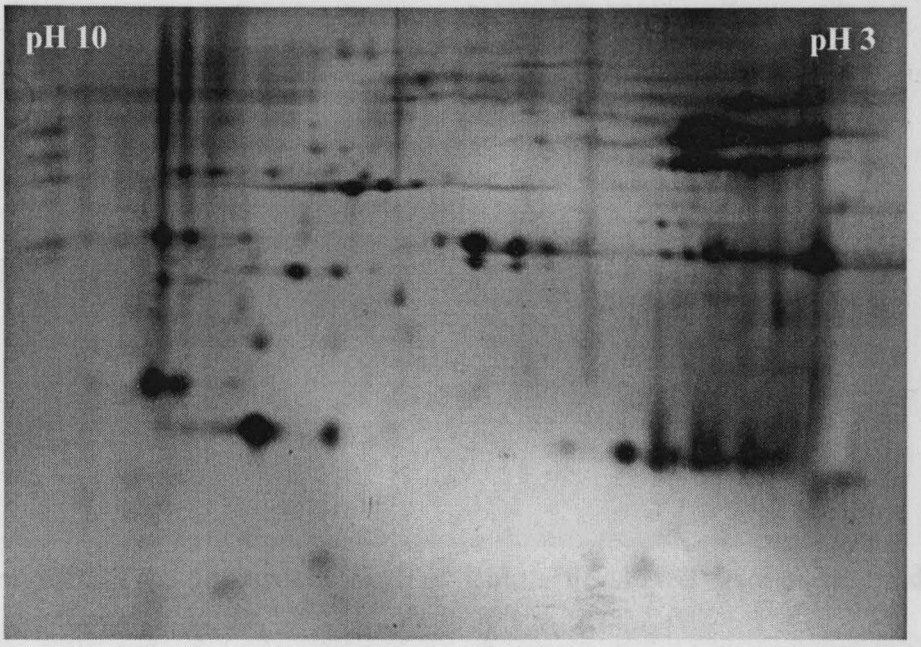

Figure B19. Two-dimensional gel of isolate 06TLND14 grown in modified Fries media on 7-16-09.

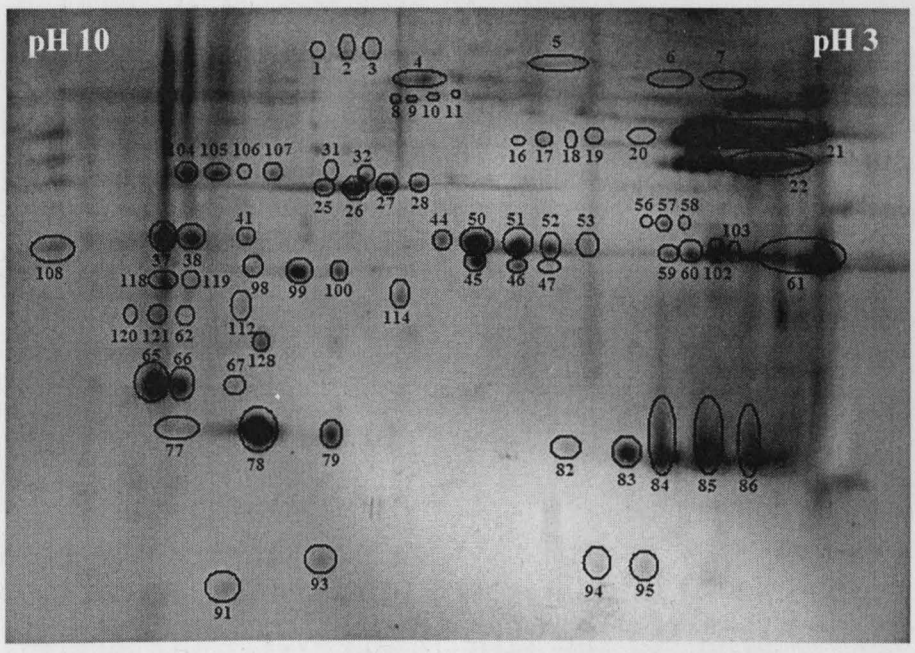

Figure B20. Modified image of the two-dimensional gel of isolate 06TLND14 grown in modified Fries media on 7-16-09.

Table B2. (continued)

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
41	5	2	2	3	12
42	4	1	1	2	8
43	4	0	0	1	5
44	4	1	2	2	9
45	5	3	2	1	11
46	5	3	2	2	12
47	4	3	2	2	11
48	3	3	0	2	8
49	1	1	0	0	2
50	5	3	2	2	11
51	5	3	2	3	13
52	5	3	2	3	13
53	5	3	2	3	13
54	5	3	0	0	8
55	5	3	0	0	8
56	5	2	2	2	11
57	5	2	2	2	11
58	5	2	2	3	12
59	4	1	2	1	8
60	4	1	2	3	10

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
61	5	3	2	2	12
62	5	2	2	2	11
63	5	0	1	0	6
64	5	0	1	0	6
65	5	3	2	3	13
66	5	3	2	3	13
67	5	2	2	3	12
68	2	1	0	1	4
69	1	0	0	0	1
70	1	0	0	0	1
71	3	0	0	0	3
72	1	1	1	0	3
73	1	1	1	0	3
74	3	0	0	1	4
75	3	0	0	1	4
76	2	0	0	0	2
77	4	1	2	0	7
78	5	3	2	2	12
79	5	3	2	3	13
80	5	2	1	0	8

Table B2. (continued)

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
81	4	1	1	0	6
82	2	1	1	0	4
83	5	3	2	2	12
84	5	3	2	3	13
85	5	3	2	3	13
86	5	3	2	3	13
87	1	0	0	0	1
88	1	1	0	0	2
89	4	3	1	2	10
90	1	0	1	0	2
91	4	3	2	3	12
92	2	1	1	0	4
93	3	2	2	1	8
94	1	1	2	1	5
95	4	2	2	1	9
96	1	0	1	0	2
97	4	1	1	2	8
98	5	2	2	1	10
99	5	2	2	3	12
100	5	1	2	2	10

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
101	4	0	0	0	4
102	5	1	2	2	10
103	5	1	2	2	10
104	3	1	1	1	6
105	3	1	2	1	7
106	3	1	2	0	6
107	2	0	2	1	5
108	4	0	2	2	8
109	1	0	1	1	3
110	3	0	0	0	3
111	2	0	0	0	2
112	2	0	2	3	7
113	2	0	1	1	4
114	2	1	2	0	5
115	2	0	0	0	2
116	1	0	0	0	1
117	3	0	0	0	3
118	3	0	1	0	4
119	3	0	1	0	4
120	1	0	2	1	4

Table B2. (continued)

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
121	4	1	2	2	9
122	2	0	0	0	2
123	1	0	0	1	2
124	3	0	0	1	4
125	2	0	0	0	2
126	2	0	0	0	2
127	1	0	0	0	1
128	0	0	1	0	1

Table B3. Frequency of Spots Appearing in 2D Gels From Four *A. rabiei* Isolates Grown in Czapek Dox Media.

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
1	1	0	1	1	3
2	1	0	1	1	3
3	2	0	1	1	4
4	3	0	0	0	3
5	1	1	2	2	6
6	3	3	3	2	11
7	3	3	3	2	11
8	2	1	1	2	6
9	3	2	3	2	10
10	3	1	3	2	9
11	3	2	3	2	10
12	3	3	3	2	11
13	3	1	1	2	7
14	2	0	1	2	5
15	1	0	1	2	4
16	2	0	0	0	2
17	3	3	3	2	11
18	3	3	3	2	11
19	3	1	3	0	7
20	2	1	1	2	6
21	3	3	2	2	10
22	3	3	2	2	10

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
23	2	2	2	2	8
24	2	2	2	2	8
25	2	2	1	2	7
26	3	3	2	2	10
27	3	1	3	2	9
28	3	3	3	2	11
29	3	3	3	2	11
30	3	3	3	2	11
31	3	2	3	2	10
32	3	2	2	2	9
33	3	1	3	2	9
34	3	3	3	2	11
35	3	1	3	2	9
36	3	2	3	2	10
37	3	3	3	2	11
38	3	3	3	2	11
39	3	3	3	2	11
40	3	3	3	2	11
41	3	3	3	2	11
42	1	0	0	0	1
43	3	1	2	1	7
44	3	1	2	1	7

Table B3. (continued)

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
45	3	1	1	0	5
46	1	1	2	2	6
47	2	1	2	2	7
48	3	2	3	0	8
49	3	1	3	2	9
50	3	2	3	2	10
51	1	1	2	0	4
52	3	2	3	2	10
53	1	0	1	1	3
54	1	2	0	0	3
55	1	0	1	0	2
56	3	3	2	2	10
57	3	2	3	2	10
58	3	1	3	1	8
59	3	3	3	1	10
60	3	3	3	1	10
61	3	3	3	2	11
62	3	2	3	2	10
63	3	3	3	2	11
64	3	3	3	2	11
65	3	3	3	2	11
66	3	3	3	2	11

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
67	2	1	1	1	5
68	2	1	2	1	6
69	2	3	3	1	9
70	2	3	3	1	9
71	1	2	3	1	7
72	3	2	3	0	8
73	3	3	3	1	10
74	3	3	3	1	10
75	2	3	3	1	9
76	2	3	2	1	8
77	1	2	3	1	7
78	2	0	1	0	3
79	1	2	0	0	3
80	1	2	1	0	4
81	1	2	0	0	3
82	1	2	1	0	4
83	1	0	1	2	4
84	1	0	0	0	1
85	1	0	0	0	1
86	1	0	1	1	3
87	1	0	1	1	3
88	1	3	2	1	7

Table B3. (continued)

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
89	2	1	3	1	7
90	2	2	2	1	7
91	1	0	0	0	1
92	1	1	0	2	4
93	2	2	2	2	8
94	2	1	0	0	3
95	2	1	0	0	3
96	2	1	0	0	3
97	1	0	0	0	1
98	1	1	0	0	2
99	1	0	0	0	1
100	1	0	0	0	1
101	0	1	0	0	1
102	0	0	1	0	1
103	0	0	1	0	1
104	0	0	1	0	1
105	0	0	1	1	2
106	0	0	1	1	2
107	0	0	1	1	2
108	0	0	1	1	2
109	0	0	1	1	2
110	0	0	1	1	2

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
111	0	0	1	2	3
112	0	0	1	2	3
113	0	0	1	1	2
114	0	0	1	1	2
115	0	0	1	2	3
116	0	0	1	2	3
117	0	0	0	0	0
118	0	1	0	0	1
119	0	2	2	1	5
120	0	2	2	1	5
121	0	2	1	1	4
122	0	1	0	0	1
123	0	1	0	0	1
124	0	1	0	0	1
125	0	1	0	0	1
126	0	1	0	0	1
127	0	1	0	0	1
128	0	1	0	0	1
129	0	1	0	0	1
130	0	0	0	1	1
131	0	0	0	1	1
132	0	0	0	1	1

Tables B3. (continued)

Spot	Isolate				
	05105 M2 3 gels	05202 STM2 3 gels	O6TL ND14 3 gels	05 APNS3 2 gels	Total
133	0	0	0	1	1
134	0	0	0	1	1
135	0	0	0	1	1
136	0	0	0	0	0
137	0	0	0	0	0
138	0	0	1	0	1
139	0	0	1	0	1
140	0	0	1	0	1
141	0	0	1	0	1
142	0	0	1	0	1
143	0	0	1	0	1
69A	1	0	3	1	5

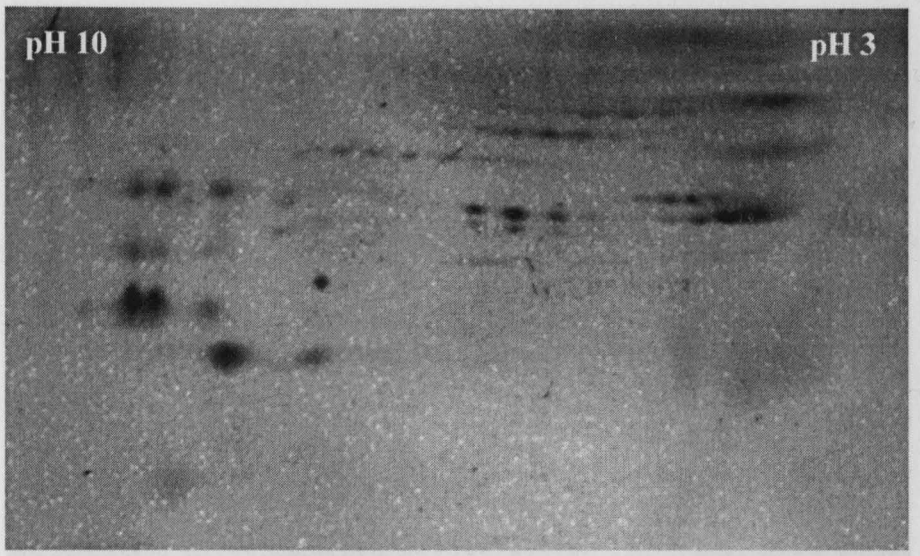

Figure B21. Two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 12-30-08.

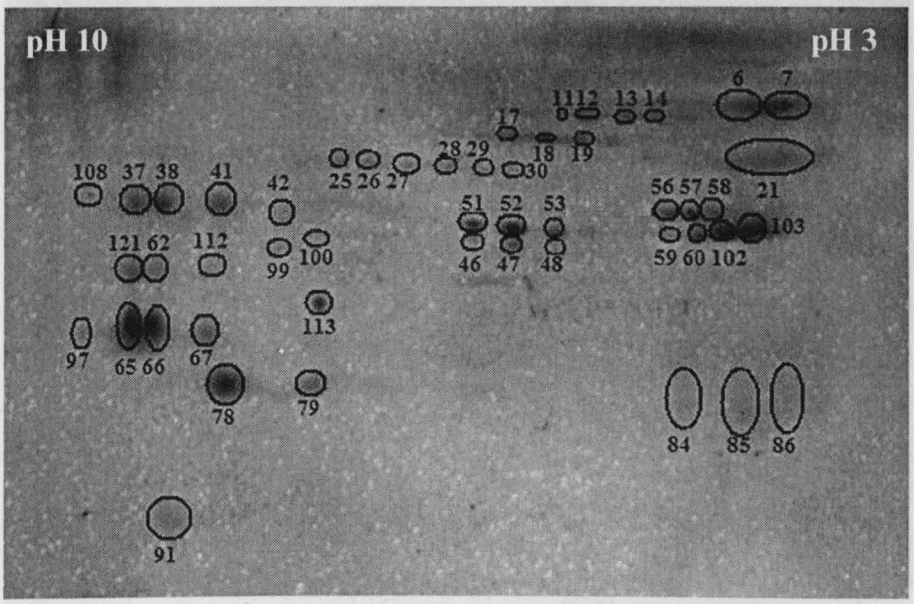

Figure B22. Modified image of the two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 12-30-08.

Figure B23. Two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 7-23-09.

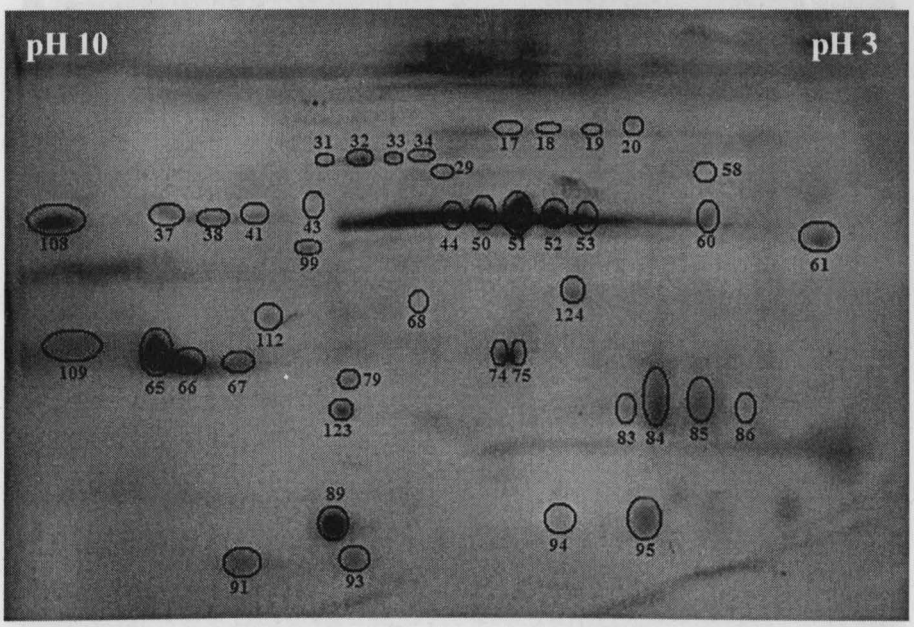

Figure B24. Modified image of the two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 7-23-09.

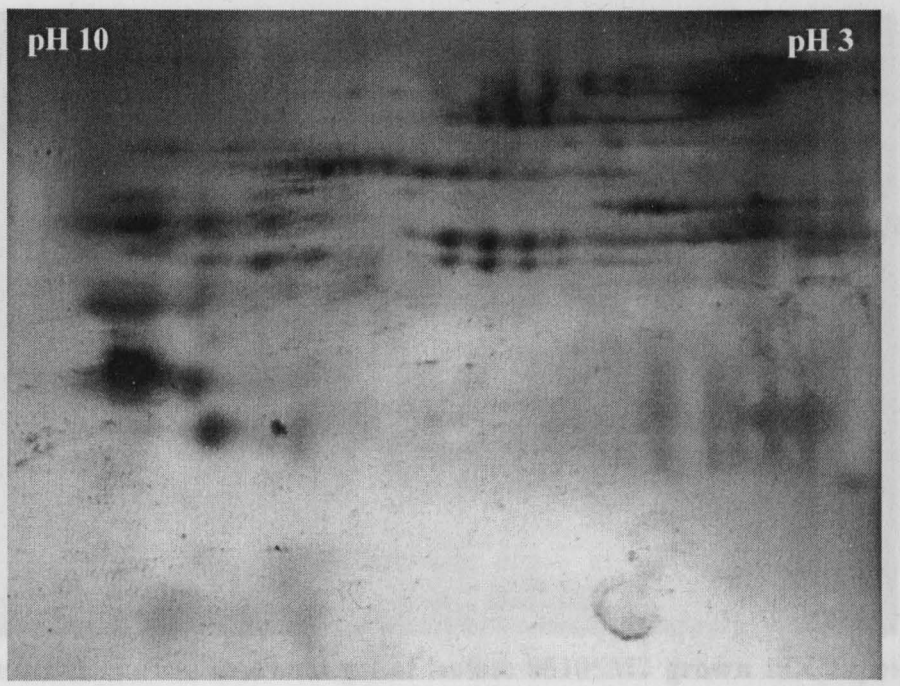

Figure B25. Two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 8-4-09.

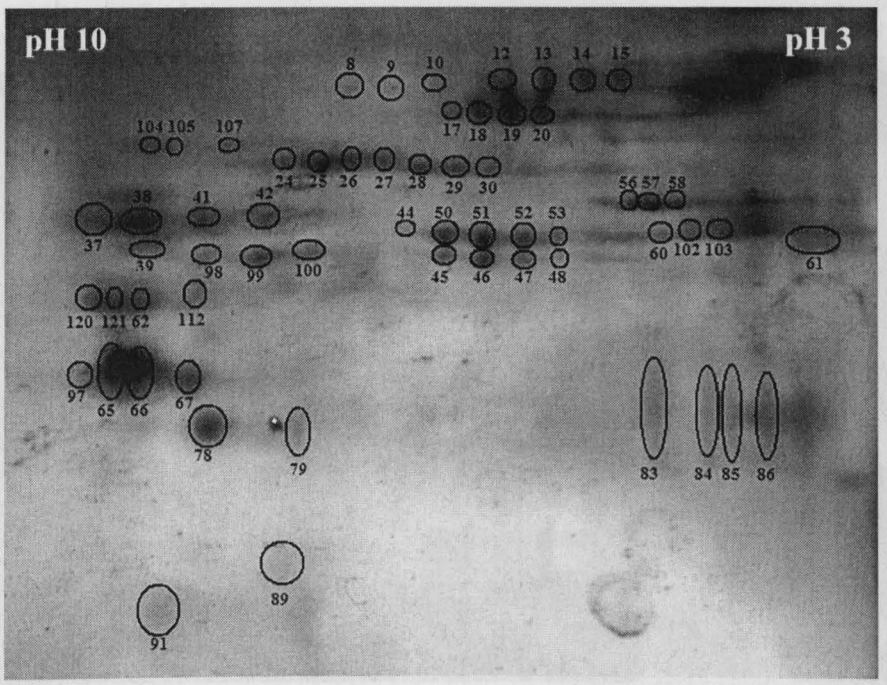

Figure B26. Modified image of the two-dimensional gel of isolate 05APNS3 grown in modified Fries media on 8-4-09.

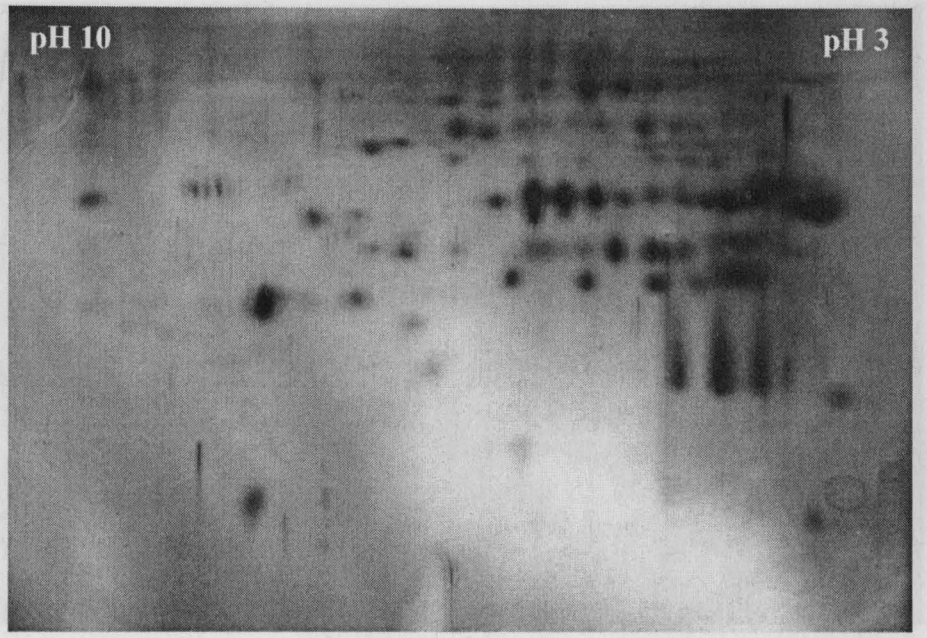

Figure B27. Two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 2-05-09.

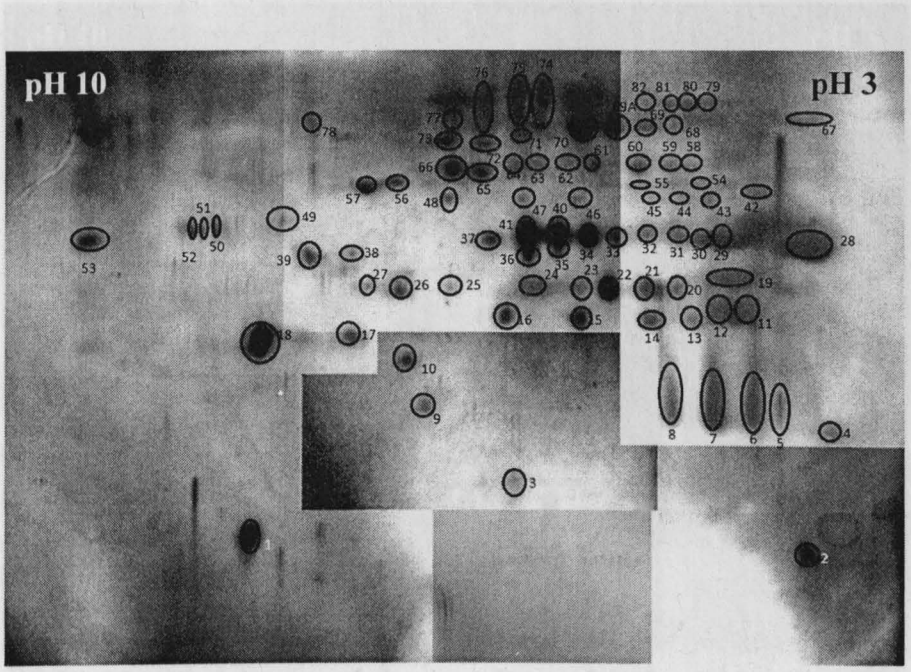

Figure B28. Modified image of the two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 2-05-09.

Figure B29. Two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 4-10-09.

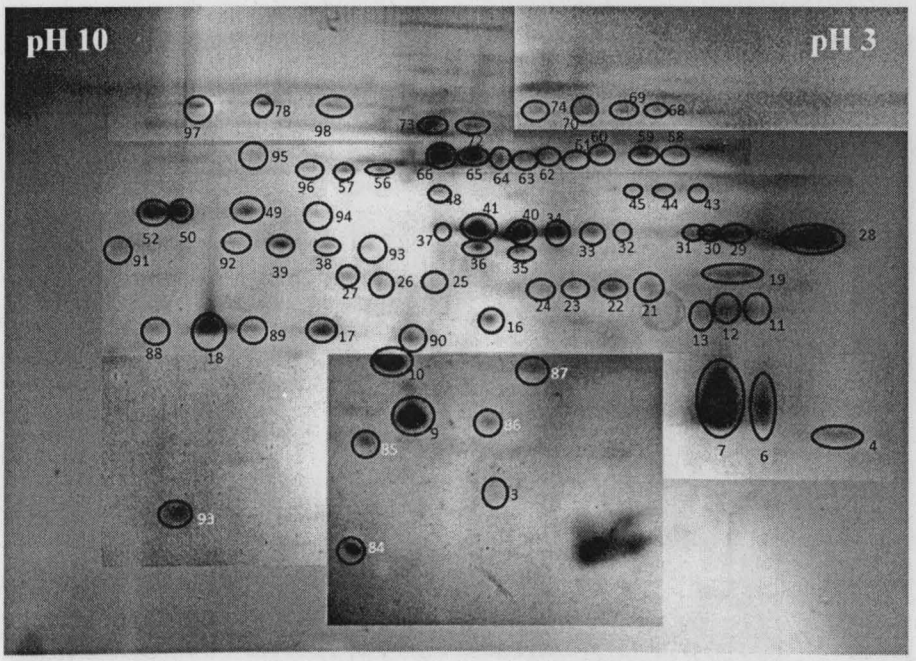

Figure B30. Modified image of the two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 4-10-09.

Figure B31. Two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 7-30-09.

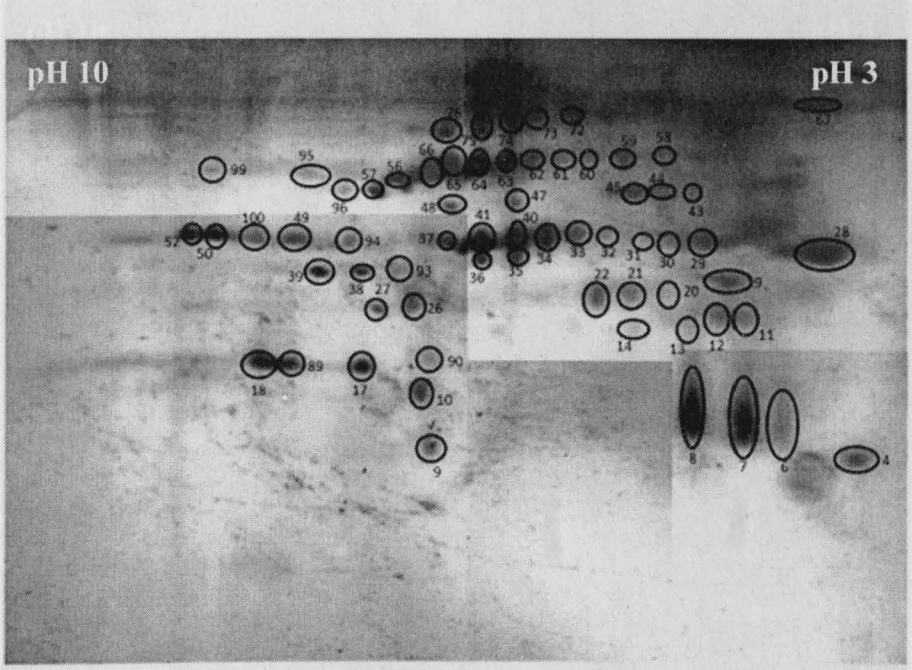

Figure B32. Modified image of the two-dimensional gel of isolate 05105M2 grown in Czapek Dox media on 7-30-09.

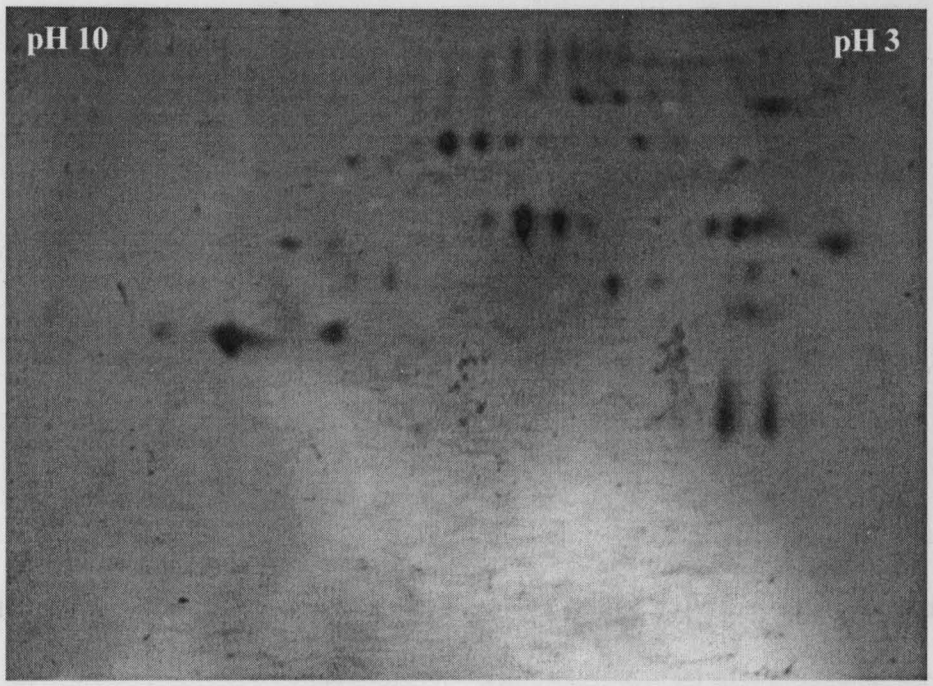

Figure B33. Two-dimensional gel of isolate 05202STM2 grown in Czapek Dox media on 2-06-09.

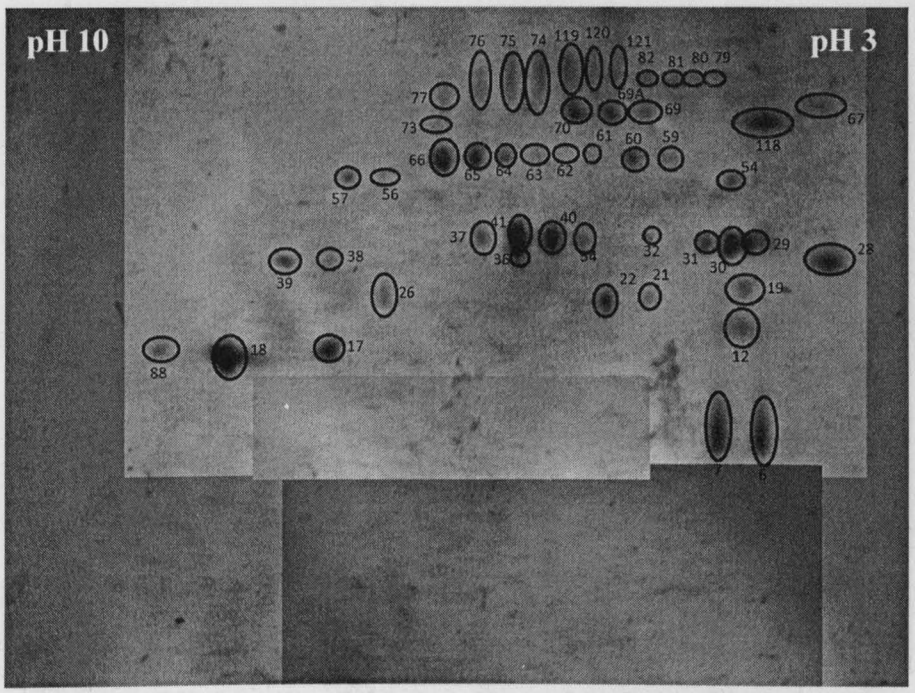

Figure B34. Modified image of the two-dimensional gel of isolate 5202STM2 grown in Czapek Dox media on 2-06-09.

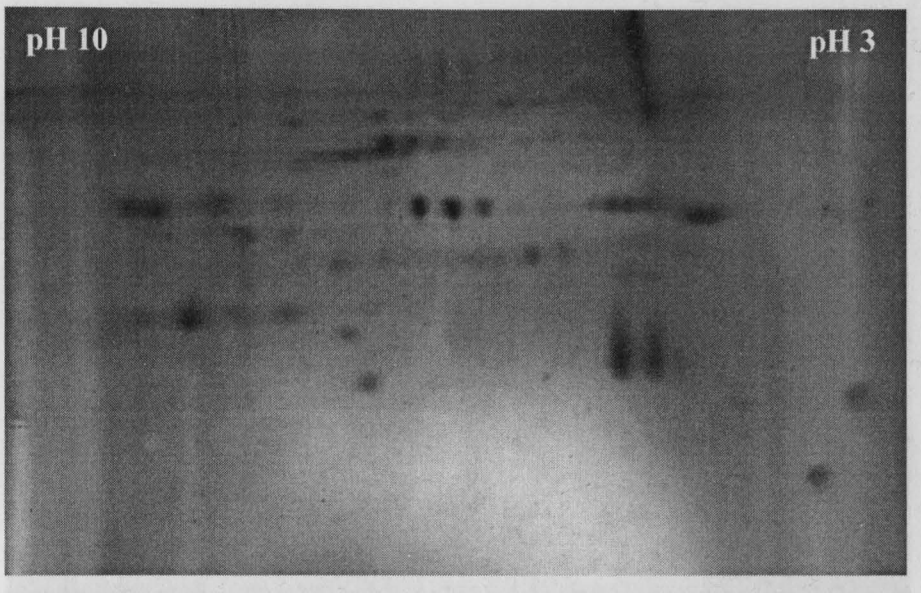

Figure B35. Two-dimensional gel of isolate 05202STM2 grown in Czapek Dox media on 3-10-09.

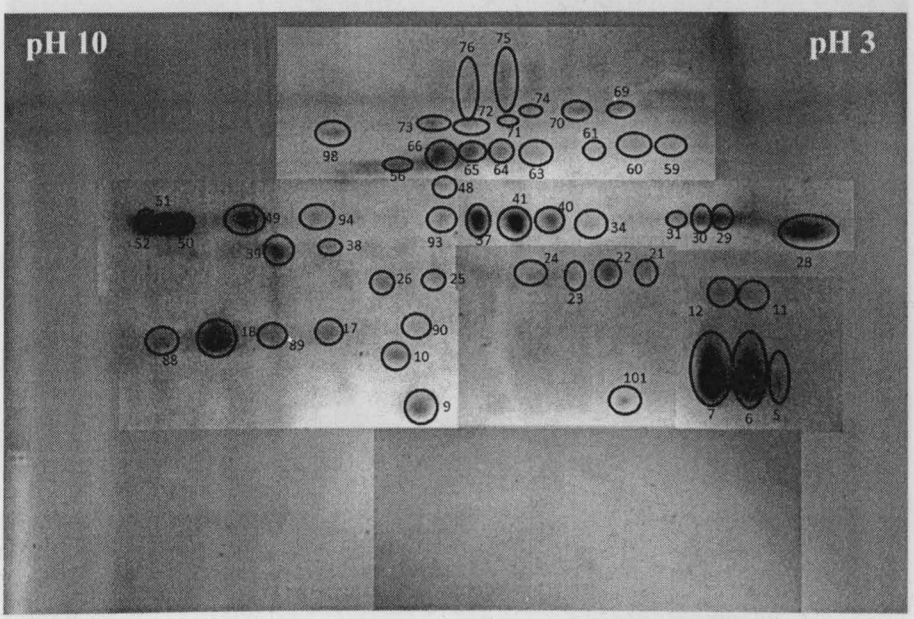

Figure B36. Modified image of the two-dimensional gel of isolate 05202STM2 grown in Czapek Dox media on 3-10-09.

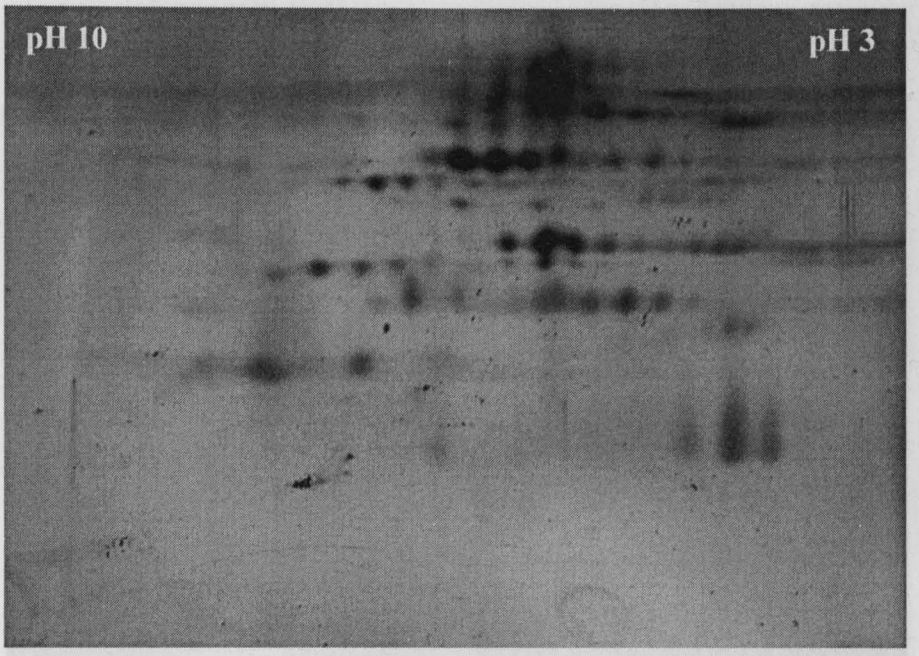

Figure B37. Two-dimensional gel of isolate 05202STM2 grown in Czapek Dox media on 8-04-09.

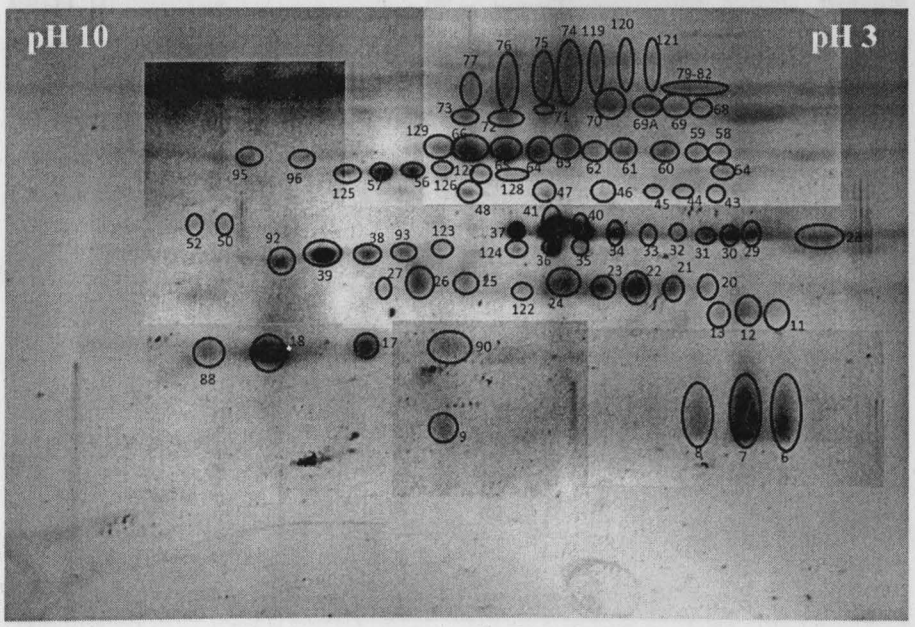

Figure B38. Modified image of the two-dimensional gel of isolate 05202STM2 grown in Czapek Dox media on 8-04-09.

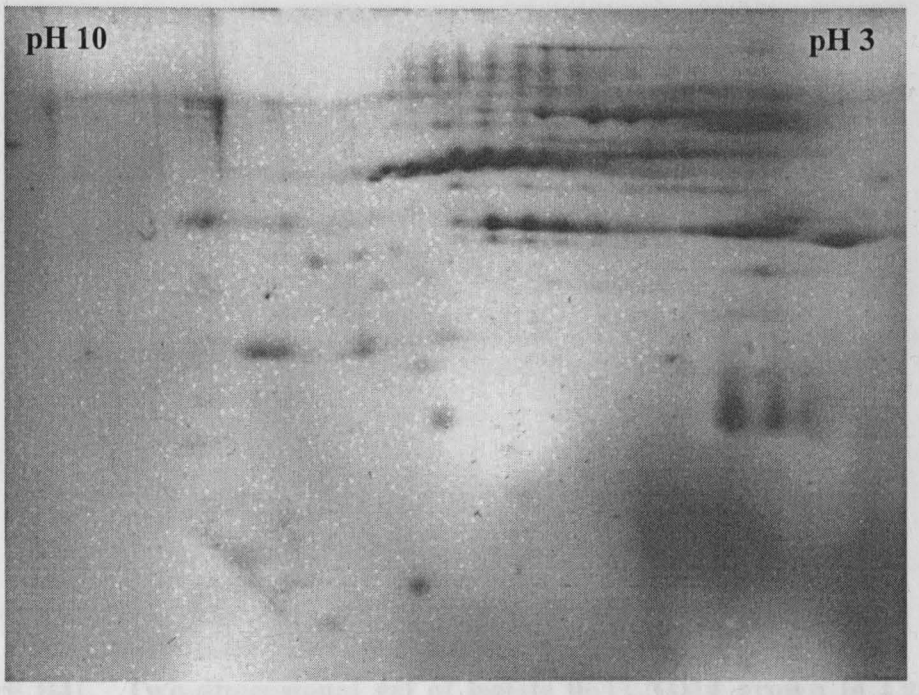

Figure B39. Two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 12-24-08.

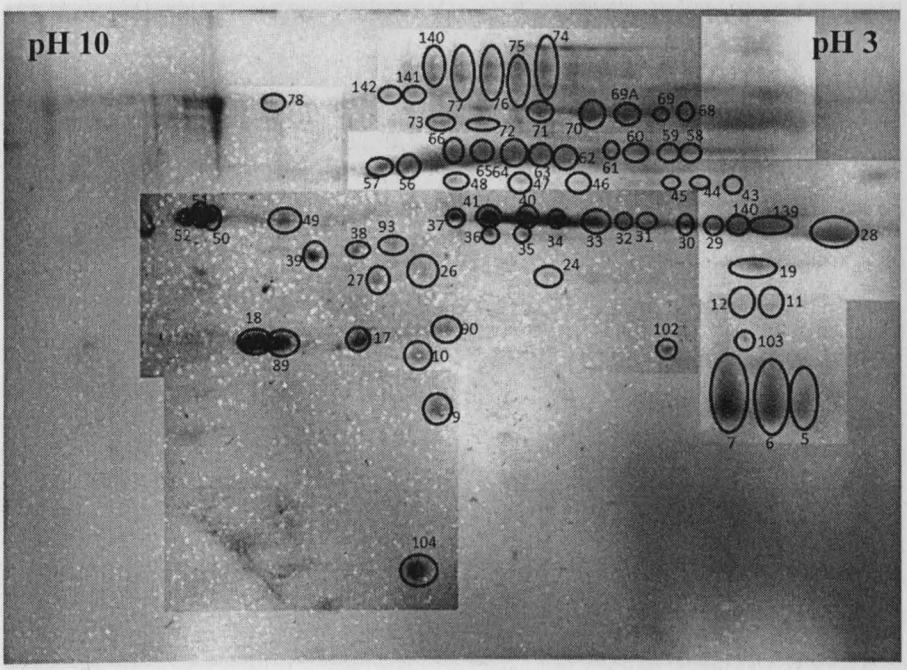

Figure B40. Modified image of the two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 12-24-08.

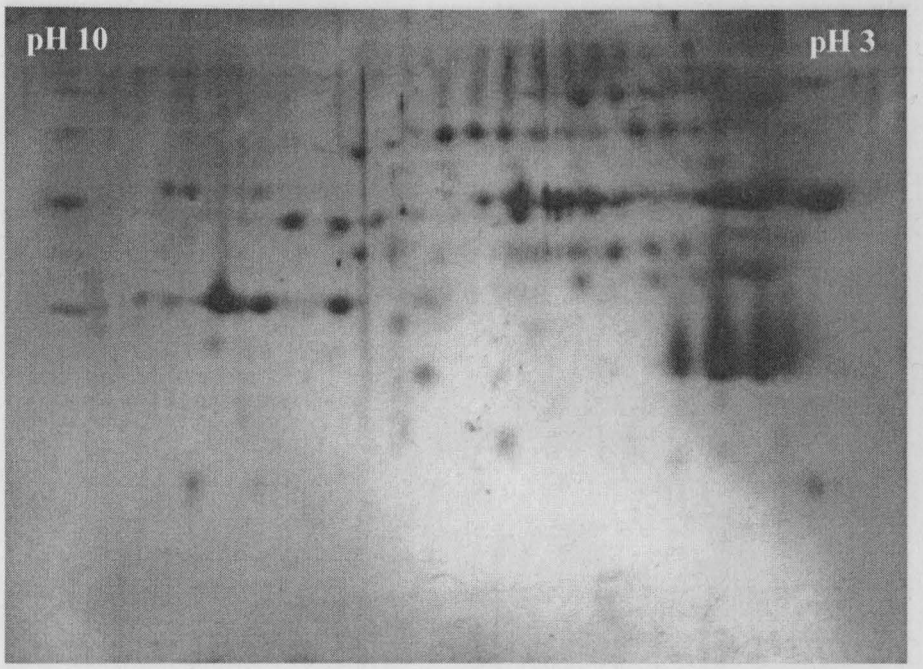

Figure B41. Two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 02-05-09.

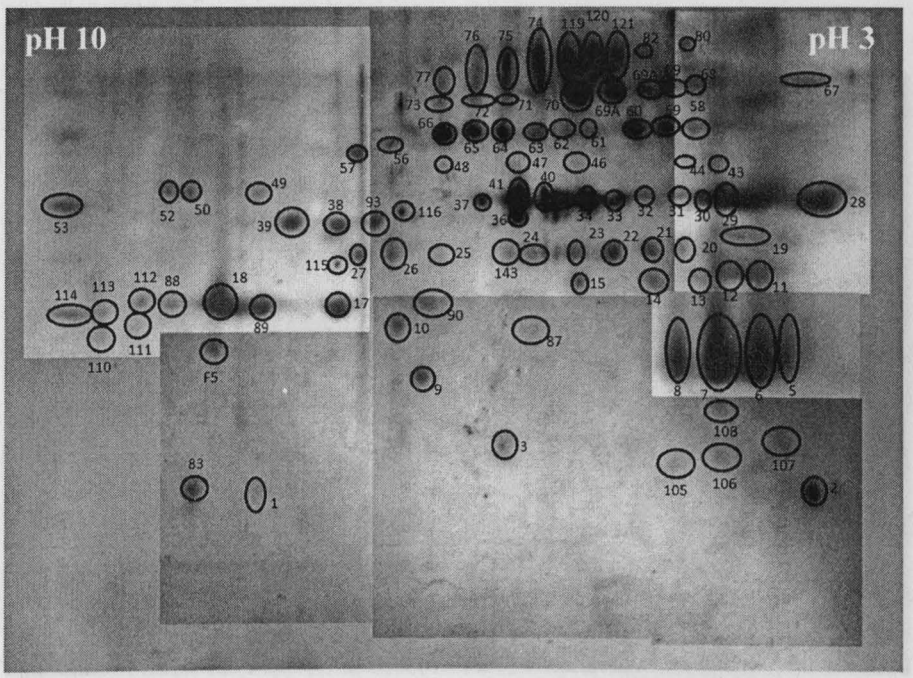

Figure B42. Modified image of the two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 02-05-09.

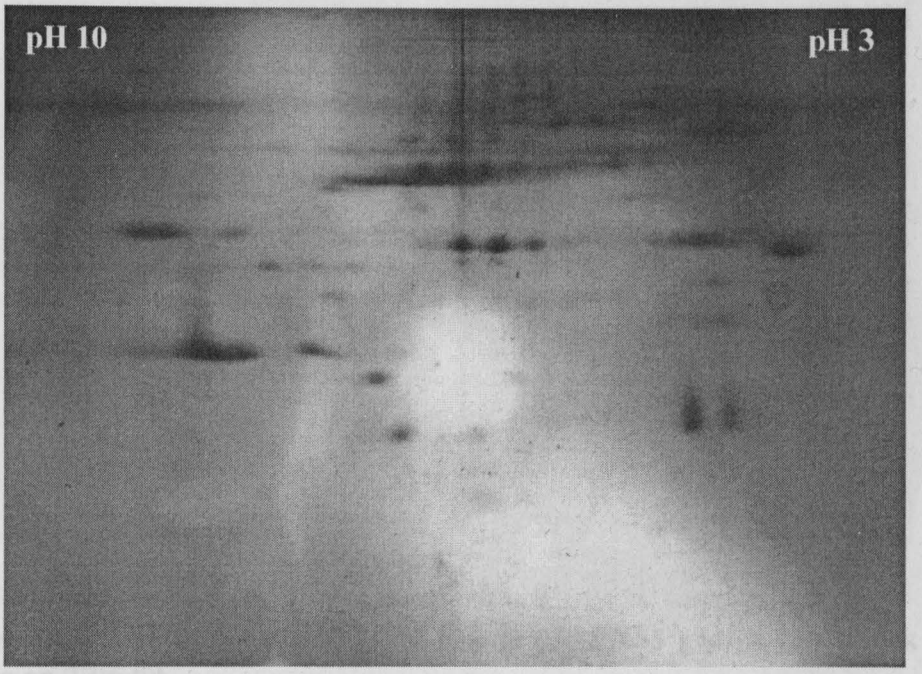

Figure B43. Two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 4-10-09.

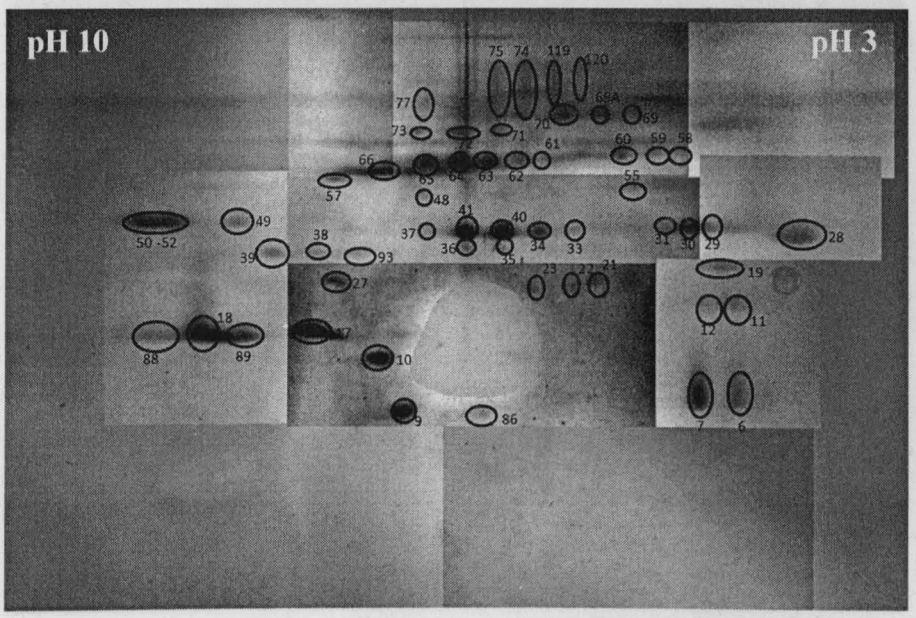

Figure B44. Modified image of the two-dimensional gel of isolate 06TLND14 grown in Czapek Dox media on 4-10-09.

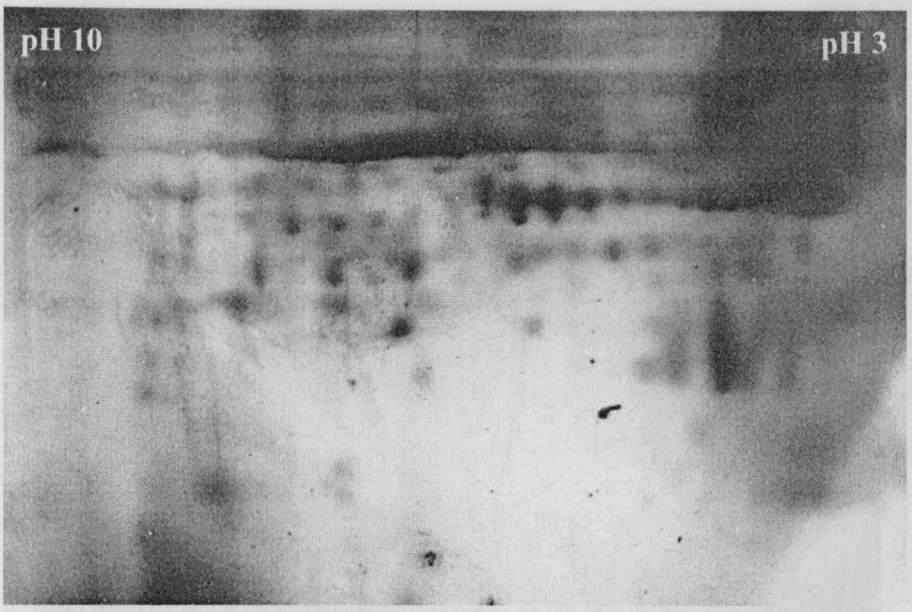

Figure B45. Two-dimensional gel of isolate 05APNS3 grown in Czapek Dox media on 2-05-09.

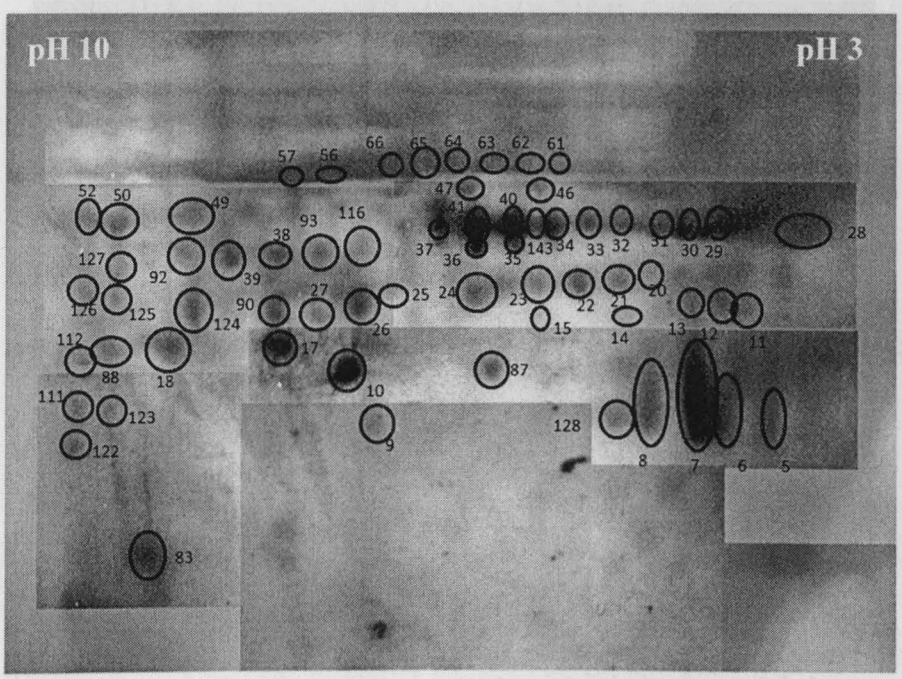

Figure B46. Modified image of the two-dimensional gel of isolate 05APNS3 grown in Czapek Dox media on 2-05-09.

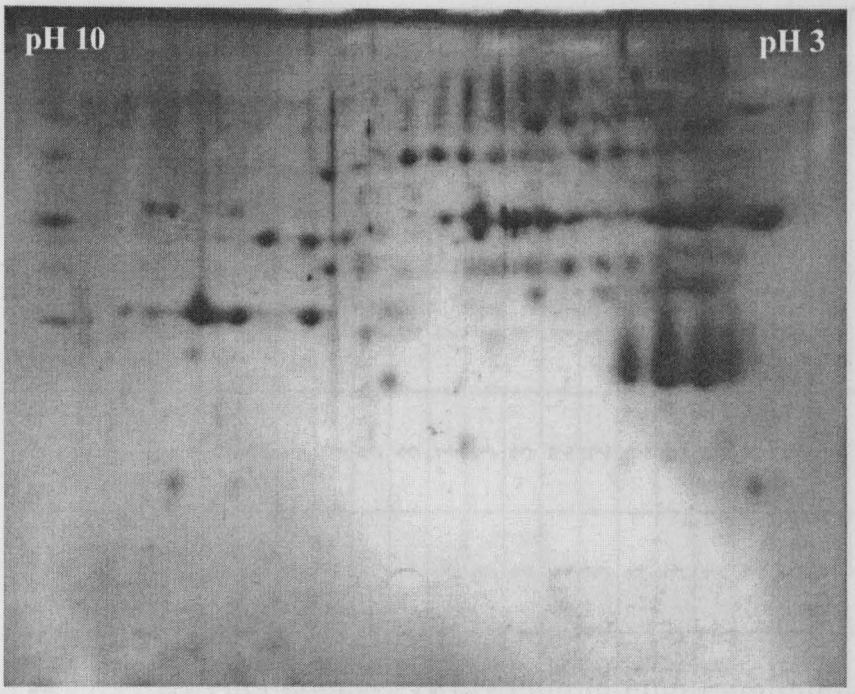

Figure B47. Two-dimensional gel of isolate 05APNS3 grown in Czapek Dox media on 8-04-09.

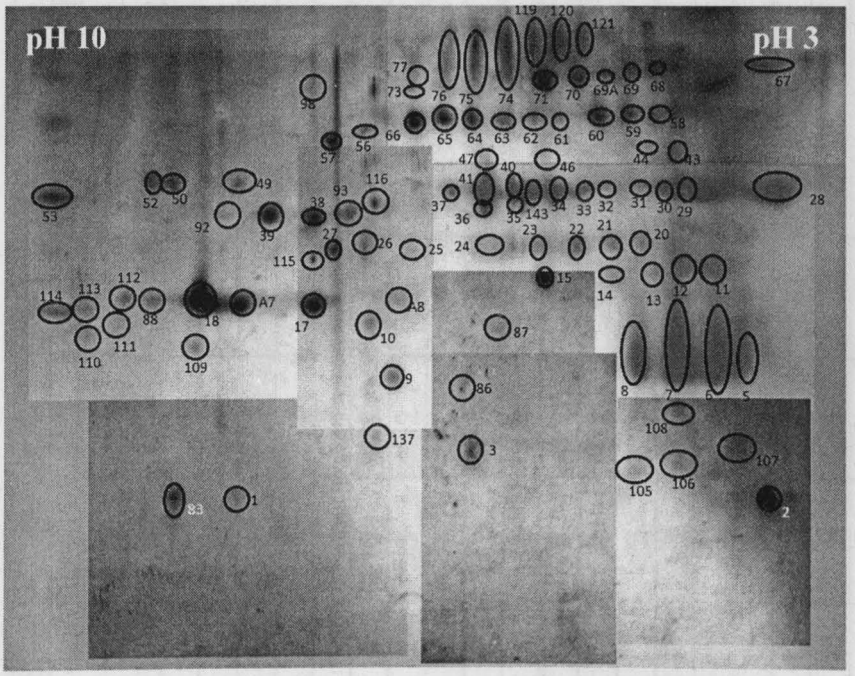

Figure B48. Modified image of the two-dimensional gel of isolate 05APNS3 grown in Czapek Dox media on 8-04-09.

Table B2. Frequency of Spots Appearing in 2D Gels From Four *A. rabiei* Isolates Grown in Modified Fries Media.

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
1	3	1	2	0	6
2	3	1	2	0	6
3	3	1	2	0	6
4	2	2	2	0	6
5	2	3	1	0	6
6	5	2	2	1	10
7	5	2	2	1	10
8	1	0	1	1	3
9	2	0	1	1	4
10	2	0	1	1	4
11	3	1	2	1	7
12	4	2	1	2	9
13	3	1	1	2	7
14	3	3	1	2	9
15	1	2	0	1	4
16	4	0	1	0	5
17	5	2	2	3	12
18	5	2	2	3	12
19	5	3	2	3	13
20	3	3	2	2	10

Spot	Isolate				
	05105 M2 5 gels	05202 STM2 3 gels	O6TL ND14 2 gels	05 APNS3 3 gels	Total
21	5	2	2	1	10
22	1	0	1	0	2
23	1	0	1	0	2
24	2	1	1	1	5
25	4	3	2	2	11
26	4	3	2	2	11
27	4	3	2	2	11
28	3	3	2	2	10
29	3	3	1	3	10
30	2	3	1	2	8
31	5	2	1	1	9
32	5	2	1	1	9
33	4	2	0	1	7
34	3	2	0	1	6
35	2	0	0	0	2
36	2	0	0	0	2
37	5	3	2	3	13
38	5	3	2	3	13
39	4	0	1	1	6
40	4	0	0	0	4