Relatedness of Haemophilus parasuis strains and their proteins' possible roles as virulence factors

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Relatedness of *Haemophilus parasuis* strains and their proteins’ possible roles as virulence factors

by

Emilie Susanna Noel Zehr

A dissertation submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

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Dedicated to my family,
Doug, Beth, and Jay
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ABSTRACT

_Haemophilus parasuis_, the causative agent of Glässer’s disease, is considered to be a remerging pathogen of swine in high-health status herds. Several molecular typing methods have been suggested as alternatives to serotyping, which failed to identify up to 30% of field isolates. This study compared random amplification of polymorphic DNA (RAPD) profiles and protein profiles of _H. parasuis_ reference strains and field isolates as methods for identifying and grouping virulent and avirulent isolates. DNA and protein profiles of 15 reference strains and 31 field isolates of _H. parasuis_, analyzed with two computer-based similarity analyses, revealed unique and reproducible DNA and protein fingerprints among the reference strains and field isolates studied. Similarities and differences existed among avirulent, virulent, and highly virulent strains, which grouped according to their pathogenicity. The combination of RAPD and whole cell lysate SDS-PAGE analyses may be useful for studying the epidemiology of _H. parasuis_.

This is the first report of the isolation and characterization of a myophage, SuMu, from a field strain of _H. parasuis_. Electron microscopy showed that the bacteriophage had an iscosahedral head and contractile tail. Mass spectrometry of proteins separated by 1-D and 2-D electrophoresis identified twenty four homologues of bacteriophage proteins. Partial DNA sequencing revealed twenty open reading frames corresponding to fourteen proteins, including eight Mu-like homologues. Epidemiology of _H. parasuis_ may be affected by this phage.

Iron-restricted growth studies of _H. parasuis_ were done to explore potential protein differences between virulent and avirulent isolates. Twenty seven iron-regulated proteins that are homologues of other _Haemophilus_ proteins were identified using 2-D electrophoresis when _H. parasuis_ was grown in stressed (iron-restricted) and nonstressed (iron-replete) conditions. Expressed proteins were further analyzed using mass spectrometry, Western blotting, Far Western blotting, and stable isotope labeling with amino acids in cell culture
(SILAC). SILAC experiments confirmed the presence of a homologue of \emph{H. influenzae}'s hemoglobin binding protein (HgbA) which was confirmed by probing blots with biotinylated porcine hemoglobin. Some identified \emph{H. parasuis} proteins were homologues of other known virulence factors of the Pasteurellacae family and may be used in future virulence studies of \emph{H. parasuis}. 
CHAPTER 1. GENERAL INTRODUCTION

Introduction

*Haemophilus parasuis* is the causative agent of Glässer’s disease in pigs. Fibrinous polyserositis, pericarditis, polyarthritis, and meningitis are the main symptoms of this disease (85). *H. parasuis* can also cause septicemia or pneumonia without polyserositis, and can be isolated from healthy nasal passages of swine. Glässer’s disease causes high morbidity and mortality in nonimmune pigs, such as specific pathogen free (SPF) animals. Mixing of conventionally raised pigs with segregated early weaning herds may result in high economic losses due to the lack of immunity to *H. parasuis* (68, 105).

It has been difficult to develop effective vaccines which protect pigs from *H. parasuis* infection or disease. Trivalent (75) and bivalent (122) inactivated commercial vaccines elicited cross-protection to the bacteria present in the respective vaccines; however, were not protective against heterologous *H. parasuis* isolates. The authors questioned the efficacy of the intramuscular route of vaccination and speculated that an intranasal live vaccine might be more appropriate for inducing antibodies against common antigens of *H. parasuis* serovars, resulting in better cross-protection. Autogenous vaccines prepared from central nervous system isolates are preferred over vaccines made from organisms isolated from arthritic or systemic sites or heterogeneous lung isolates. However, these vaccines are herd-specific and serovar-specific and probably do not protect the animals from a freshly introduced heterologous serovar. A common management practice is to vaccinate sows prior to farrowing in order to protect their piglets after weaning through colostrum passive antibodies (85).

*Haemophilus parasuis* strains have been classified into 15 serovars based on immunodiffusion of heat-stable polysaccharide (soluble, precipitating) antigens (59). A second method for serotyping, the indirect hemagglutination (IHA) test, was developed
because of the high incidence of nontypeable (NT) isolates and cross-serotype reactivity (30%) with the immunodiffusion test (120). The IHA test was based on particulate lipopolysaccharide antigen and has less than 10% cross-reactivity. Some scientists prefer to combine both tests to improve the sensitivity and specificity of the tests and decrease the problems due to cross-reactive samples. On the other hand, there are still many NT isolates that do not have characterized serovar-specific reagents. Moreover, reagents for serotyping field isolates are not readily available.

Partially due to lack of reagents and also because serotyping reagents cannot identify NT strains, other diagnostic methods have been employed to identify field isolates. These methods include restriction enzyme analysis DNA fingerprinting (114, 115), enterobacterial repetitive intergenic concensus-polymerase chain reaction (ERIC-PCR) (91, 102), whole cell lysate protein profiles (87, 93), and outer membrane protein (OMP) profiles (109). This study compared the relatedness of 15 reference serovars and 31 field strains of *H. parasuis* by random amplified polymorphic DNA (RAPD) and by whole cell lysate profiles. A relationship between passage number of isolate and its whole cell lysate pattern was established.

Because no efficacious commercial vaccines or easy-to-perform diagnostic tests for *H. parasuis* are available, it is of interest to identify proteins present in virulent strains which are not expressed in avirulent strains. Some potential virulence factors that have already been studied in *H. parasuis* include seven up-regulated genes expressed when the organism was cultured at 40°C (53), a 60 kDa heat shock protein (95), a 42 kDa porin (51), a 35 kDa invasion, a lipopolysaccharide which may be involved in adherence (121), an endotoxin (2), and a neuraminidase (66).

In this study, virulence-related proteins were examined after they were expressed by bacteria grown under stress related conditions induced by iron limitation. The rational for these studies are that stress responses due to lack of iron in the growth medium mimic the effect of pathogenic bacterial invasion and resulting sequestration of iron in the
animal host, whose tissues are also iron-limiting. These iron-regulated responses can result in the expression of new bacterial proteins or other virulence factors that enable the pathogen to evade the host’s immune surveillance system and allow the pathogen to survive in the host. The assumption was made that these newly synthesized proteins may be used to develop improved vaccines and companion diagnostic tests. This work identified upregulated, immunoreactive; downregulated, immunoreactive; and upregulated, nonimmunoreactive; and downregulated, nonimmunoreactive homologues to other Haemophilus proteins when H. parasuis was grown in stress (iron-deplete) and nonstress (iron-replete) conditions. Expressed proteins were analyzed by 2-D electrophoresis, Western blotting, and Matrix Assisted Laser Desorption /Ionization-Time of Flight (MALDI-TOF) mass spectrometry. Stable isotope labeling with amino acids in cell culture (SILAC) was used to confirm the presence of a hemoglobin binding protein which might allow hemoglobin to bind to a receptor where heme is subsequently removed and enters the bacterial cell through an H. influenzae HxuCBA-like iron-specific transport system (44) in H. parasuis. One-dimensional Far Western blots of outer membrane proteins probed with biotinylated porcine transferrin or biotinylated porcine hemoglobin showed that H. parasuis outer membrane proteins of strains used in the original 2-D electrophoresis study reacted with the specific iron-related probes.

Interestingly, one of this study’s H. influenzae homologues (iron downregulated, immunoreactive protein) was phage-related (Table 4.1). A microarray analysis of H. parasuis gene expression under iron-limiting conditions also identified phage proteins (73). The present report describes a phage from a virulent serotype 5 field isolate, which was initially observed in whole cell lysate profiles. The Mu-like phage was characterized by electron microscopy, DNA sequencing, and proteomics including 1-D and 2-D gel electrophoresis followed by Mascot, Protein Prospector, and BLAST analysis.
**Dissertation Organization**

Chapter 1 contains the general introduction, organization, and literature review sections of the dissertation. Chapter 2 of the dissertation deals with the relatedness of *H. parasuis* reference and field strains as evidenced by protein whole cell lysate sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and random amplified polymorphic DNA (RAPD) analysis. The presence of a Mu-like bacteriophage in an *H. parasuis* serotype 5 field strain detected by using DNA sequencing, mass spectroscopy, and electron microscopy is reported in Chapter 3. Chapter 4 compares *H. parasuis* proteins of an avirulent serovar 6 reference strain to a virulent serovar 5 reference strain grown under iron-replete and iron-deplete conditions by 1-D and 2-D gel electrophoresis, Western blotting, MALDI-TOF, and SILAC. Chapter 5 ends with the general conclusions and gives examples of future work in this research area. References for the general introduction and literature review are at the end of Chapter 1. References for each journal paper follow each paper’s discussion. References for the general conclusions follow the end of Chapter 5.

**Literature Review**

**Description of the organism.** *Haemophilus parasuis* is a Gram-negative pleomorphic bacterium, varying in morphology from single coccobacilli to long, thin, filamentous chains (104). Glässer (45) associated this organism with polyserositis and polyarthritis in pigs in 1910. The causative organism is spread by direct contact from pig to pig and colonizes the nasal cavity (50). Originally, it was identified as *H. suis* because it was believed that it required both factor X (protoporphyrin IX or hemin) and factor V (β-nicotinamide adenine dinucleotide or β-NAD) for growth. Later, it was renamed *parasuis* meaning “suis-like” because it did not require factor X (hemin) for growth as did other *Haemophilus* species (9). *H. parasuis* does require factor V, because they can be observed as satellite colonies that grow along a nurse streak of *S. aureus* on blood
agar. The *S. aureus* secretes β-NAD and the *H. parasuis* uses it as a growth factor. The colonies are small, translucent, and nonhemolytic and the bacteria are nonmotile (104). The requirement for serum in broth media may be needed for detoxification of the media constituents rather than an actual growth requirement (60).

The presence of a capsule on an isolate establishes the basis for identifying the serovars and virulence of the organism. It has been documented that non-encapsulated or rough isolates tend to be nontypeable (NT) and that the antigens in a polysaccharide capsule are serovar-specific (60). Other antigens, the outer membrane proteins, also play a role in virulence. Morozumi and Nicolet (76) concluded that the pathogenicity of *H. parasuis* was correlated to the presence or absence of acidic polysaccharide capsules and PAGE type II protein profiles.

Taxonomically, *H. parasuis* is classified as a member of the phylum Proteobacteria (58), described mainly by 16S ribosomal RNA (rRNA) sequences. All Proteobacteria are Gram-negative, with an outer membrane made up primarily of lipopolysaccharides. The class *H. parasuis* belongs to is Gammaproteobacteria, which includes many veterinary and medically important bacteria. The organisms in the family Pasteurellaceae (58) of the order Pasteurellales are commensals of the upper respiratory tracts of birds and mammals. They are facultative anaerobes, possess oxidase, and have been classified based on metabolic properties, including urease negative, mannitol negative, catalase positive, and alpha-fucosidase positive (115). There are five genera in the family Pasteurellaceae: *Actinobacillus, Haemophilus, Lonepinella, Pasteurella*, and *Mannheimia* (37). However, these classifications are not true representations of their evolutionary relationships. For example, DNA-DNA rehybridization and 16S rRNA sequence data implies that *H. parasuis* is more closely related to *Actinobacillus indolicus* than to other *Haemophilus* species (60). The mol % G + C content of *H. parasuis* DNA is 41-42 % (60).
Serotyping. Although Glässer discovered the bacteria in 1910 (45), Biberstein and White (8), then Kilian (61) proposed that it be classified as a new species of *Haemophilus*, based on its ability to grow without hemin (X factor). Morozumi and Nicolet (77) based their classification of 5 serovars of *H. parasuis* on capsular acidic polysaccharide antigen for serovars 1-3 and cell outer membrane protein antigen for serovars 4 and 5. They believed that any of the 5 serovars could be virulent based on protein profiles by PAGE type II except with the exception of serovar 3.

At the present time, there are 15 recognized serovars of *H. parasuis*, based on immunodiffusion of heat-stable polysaccharide antigens (59, 103), which probably include some proteins, capsular, and LPS determinants (68), and on virulence observed in SPF pigs (59). However, the expression of the polysaccharide capsule can be lost by *in vitro* passage and influenced by growth medium (59, 76, 106, 107). Virulence in SPF pigs was high based on causing death or morbidity with serovars 1, 5, 10, 12, 13, and 14; low due to induction of polyserositis, but not death with serovars 2, 4, and 15; mild in serovar 8; and serovars 3, 6, 7, 9, and 11 are considered avirulent (59). The authors postulated that differences in strain phenotypes could be associated with isolates from systemic sites and respiratory sites (59). However, two serovar 14 strains produced variable virulence in caesarean-derived, colostrum-deprived (CDCD) pigs, which indicates that there are factors other than an isolate’s serotype that contribute to the virulence potential of a strain (104).

Another method for serotyping *H. parasuis* is the indirect haemagglutination (IHA) test (30, 125), used by some labs as a secondary technique when the immunodiffusion test gave inconclusive results. Initially, IHA results were frequently reported to be negative or inconclusive due to the method of antigen preparation (94). However, the immunodiffusion test had interfering cross-reactions when field strains were tested (120). Recently, the IHA method identified more than 90% of the *H. parasuis* field strains, including cross-reactive immunodiffusion NT strains (120).
Combining the IHA and immunodiffusion serotyping methods reduced the number of NT isolates from 36.7% to 9.6% in a Chinese report (19) and from 47% to 15% in a Danish study (4). Both methods require the availability of proper typing reagents, namely antisera and antigens.

**Prevalence of *H. parasuis***. When Glässer’s disease was first discovered in 1910, it was not as prevalent as it is today. Nursery-age piglets were diagnosed with *H. parasuis* by veterinarians or laboratories during the previous twelve months in 73.4% of the sites raising 100 or more hogs in 2006 in the United States. Large sites with 5000 hogs or more had the most occurrences of *H. parasuis*. Grower/finisher pigs were diagnosed with *H. parasuis* in 60.2% of the sites (126). In Germany, *H. parasuis* was recently isolated from bronchoalveolar lavage fluid of 43% of healthy animals and 53.1% of pigs showing clinical signs of pneumonia (98).

There has been an increase in new respiratory diseases, such as porcine reproductive and respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), *Mycoplasma hyopneumoniae*, or *Streptococcus suis*, that also compromise the pig’s ability to fight infections. Brockmeier (16) showed that intranasal coinfection of pigs with *Bordetella bronchiseptica* and *H. parasuis* increased the pigs’ upper respiratory tract colonization by *H. parasuis*. Narita et al. (84) reported that pseudorabies virus (PRV) infection of respiratory epithelial cells allowed the proliferation of *H. parasuis* in the lung, possibly due to a shift from macrophage to more neutrophils in the bronchioalveolar lavage fluid. There has also been a trend toward segregated SPF facilities which are more prone to disease if new pigs carrying different strains of *H. parasuis* or other respiratory pathogens are introduced.

Historically, serotyping has been the method of choice for identification of isolates. In 1992 the most prevalent serovars were 5, 4, and nontypeable in North America and Europe (59). There did not seem to be a correlation between serovar, site of isolation, or pathogenic potential of the isolates. However, an isolate was categorized as
respiratory if it originated from the respiratory tract of a pig without evidence of polyserositis and systemic if it was isolated from the lungs or another site of a pig with polyserositis (106). In 1999 the most prevalent strains of *H. parasuis* in Japan, Germany, the United States, Canada, and Australia were serovars 5, 4, 13, and NT isolates (104). However, by 2005, combining the immunodiffusion and IHA serotyping methods showed that serovars 4, 5, 13, and NT isolates were the most prevalent in China (19). In 2004, a Danish study (4) that utilized IHA and immunodiffusion showed serovars 5, 4, and NT isolates as the most prevalent isolates. Both the 2004 and 2005 studies reported serovar 4 as more frequently isolated from the respiratory tract while NT isolates were usually from systemic sites. Two other studies done in 2003 and 2004 in Canada and in the United States, respectively, confirmed that serovar 4 had become more prevalent than serovar 5 (91, 120).

Two serovar 2 isolates were most recently isolated from two geographically separated wild boars in Slovenia (97). Instead of the immunodiffusion serological method, this study utilized IHA plus two genotyping methods, ERIC-PCR and multilocus sequence typing to characterize the isolates. The authors hypothesized that wild boars may serve as a reservoir of *H. parasuis* which could be transferred to domestic pigs. They also assumed that the wild pig population does not show as much disease due to *H. parasuis* because piglets are allowed to nurse long enough to absorb passive antibodies and later can produce their own antibodies.

**Protein analysis of isolates.** Nicolet et al. (86, 87) were the first to use sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of *H. parasuis* whole cell preparations in order to correlate protein patterns with the type of isolate they were studying. They observed three protein profiles: respiratory isolates (PAGE type I); a Glässer’s syndrome (PAGE type II), and a third atypical field strain protein band pattern. They also predicted the heterogeneity of *H. parasuis* strains. Ruiz et al. (109) compared the OMP and DNA profiles from *H. parasuis* isolated from respiratory and systemic
sites. They found that the OMP and DNA profiles were unrelated, because the same genotype could produce different OMP patterns. However, they suggested that OMP and DNA profiles were more homogeneous from strains isolated from systemic sites and that certain OMP patterns were related to virulence.

**DNA analysis of isolates.** Smart et al. (114, 115) were the first to use a genome-based technique, restriction endonuclease fingerprinting (REF) analysis, to classify *H. parasuis*. They compared isolates from healthy, SPF, conventional, and diseased pigs and looked for the occurrence and distribution of strains. An advantage of REF analysis is that all isolates can be classified, i.e., there are no NT isolates. This method was more sensitive than serotyping and allowed the identification of more unique strains, 24 of the 69 isolates were unique, within groups of related organisms. A subsequent REF analysis study (114) was able to detect the source of an *H. parasuis* infection in piglets moved from one SPF farm to another and showed that a commercial bacterin was not cross-protective nor as effective as an autogenous vaccine was.

The second method of genotyping, a PCR-based method that could differentiate *H. parasuis* isolates, was ERIC-PCR (102). All 15 reference serovars gave unique, reproducible fingerprints after repeated tests. Twelve clinical isolates from 3 related multi-site farms yielded identical fingerprints which were different than the pattern of any of the reference strains, making this a viable method for epidemiology studies. There were no NT isolates when this method was used as compared to serotyping. There is no need for pure DNA preparations with this method, as there is with REF analysis. The basic tools used are primers, a thermocycler, and a horizontal agarose electrophoresis apparatus. Oliveira et al. (91) expanded ERIC-PCR to characterize the genetic variability of 98 field isolates from 15 North American farms and compare those results to serotyping. They determined that serovar 3 was mainly isolated from the respiratory tract, serovars 1, 4, 12, 14, and NT isolates were isolated from pneumonic lungs or other systemic sites, and serovars 2, 5, and 13 were isolated only from systemic sites. The use
of computer software rather than visual inspection revealed that similar genotypes clustered within the same serovar group, which implied that the genotype of an isolate might predict its serovar. Although there was high genetic diversity among isolates, only a few caused disease and the disease-causing organisms were recommended for use in autogenous vaccines.

A species-specific PCR assay based on amplification of a specific target, the *H. parasuis* *tbpA* gene, which codes for an OMP involved in iron uptake, followed by digestion of the amplicon with 3 restriction endonucleases created 12 restriction fragment length polymorphism (RFLP) groups in the 15 reference serovars and 33 RFLP profiles in 101 clinical isolates, including 23 new RFLPs not found in the reference serovars (28). These authors concluded that PCR-RFLP could type 100% of the highly heterogeneous isolates compared to the 65% typed by immunodiffusion. The reproducibility of their technique was an improvement over previous PCR methods which used nonspecific primers and pure cultures of organisms were not required to analyze the isolates with this method. They could see no correlation between the serotyping and PCR-RFLP methods. A subsequent PCR-RFLP study (31) related to the *aroA* gene and two restriction endonucleases only grouped serovars 3 and 7 with the same RFLP pattern while all the other reference serovars showed a second identical pattern. Therefore, since these results were not sufficient to even discriminate *H. parasuis* reference serovars, future experiments should utilize more than two restriction endonucleases after amplifying a possibly more heterogeneous gene target.

Using another genome-based technique, multilocus enzyme electrophoresis, and assaying 17 enzyme loci in 40 Australian field isolates and 8 reference serovars, Blackall et al.(12) observed 34 different electrophoretic types (ET) of *H. parasuis*. Although there appeared to be a high level of genetic diversity, and the ETs clustered into two major subdivisions, the authors did not conclude that there was a correlation between virulence and genetic grouping. However, they asserted that serotyping was not appropriate for
epidemiological studies, especially since they found diversity within serovars. In a more recent study, a multilocus sequence typing system (96) used partial sequences of seven housekeeping genes to separate 11 reference serovars and 120 field isolates into 13 lineages, 69 singletons and 6 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clusters of related genotypes. One cluster was associated with nasal, avirulent isolates while another cluster contained mostly systemic, virulent isolates. A neighbor joining analysis confirmed that the Glässer’s disease-associated cluster was divergent from the other *H. parasuis* isolates.

**RNA analysis of isolates.** Oliveira et al. (92) introduced a species-specific PCR test based on *H. parasuis* 16S rRNA. Advantages of this test were that nonviable organisms and tissues from animals that had been treated with antibiotics could be analyzed. However, not all serovars were amplified and some *Actinobacillus* species related to *H. parasuis* that colonize the swine upper respiratory tract were also detected. The authors recommended that diagnostic samples only be taken from systemic sites. Angen et al. (3) developed an improved species-specific 16S rRNA test which used multiplex PCR and 3 different primers. Because of its 100% specificity for *H. parasuis*, clinical samples with contaminating bacteria did not give false-positives. These authors found significant heterogeneity between the 15 reference serovars of *H. parasuis* as well as duplications of sequences between *rrn*-operon clusters. Nasal swabs from clinically healthy piglets from multi-site farms revealed that 54% were PCR positive for *H. parasuis* at 4 weeks old and 87% were positive at 10 weeks old. A drawback of both of the latter two PCR tests is that they can only give a “yes or no” answer to the presence of *H. parasuis* rRNA and no classification of the isolates can be done.

**Vaccines.** Ways to control the spread of *H. parasuis* disease include (104) vaccination and antibiotic treatment of all pigs in the facility if any are found to be diseased, and management procedures which decrease herd exposure to respiratory pathogens, eliminate the mixing of pigs, and wean piglets at an older age and over shorter
lengths of time. Sanitation of the facility, biosecurity, and source and type of food should be controlled (50).

Stressful events that might make the piglets more prone to *H. parasuis* infection include weaning, weather changes, and movement to new housing (60). The effects of vaccination against *H. parasuis* serovar 5 and *E. coli* during outdoor summer and winter conditions was studied in Hungary (57). Winter vaccinates had better average daily gain, feed efficiency, and less nursery mortality than nonvaccinates. Summer pigs were significantly heavier than winter pigs during the nursery period. Since it has been proposed that coinfection with either or both porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae* may cause immunosuppression, vaccination against these agents is suggested as well as vaccination against *H. parasuis* (50). Along with vaccination of the herd, replacement gilts should be quarantined to make sure they are not carrying any undesirable diseases before they are mixed with the herd.

The lack of herd immunity has been attributed to weaning piglets before they are 21 days old (50). Disease follows because either the piglets received no maternal antibodies or the antibodies decayed and were not protective. On the other hand, some carrier piglets actually get infected by nursing their mothers. Other suspected sources of infection include caretakers and rodents carrying the organism into the facility. Blanco et al. (13) showed that piglets getting maternal colostrum for 4 days, then using 2% iodine as a disinfectant, and antibiotics to reduce piglet diarrhea after transport to the nursery were important in protecting newborn piglets from systemic disease.

Blackall et al. (11) suggested that inactivated vaccines should at least contain the serovar (or serovars) prevalent in the target pig population or be produced from *H. parasuis* isolates cultured from the target herd. Rapp-Gabrielson et al. (105) found that protective antigens of *H. parasuis* inactivated bacterins seemed to include both serovar-specific and cross-protective determinants. However, they could not identify which
antigens might give broad cross-protection against all serovars or field strains. Takahashi et al. (122) found that monovalent vaccines protected against homologous challenge, but not against heterologous challenge. On the other hand, a bivalent vaccine protected against challenge with both *H. parasuis* serovars 2 and 5. Upon vaccination of SPF pigs with a monovalent or a trivalent vaccine, pigs as early as 2 to 4 weeks of age were protected and the only antibodies detected by immunoblot were to a 37 kDa OMP (74, 75). No antibodies were detected by ELISA or IHA, inferring additional mechanisms of immunity in immature piglets.

Nedbalcova et al. (85) recommended autogenous vaccines prepared from central nervous system isolates that were infecting that particular herd. Gilts should be vaccinated 3 to 4 weeks before farrowing and piglets should be vaccinated at 7-8 weeks (50). However, serovar diversity and the large number of NT isolates contribute to the lack of cross-protective vaccines (94).

Oliveira et al. (90) showed that direct and early colonization of 5 day old piglets via tonsillar or oral routes with the herd’s systemic isolates, while the maternal antibodies were still present, protected piglets twice as well as natural colonization via nose to nose contact with sows. In a promising advancement, Bigas et al. (10) developed a thyA mutant of *H. parasuis* which lacked colonization capacity but still induced wild type levels of serum bactericidal activity in infected guinea pigs.

Pathogenesis. Kielstein and Rapp-Gabrielson (59) assigned virulence to the 15 reference serovars of *H. parasuis* after intraperitoneal inoculation of SPF pigs. The strains were classified as highly virulent resulting in death, low virulent that caused polyserositis but not death, or avirulent. Clinical symptoms of an *H. parasuis* infection include neurological signs, anorexia, lameness, swollen joints, difficulty in breathing, fever, wasting, lethargy, cyanosis, unilateral ear inflammation, abortion, and sudden death (37). Gross pathological lesions include arthritis, pleuritis, pericarditis, peritonitis, meningitis, polyserositis, rhinitis, periorchitis, and sometimes pneumonia (68).
Amano et al. (2) studied the effect of *H. parasuis* endotoxin on the coagulation system of SPF pigs inoculated intratracheally. The fibrinous microthrombi which formed in the kidney, liver, and lungs may have led to septicemia, disseminated intravascular coagulation, endotoxic shock, and death within 28-42 hours. The authors noted that intranasal inoculation did not lead to systemic infection as quickly as the intratracheal route, which may have allowed the bacteria to avoid the mucosal immunity of the upper respiratory tract.

Another group (20) examined the ability of rabbit or porcine serum to kill *H. parasuis* isolates obtained from nasal cavities of healthy swine, pulmonary tissues, and systemic sites. All of the nasal strains were serum-sensitive, pulmonary strains that were associated with bronchopneumonia but not systemic disease were serum-sensitive, while all systemic isolates were resistant to serum killing, including pulmonary strains isolated from pigs with systemic infections. The authors surmised that bacterial serum resistance is required for spreading to internal organs and for systemic disease. Additionally, coinfection with other pathogens may have caused the clinical lesions in bronchopneumonia cases. It was initially predicted that PRRSV might have an immunomodulatory effect on *H. parasuis* infection because alveolar macrophages had a decreased ability to produce superoxide anion (116). Later, it was shown that pigs dually infected with the virus and bacteria did not result in an increased polyserositis when compared to *H. parasuis* alone was used as the inoculum (113, 117). However, Brockmeier (16) was able to show that *B. bronchiseptica* increased colonization and/or aggravated disease with *P. multocida, S. suis*, or *H. parasuis*.

Even though Vahle et al. (127) failed to isolate *H. parasuis* from the middle part of the swine nasal cavity which had rhinitis and loss of mucocilliary cells, it was still suggested that the bacteria may have entered the blood circulation through nasal mucosal changes after colonizing the upper respiratory tract. Recently, all serotypes of *H. parasuis* were shown to adhere to porcine brain microvascular endothelial cells
Additionally, serotype 4 and 5 strains displayed higher invasive capacity than strains of the other serotypes. Mechanisms involved include the interaction of a pre-existing bacterial putative invasin with a cell receptor; a nonproteinaceous surface component such a lipooligosaccharide (LOS) implicated in adhesion and invasion; and reorganization of host cytoskeleton components such as actin microfilaments and microtubules. The entry of *H. parasuis* into the endothelial cells of the blood brain barrier may be a key step in the pathogenesis of meningitis.

Another pathogenic mechanism is antigenic variation of the bacteria’s surface receptors (38). This occurs when a strain changes the outer antigens presented to the host enabling persistence in or reinfection of the same host because the new antigens are not recognized by previously synthesized antibodies. Various forms of antigenic variation include on/off switches for different copies of an antigen, many silent copies of a gene which can be expressed at random, and highly variable genetic regions which are constantly changing their expression through point mutations, deletions, insertions, or ribosomal frameshifts (39, 41). Tandem repeated DNA sequences of surface-exposed proteins can allow for antigenic variation through intragenic homologous recombination (101). Examples of antigenic variation include varying the amount of sialylation of surface glycoproteins or LPS and changes in pili, flagella, and several OMPs, such as the Opa proteins in *Neisseria*. *H. influenzae* can also express more than 30 variants of IgA protease (39, 131).

In *H. influenzae*, mutations produced by altered amino acid substitution which cause change in surface exposed epitopes of OMPs P2 and P5, respectively, are observed. After these alterations, complement-mediated bactericidal antibodies failed to recognize the P2 and P5 colonization factors and lyse the pathogen. Persistent infections ensued because the organism could evade the immune response (34, 35). *H. parasuis* also appears to have antigenically variable adhesins, OMPs P2 and P5 (71, 72). A cross-reactive monoclonal antibody (4BF8) produced against P5 from *H. parasuis* reacted
against 2 forms of the P2 protein: a 48 kDa protein in most virulent serovars and a 55 kDa protein in most avirulent serovars. In addition, the antibody reacted against a 32 kDa P5 protein (71). The difference in *H. parasuis* P2 protein sizes derived from virulent and avirulent isolates may be due to differences in variable exposed loop surfaces of each protein as described in *H. influenzae* (35, 71). The P2 protein is recognized as a carcinoembryonic antigen while P5 is not, probably due to P5’s antigenic variability (72).

Other examples of bacterial exposed surfaces which probably undergo antigenic variation are outer membrane receptors for heme, hemoproteins, transferrin, and siderophores, all of which are highly immunogenic. The latter three OMPs show phase variability, meaning their expression is up- or downregulated, depending on the environment of the bacteria. Phase variability provides a way for the pathogen to escape the host’s immune system because as an OMP changes its immunogenicity, the host’s acquired immune system might not have the same memory for that antigen (39, 129).

A change in nutrients, such as nicotinamide adenine dinucleotide (NAD) or iron, may be growth limiting to *H. parasuis* when the organism invades tissues of the host (88). At this point, there might be production of fimbriae, altered outer membrane profiles, and enhanced adhesion. In normal tissues, iron is sequestered in iron-binding molecules such as transferrin or lactoferrin, or in heme-binding molecules such as hemoglobin. The free ionic iron in host tissues is $10^{-18}$ M, which is insignificant. However, Bullen et al. (17) proposed that large numbers of bacteria can produce reducing conditions which could cause loss of normal vascular permeability, allow hemoconcentration, and end with shock in the host. During an infection, the bacteria and the host must compete for iron which is normally either enclosed in erythrocytes or is restricted in iron-binding proteins.

**Bacterial Iron Acquisition.** Somehow the invading bacteria must acquire iron in order to survive in the host. Most of the bacterial proteins involved in iron and heme acquisition are up-regulated during iron-limiting conditions. Extra cytoplasmic function
(ECF) sigma factors (129) control positive regulation of iron/heme-uptake genes. In most cases, iron-starvation sigmas are repressed by the iron-loaded Fur protein. In iron-rich media, Fur negatively regulates the iron/heme-uptake genes as well as various virulence factor genes, such as lactoferrin, transferrin, and hemoglobin. During iron-limiting conditions, the sigma factor is released from the antisigma inhibition and induces transcription of the target operons.

All cells have to protect themselves from reduced Fe$^{2+}$, which activates the Fenton reaction and the production of hydroxyl radicals. The cells sequester iron within lactoferrin, transferrin, ferritins, or protoporphyrin rings. Bacteria have multiple systems through which they can obtain the essential element of iron (129). For instance, there can be direct contact between the bacteria and the extracellular iron or heme source through receptors at the cell surface. Additionally, there can be indirect scavenging of iron by siderophores which chelate ferric ion or by hemophores which chelate heme, both of which are synthesized and released by the bacteria. The chelated iron compounds are returned to specific bacterial outer membrane receptors.

An example of a hemophore is *H. influenzae*’s heme-hemopexin utilization system (HxuCBA). Found only in Gram-negative bacteria, they are specialized extracellular proteins that acquire heme from multiple sources, then bring it back to a specific outer membrane receptor. Soluble HxuA (100 kDa) associates with a helper protein, HxuB (60 kDa), which are secreted from the cell; HxuA binds to heme-hemopexin and the resulting complex is presented to HxuC (80 kDa), the outer membrane-specific receptor (24, 25, 78, 129). HuxC has been described as a TonB-dependent protein involved in protein transport (25). Not only was HuxC involved in the transport of heme, but it could also utilize hemoglobin and hemoglobin-haptoglobin (Hgp) (24). Alternatively, the phase-variable HgbA, HgbB, and HgbC TonB-dependent outer membrane proteins could also bind hemoglobin or hemoglobin-haptoglobin. Morton et al. (79) described a TonB-dependent 101-103 kDa general haem-utilization
protein (Hup) that could utilize heme, heme-hemopexin, heme-albumin, or hemoglobin-haptoglobin in \textit{H. influenzae}. After heme is removed from the outer membrane receptor, it is transported into the cell through a TonB-induced conformational change in the receptor and an ABC transport system. The iron is released from the heme by a heme oxygenase or by a reverse ferrochelatase reaction (44). Under iron-limiting conditions, \textit{H. influenzae} has accumulated redundant mechanisms to obtain heme and iron from host sources as well as a mechanism to shift cells into gluconeogenesis (132).

\textit{Mannheimia haemolytica}, another member of the Pasteurellaceae which colonizes the bovine lung, up-regulates numerous proteins under iron-limiting conditions, including two hemoglobin receptors, HmbR1 and HmbR2; transferrin-binding protein, TbpA and TbpB receptors; a hemin receptor; a putative periplasmic hemin transport system; TonB; ABC transporters; and siderophores for ferrichrome transport homologous to \textit{fhu}BCD of \textit{A. pleuropneumoniae} (108). \textit{Actinobacillus pleuropneumonia} has two TonB systems that work together to take up transferrin (7). Isolates with \textit{tonB2}, most homologous to other Pasteurellaceae gene clusters, are necessary for heme, porcine hemoglobin, and ferrichrome assimilation and \textit{tonB2} mutants have attenuated virulence \textit{in vivo}. On the other hand, \textit{tonB1} mutants seemed to be able to utilize transferrin-iron at low doses of infection and may be essential for virulence at those doses.

The transport of iron into the periplasm of \textit{Neisseria meningitidis} depends on TonB, ExbB, and ExbD, located in the cytoplasmic membrane. The TonB complex provides proton motive force in order to internalize the substrate. In \textit{N. meningitidis}, specific binding of transferrin to the TbpA receptor or hemoglobin to the HpuAB receptor depends on TonB and a functional proton motive force. Transport across the inner membrane is carried out by ABC permeases. Once inside the bacteria, the heme is either degraded by heme oxygenase or is stored in ferritins (129). In the case of \textit{N. meningitidis'} lactoferrin uptake, the system consists of a two-protein receptor, LbpB (bilobed lipoprotein) and LbpA (siderophore receptor homologue), which removes iron
from lactoferrin; TonB, and FbpA (ferric ion-binding protein A), which aids in iron removal and transport to the periplasmic space (36).

Two mechanisms for iron acquisition in *H. parasuis* have been described: 1) a Fur-independent *tonB*-like constitutive siderophore receptor system for inorganic iron uptake (32, 80); and 2) a Fur-dependent siderophore-independent outer membrane receptor system for binding and uptake of protein-bound iron compounds (21, 29, 80). Morton and Williams (80) detected siderophores by production of orange halos around colonies on CAS (Chrome Azurol S) agar; 74, 82, and 100 kDa iron-repressible outer membrane proteins; and utilization of iron from porcine transferrin in *H. parasuis*. A constitutively expressed ferric hydroxamate uptake (Fhu) ferrichrome siderophore system was reported by del Rio (32) in all serotypes of *H. parasuis*. However, the authors found no Fur homologue for this gene cassette. Four *H. parasuis* Fhu proteins, FhuCDBA, were 99-100% homologous to *A. pleuropneumonia* fhu genes. Although FhuA protein expression was not changed under iron-restricted conditions, it was shown to be an immunogenic protein after probing Western blots with convalescent serum.

Charland et al. (21) determined that *H. parasuis* binds porcine transferrin via direct contact between the protein and bacteria. The molecular weights of TbpA and TbpB were reported to be 102 and 59.6 kDa, respectively. A Fur box was identified upstream of the *TonB* gene of *H. parasuis*, which confirmed that the expression of the ExbB, ExbD, TbpB, and TbpA proteins was regulated by Fur (29). PCR amplification of the *tbpB* gene showed heterogeneity between *H. parasuis* reference serovars, whereas amplification of the *tbpA* gene yielded homogeneous products. On the basis the size of the PCR amplicons, the heterogeneous *tbpB* gene products could be separated into two groups.

**Virulence in bacteria.** Some of the first virulence factors used by bacteria after infection of the host are adhesins or receptors, which attach the pathogen to the host surfaces (39). Capsules on Gram-negative bacteria prevent antibody recognition and
complement-mediated lysis or opsonization of bacteria. Later, invasins, hemolysins, proteases, toxins, outer membrane proteins, and secretion systems allow the pathogen to enter the host cell. Type III secretion systems (81) inject toxins directly into the host cells and kill them, while Type IV secretion systems (23) inject molecules into the host cell, which affect the vesicular transport system and allow intracellular survival of the pathogen.

**Surface constituents.** Gram-negative bacteria have capsules made up of lipopolysaccharide (LPS), which prevents antibody and the complement membrane attack complex from inserting on the cell membrane, yet allows fimbriae to protrude through the capsule in order to bind to host surfaces (39). A prerequisite for infection of the host cell is bacterial adhesion to the mucosal epithelium. Münch et al. (82) demonstrated the presence of fimbriae in *H. parasuis* after culturing the bacteria at elevated temperatures and embedding the colonies in stabilizing Epon resin prior to electron microscopy.

Stressed or nutrient-limited bacteria upregulate various OMPs. These include the iron-regulated proteins mentioned above, such as lactoferrin-, transferrin-, hemoglobin-, and hemoglobin-haptoglobin-binding proteins, heme-hemopexin utilization proteins, and siderophores. Stress to bacteria caused by host defenses, such as antibiotics or antimicrobial agents, can even activate deletions and rearrangements in the bacterial genome which maintain virulence and broaden host range (5). Additional surface constituents include neuraminidase and adhesins like P2 and P5.

**Adhesins.** Recently, McVicker and Tabatabai (71, 72) characterized two *H. parasuis* OMPs homologous to *H. influenzae*’s P2 and P5 adhesins. Besides adhering to the host cells, the adhesins are antigenically variable from one strain to another and even within the same strain over the course of a chronic infection. This mechanism helps the organism avoid the host’s immune response.

**IgA proteases.** Mucosal pathogens, such as *Haemophilus influenzae*, secrete IgA proteases (220 kDa) through a bacterial autotransporter, which then degrade secretory
IgA antibody found at mucosal surfaces which plays a role in humoral antibody defense of the host (39). IgA proteases also degrade pathogen-induced antimicrobial peptides, such as defensins and cathelicidins of the host’s innate immune system, thereby preventing lysis of the invading pathogen (49) and allowing the pathogen to infect its host. IgA protease cleavage at the IgA hinge region results in the production of specific Fabα fragments, which can subsequently coat the bacteria and enable its adherence to host tissues or aid in its evasion of the host immune system by blocking access of intact antibody to the bacterial surface (69, 123). In chapter 4, an IgA protease of *H. parasuis* is described.

**Invasins.** *H. parasuis* causes meningitis and invasins have been implicated in its crossing of the blood brain barrier. Serotypes 4 and 5 as well as most field strains of *H. parasuis* were found to adhere to and invade porcine brain microvascular endothelial cells (PBMEC) better than the other serotypes (128). Invasion of PBMEC was both time and bacterial concentration dependent. Moreover, *H. parasuis* was protected from antibiotics while it was within the PBMEC for 8 hours. Actin microfilaments and microtubules, host cytoskeleton components, were rearranged prior to PBMEC invasion. This invasion process may be the key step in *H. parasuis*’s crossing the blood brain barrier and causing meningitis.

Neuraminidase (sialidase) (82 kDa) has been characterized as a possible invasin and as one of the potential virulence factors in *H. parasuis* (65). The bacterium hydrolyzes sialic acid from sialoglycoconjugates and utilizes sialic acid for growth. Possible functions of neuraminidase include unmasking receptors for adhesion or invasion and allowing colonization or interfering with innate or humoral immunity (66). IgA-like proteases named hap (*Haemophilus* adherence and penetration) proteins (155 kDa) have also been implicated in invasion of host cells by *H. influenzae*, which may allow the pathogens to persist in the respiratory tract (118).
Altering phagocytosis. Pathogens can also change transcriptional and host cell death pathways. Phagocytized bacteria skew the type of neutrophil death to apoptosis or autophagy which are less inflammatory because of impaired production of reactive oxygen species and therefore less harsh on the pathogen than cytotoxic cell death (46). Moreover, apoptotic neutrophils are usually phagocytosed by macrophages, which allow the pathogen to replicate, survive, and cause disease longer intracellularly. If the neutrophil lyses, the bacteria are released and the host tissue is damaged by inflammation (33). In the only phagocytosis study done with \textit{H. parasuis}, a prior infection with PRRSV decreased macrophage killing of the bacteria, possibly due to the porcine alveolar macrophages’ decreased ability to produce superoxide anion (116).

Toxins. Bacteria utilize toxins to colonize and infect the host. Exotoxins, such as ADP-ribosyltransferase, mimic the structure of 18S rRNA and translation elongation factor 2 (eEF2) and inhibit the binding of transfer RNA during protein translation (134). Protein synthesis in the host cell is terminated because neither eEF2 nor rRNA can effectively mutate to overcome translation inefficiencies. No exotoxins have been reported in \textit{H. parasuis}. However, Amano et al. (2) discovered an endotoxin, a component of Gram-negative bacteria outer membrane LPS, in the blood of \textit{H. parasuis} septicemic pigs and postulated that it could cause coagulation system dysfunction, leading to septicemia, disseminated intravascular coagulation, endotoxic shock, and death.

Quorum sensing and biofilms. Biofilms can form on mucosal surfaces and allow for close contact between bacterial species present in the host. Recently, biofilms were discovered in \textit{H. parasuis} reference serovars and field isolates (56). However, they found that reference serovars 2, 9, 12, 13, and 15 and that 57% of the field isolates did not form biofilms \textit{in vitro}. A higher proportion of avirulent isolates formed biofilms than virulent strains did. The authors also studied the relationship between biofilm formation and virulence. When biofilm-forming isolates were passaged in pigs, strains that were
reisolated from the lung and brain had lost the ability to form biofilms. However, nasal isolates retained the ability to form biofilms, possibly elucidating the mechanism of persistent infection of the porcine respiratory tract. The virulent isolates may switch their characteristics from biofilm establishment to invasive properties in vivo.

Biofilm formation can be characterized by interkingdom signaling or “quorum sensing” of cells through secreted peptide (Gram-positive bacteria) or lipid (Gram-negative bacteria) hormones. The concentration of signaling molecules is related to the cell density of the bacteria (42). Signal transduction cascades act through second messengers, such as cyclic AMP or intracellular calcium which are up-regulated after an outer membrane receptor is bound by a substrate. Alternatively, a hormone’s diffusion through the cell membrane and direct binding to nuclear receptors can cause changes in bacterial or host gene transcription (110). Induced virulence factors, such as elastase, exotoxin A, alkaline protease, neuraminidase, hemolysin, catalase, superoxide dismutase, hydrogen cyanide, chitinase, lectins, rhamnolipid biosurfactants, and pyoverdine in *Pseudomonas aeruginosa*, allow the biofilms to adapt to changes in temperature, osmolarity, pH, and nutrients (133). Homologues of the *luxS* bioluminescence gene of *Vibrio harveyi* from *H. influenzae* and *Deinococcus radiodurans* also possess autoinducer activity (119).

Acyl homoserine lactone autoinducers from Gram-negative bacteria like *P. aeruginosa* have been shown to inhibit expression of interleukin-12 and tumor necrosis factor alpha (124). The host protective T-helper-1 response was predicted to shift to the bacterial protective T-helper-2 response by the quorum sensing factors’ ability to affect T cell proliferation and the release of interleukin 2 (55).

It is projected that 99.9% of bacteria in nature are attached to a surface in the manner of biofilms (83). *P. aeruginosa* forms biofilms around blood vessels (52) and this may lead to systemic spread to the liver or the bloodstream (112). Concentrations of autoinducers are higher locally within biofilms where apoptosis and proinflammatory
responses occur, while concentrations are lower away from biofilms where anti-inflammatory responses take place (22). The DNA in biofilms acts as a part of the extracellular polysaccharide matrix at the outer edge of the stalks and at the junction of the stalks and the cap and may be used as a supporting framework for twitching cells as they progress to form the cap (1) and as a means of bacterial transformation and horizontal gene transfer (99).

Extensive research has been done with nontypeable *H. influenzae* (NTHi) biofilms (83, 130). OMPs P2, P5, and P6 are expressed on the bacterial cell membranes and the lipooligosaccharide (LOS) expression is altered during growth of the bacteria in the biofilm. HMW proteins were distributed throughout the entire biofilm but were close to both the extracellular matrix (ECM) and the bacteria. Both high molecular weight (HMW) (120 and 125 kDa) and hap proteins, all adhesins, were believed to be involved in microcolony formation. Interestingly, the IgA protease was located on top of the biofilm, where it was hypothesized that it could best degrade host antibodies.

**Pathogenicity islands.** Pathogenicity islands (PAIs) are distinct genetic elements on the chromosomes of pathogens which encode virulence factors (43). PAIs are obtained through horizontal gene transfer via phage transduction, plasmid conjugation, or naked DNA transformation. Horizontal or lateral gene transfer was discovered in the 1950’s when multidrug resistance was observed to be transferred among bacteria rather than by individual mutations (89). The DNA sequences of ancestral (vertically transmitted) genes differ from horizontally transmitted genes which have a distinct phenotype and restricted phylogenetic distribution. Ways to identify PAIs are their different % mol G + C content compared to the rest of the bacterial DNA, their codon usage bias, their association with mobile genetic elements such as integrases or insertion sequences, and their links to tRNA genes (47). Some functions of PAIs include adherence mechanisms, siderophores or iron uptake systems, toxins, invasins, and protein secretion systems (43, 48). Loss of unstable PAIs by specific excision at certain tRNA
sites may cause a shift from acute disease to chronic infections (14). Bacterial genomes can only retain a finite amount of DNA and chromosomal deletions get rid of genes with no function. Moreover, PAIs are absent in nonpathogenic or closely related bacterial species. Horizontal gene transfer can provide a new ecological niche and promote bacterial speciation.

**Plasmids.** As stated previously, PAIs can be horizontally transferred by plasmids or bacteriophage. Lancashire et al. (62) did not imply that there were PAIs in *H. parasuis*, but they did identify a native *H. parasuis* plasmid that encoded tetracycline resistance and carried mobilization genes. They surmised that the use of tetracycline as an antibiotic and feed additive for growth of swine may contribute to selection of tetracycline-resistant *H. parasuis*. They found the same tetracycline-resistant plasmid from disease outbreaks 12 years apart on two different farms from multiple *H. parasuis* field isolates and one *A. pleuropneumoniae* clinical isolate. In another study, San Millan et al. (111) demonstrated that penicillin- and amoxicillin-resistant strains of *H. parasuis* carried a β-lactamase-encoding plasmid. Pulsed field gel electrophoresis (PFGE) illustrated that susceptible strains had widely different electrophoretic patterns, while resistant strains had identical profiles, confirming that there had been a clonal spread of the β-lactamase trait. They were concerned that continued indiscriminate use of β-lactam antibiotics might cause mutations which may then expand resistance to cephalosporins, utilized in both animals and humans. Antimicrobial susceptibility of *H. parasuis* differs dramatically in various countries (27). For instance, British isolates were primarily susceptible to a number of antibiotics, while Spanish isolates were multiresistant to many of the same antibiotics. The more recently introduced and least-used antibiotics were most likely to cause the least resistance. The use of good hygiene and management practices was recommended prior to testing for antibiotic susceptibility before the use of drug therapy.
**Bacteriophage.** Bacteriophage can also introduce foreign DNA into a bacterium by transduction. Gene sequences within certain pathogenicity islands are similar to phage integrases, including HP1’s integrase of *H. influenzae*, suggesting that the PAI was acquired through phage-mediated events (89). Double-stranded DNA tailed bacteriophages, Podoviridae (short tail stub), Siphoviridae (long non-contractile tail), and Myoviridae (contractile tail), as well as Inoviridae (filamentous tail with single-stranded DNA) all encode virulence factors and can change a nonpathogenic bacteria to a virulent one by lysogenic conversion of the bacteria through recombination events (15). For example, pyogenic exotoxins were upregulated and bacteriophages were produced when lysogenic group A streptococci and pharyngeal cells were co-cultured, which suggested cross-talk between the two entities. The virulence genes are commonly located near the recombination sites, the tail genes, or the capsid protease of the bacteriophage. Through their virulence factors, the bacteriophages contribute to pathogenesis, strain differentiation, and genomic diversity of the bacterial species. A few of the virulence determinants found thus far in bacteriophage consist of immunoglobulin-binding regulators; many toxins including an enterohemolysin; membrane proteins, glycosylation proteins, glucosyl transferase, and O-antigen acetylase which alter antigenicity; type III effectors involved in invasion; superoxide dismutase, neuraminidase, and hyaluronidase involved in intracellular survival; OMPs involved in serum resistance; adhesins and others (15, 70). Double-stranded DNA bacteriophages also express immunoglobulin-like domains, including hydrolases, peptidases, and endopeptidases that bind to or degrade bacterial cell polysaccharides or LPS, and which increases bacterial infectivity (40, 41). To date, there has been one report (73) on the presence of bacteriophage genes in *H. parasuis* after growth under iron-limiting, oxygen-limiting, heat, and acidic stress conditions.

**Detection of virulence factors.** Signature-tagged mutagenesis is a genetic method used to identify virulence genes whose products are needed for survival and
Virulence factors of pathogenic bacteria are expressed during various stages of the infection: 1) tropism for specific host and/or tissue; 2) host cell interactions; 3) means of survival in the host; and 4) crossing the blood brain barrier. Mutants that have lost the ability to survive in the host can be identified by a transposon inserted in a surface molecule which may be involved in colonization, tropism, crossing of barriers, multiplication, and stress adaptation. Mutant genes from biosynthetic (purine/glucose/LPS) and regulatory pathways have also been found. Inhibitors of virulence factors are also being investigated, such as two structurally related triazines, which interfere with the biosynthesis of the capsule and lipopolysaccharides of *Klebsiella pneumoniae* (8). The TraJ protein, associated with conjugation, was shown to be involved in the ability of *E. coli* to become systemic and to cross the blood brain barrier by interacting with macrophages (54).

Other ways to detect virulence factors is to use bioinformatics to compare genomes of closely related strains of bacterial pathogens and search for conserved genes in those strains. Microarrays of “complete” bacterial genomes have been compared to “mutant” genomes, which fail to survive in given conditions (18). The regulatory functions of Fur were studied by microarray transcriptional profiling concerning Fur’s binding to 200 regulator binding sites within the *Helicobacter pylori* genome. Genes were found that were associated with Fur repression as well as mutant genes that were not regulated by Fur (26). TTSS effector proteins have been studied in yeast (64). Host factors needed for intracellular infection have been identified through expressed sequence tag (EST)-based knockdown (67) and RNA- interference methods (100).

**Virulence studies in H. parasuis.** Hill et al. (53) reported seven upregulated potential virulence factors by using differential display reverse transcription-polymerase chain reaction in *H. parasuis* serotype 5, strain 1185, grown in conditions mimicking those found in swine. Genes included 1) a questionable low homology *Homo sapiens* basement membrane-specific heparin sulphate core protein precursor with
immunoglobulin domains that resembles a mammalian protein found in joints; 2) sodium- and chloride-dependent ion transporter which may influence proton motive force or adaptation to the high salt environment of plasma; 3) a pyridine nucleotide transhydrogenase which provides the NADPH for biosynthesis in the pentose phosphate shunt; 4) diadenosine tetraphosphatase which may control the heat shock response by hydrolyzing deadenosine tetraphosphate (Ap4A); 5) long chain fatty acid CoA ligase which has a role in uptake of long chain fatty acids and fatty acid chain length specificity and which controls virulence factor synthesis and activation; 6) cysteine synthetase which is sensitive to pH and salt stress; 7) phosphoenolpyruvate-protein phosphotransferase which transports carbohydrates into the cell and is involved in signal transduction. The first 3 genes above are less homologous to known sequences than the last 4 genes are. All of these genes were present in all 15 reference strains of \textit{H. parasuis} which makes them unsuitable as diagnostic markers for virulence.

Olvera et al. (95) explored the possibility of correlating a partial sequence of the 60 kDa heat shock protein gene (\textit{hsp60}), 16S rRNA \textit{H. parasuis} gene, and enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) fingerprints to virulence. Although ERIC-PCR could be utilized for characterizing close relatedness of local farm isolates, they did not find a definite relationship between the 16S rRNA and \textit{hsp60} sequences and site of isolation, virulence, serotype, or geographical origin of isolate. Moreover, the 16S rRNA and \textit{hsp60} sequences were found to be possibly horizontally transferred among \textit{H. parasuis} and \textit{Actinobacillus} strains, making them unacceptable as global molecular diagnostic tools for \textit{H. parasuis}.

Lancashire et al. (63) used representational difference analysis (RDA) and reverse Southern hybridization to genetically screen a 96-clone library of virulent (serovar 12) and avirulent (serovar 4) isolates for serovar-specificity and relatedness between serovars and virulence. They reported the identification of 13 serovar-4 specific clones after
verification by DNA sequencing. This technique appears to be complicated, labor-intensive, and expensive, especially since they only worked with 2 serovars.

Ruiz et al. (109) studied OMPs treated with sodium lauryl sarcosinate and DNA profiles of \textit{H. parasuis} from respiratory and systemic sites of swine. They concluded that repetitive element-based PCR patterns were more variable than the OMP profiles and that systemic isolates associated with polyserositis or from pneumonic lungs were more similar to each other than those of isolates from lungs without lesions. However, they noted that there was no virulence correlation between Rep-PCR and OMP profiles, but they did suggest that OMP profiles may be related to virulence.

Hartman et al. (51) described a 42 kDa outer membrane protein (porin) in \textit{H. parasuis}. Tadjine et al. (121) reported species-specific monoclonal antibodies, including those specific for a 35 kDa OMP homologous to OmpA, an invasin of brain microvascular endothelial cells and an LPS epitope, which may be involved in adherence to porcine respiratory tract cells. Lichtensteiger and Vimr (66) isolated a neuraminidase, which might be involved in colonization or invasion by \textit{H. parasuis}. The enzyme may degrade sialic acid residues of host glycoconjugates and expose receptors on the host cells. Amano et al. (2) reported an \textit{H. parasuis} endotoxin that contributed to septicemia and disseminated intravascular coagulation in specific pathogen free (SPF) pigs.

Although many potential virulence factors are known for \textit{H. parasuis}, others are yet to be determined. A limited number of studies have been done on strain relatedness of \textit{H. parasuis} by DNA and protein profiles. Little information is available on protein expression of \textit{H. parasuis} when the bacterium was cultured under iron-limiting conditions. Additionally, there has been no previous report of the characterization of a bacteriophage on \textit{H. parasuis} that may introduce potential virulence factors into the bacterium. This study will address these issues.
References


CHAPTER 2. COMPARISON OF Haemophilus parasuis REFERENCE STRAINS AND FIELD ISOLATES BY RANDOM AMPLIFICATION OF POLYMORPHIC DNA AND PROTEIN PROFILES

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Abstract

Haemophilus parasuis is the causative agent of Glässer’s disease and is a remerging pathogen of swine in high-health status herds. Reports on serotyping of field strains from outbreaks describe that greater than 30% of them are nontypeable and therefore cannot be traced. Molecular typing methods have been suggested as an alternative to serotyping. This study was initiated to compare random amplification of polymorphic DNA (RAPD) profiles and protein profiles of H. parasuis reference strains and field isolates as a method for identifying and grouping virulent and avirulent isolates. The DNA and protein profiles of 15 reference strains and 31 field isolates of H. parasuis were analyzed with a computer-based similarity program (Gel Compar II). Profiles were analyzed by calculating their differences using the Dice algorithm. Bands were also recoded as absent or present, then analyzed with a maximum parsimony method. The RAPD analysis utilized three different 10mer primers in 45 cycles of low stringency polymerase chain reaction after cells were lysed by a “hot start” step. Protein profiles were generated from SDS-PAGE gels of whole cell lysates. The results revealed unique and reproducible DNA and protein fingerprints among the reference strains and field isolates studied, including H. parasuis bacteriophage proteins identified by protein profiles and mass spectrometry. Similarities and differences existed among avirulent, virulent, and highly virulent strains, which grouped according to their pathogenicity. The combination of RAPD and whole cell lysate SDS-PAGE analyses may be useful for studying the epidemiology of H. parasuis.
Introduction

*Haemophilus parasuis* causes Glässer’s disease in pigs, with symptoms of fibrinous polyserositis, pericarditis, polyarthritis, and meningitis (20). *H. parasuis* also causes septicemia and pneumonia without polyserositis and can be isolated from healthy nasal passages of swine. Introduction of conventionally raised pigs into segregated early weaning (SEW) herds may result in infection and high economic losses because the latter lack immunity to *H. parasuis* (17, 32). *H. parasuis* also remains a problem in many high health herds. Economic losses in 2006 in the United States can be estimated at $13.6 billion dollars, based on the number of sows and gilts in the pig population, the estimated loss of 3.5 piglets/sow/year due to *H. parasuis* infection, and a hog’s average market weight of 262 pounds at $0.60/pound (Rodney B. Baker, Iowa State University, personal communication) (40).

*Haemophilus parasuis* strains are classified into 15 serovars based on immunodiffusion of heat-stable polysaccharide antigens (14, 31), and on virulence in specific pathogen free (SPF) pigs (14). However, reagents for serotyping field isolates are not readily available. Partially due to lack of reagents and also because serotyping field isolates cannot identify the nontypeable (NT) strains, other methods have been employed to identify field isolates. A second method of serotyping, the indirect hemagglutination (IHA) test, was developed due to the high incidence of NT isolates and cross-serotype reactivity (30%) with the immunodiffusion test (39). The IHA test was based on particulate lipopolysaccharide antigen and had less than 10% cross-reactivity. Some scientists prefer to combine both tests in order to improve interpretation of the tests and decrease the problems due to cross-reactivity (2, 5). Nonetheless, there are still many NT isolates that do not have serovar-specific reagents and cannot be characterized.

Smart et al. (37, 38) characterized *H. parasuis* isolates by their restriction endonuclease fingerprints and found various DNA patterns for *H. parasuis* strains isolated from pigs and farms. Distinct profiles were observed with isolates from animals
with systemic disease and also from isolates from the upper respiratory tract of healthy animals. Both Rafiee et al. (30) and Oliveira et al. (24) used enterobacterial repetitive intergenic concensus-polymerase chain reaction (ERIC-PCR), a DNA genotyping method, to evaluate the 15 reference serovars and various field isolates for their genetic relatedness and the epidemiology of field outbreaks. They concluded that the ERIC-PCR genotyping technique was sensitive to *H. parasuis* strain variation and would be the preferred technique to obtain epidemiological information compared to serotyping (24, 30).

Oliveira et al. (25) introduced a species-specific PCR test based on *H. parasuis* 16S rRNA. However, not all serovars were amplified and some *Actinobacillus* species related to *H. parasuis* that also colonize the swine upper respiratory tract were detected. Angen et al. (1) were able to improve the 16S rRNA species specific PCR test by employing multiplex PCR and 3 different primers to obtain 100% specificity for *H. parasuis* and no false-positives. A drawback to the two previous 16S rRNA PCR methods is that there can be no classification based on numerous bands because the results are shown with either one band or no bands.

Two other genome-based methods used were species-specific PCR based on *H. parasuis* target genes of *tbpA* (6) and *aroA* (7). Depending on the number of restriction endonucleases used, it appeared to make a difference in the outcome of the experiments. The first group was able to discriminate 33 restriction fragment length polymorphisms (RFLPs) among the 15 reference serovars and 101 clinical isolates, while the second report only had 2 RFLPs among 15 reference serovars. Another genomic method is multilocus enzyme electrophoresis (4, 28). Blackall et al. (4) found 34 different electrophoretic types from 40 field isolates and 8 reference serovars, which clustered into 2 major subdivisions. Olvera et al. (28) concluded that 120 field isolates and 11 reference serovars clustered into avirulent, nasal isolates and virulent, systemic isolates groups.
Another approach for evaluating *H. parasuis* isolates is to compare protein profiles of whole cell lysates (22, 26) or outer membrane proteins (34). Nicolet et al. (22) observed two main patterns of protein profiles of whole-cell lysates. They suggested that strains obtained from systemic sites were homogeneous and different from those obtained from respiratory sites. These two patterns were later identified as polyacrylamide gel electrophoresis (PAGE) type II and PAGE type I, respectively (21). Ruiz et al. (34) examined outer membrane protein (OMP) profiles and found that similar OMP profiles were found from isolates from systemic sites suggesting that some OMPs may be related to virulence. Similar conclusions were presented by Oliveira and Pijoan (26) for whole cell lysates.

This work analyzed the DNA and protein profiles of 46 *H. parasuis* reference and field isolates. Random amplification of polymorphic DNA (RAPD) is a molecular typing technique that is relatively easy to use since cells can be lysed directly on the PCR machine. It is especially sensitive to strain variation when three different primers are employed (29). The present study investigated the relatedness among *H. parasuis* strains by using the RAPD technique and by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to study the whole cell lysate patterns of the strains. The objectives of this study were to compare the relatedness of the reference strains and field isolates based on the RAPD patterns and protein profiles of whole cell lysates and to determine if virulence-related clustering occurred.


**Materials and Methods**

**Strains and growth conditions.** *H. parasuis* type strains were obtained from Richard Ross of the College of Veterinary Medicine, Iowa State University, Ames, Iowa and isolated between 1978 and 1990 (14, 22). Tables 2.1 and 2.2 describe the *H.*
parasuis strains used in this study. Field strains 30 and 31 were obtained from Vickie Rapp-Gabrielson in 1999 (Schering-Plough, Omaha, NE) and were originally isolated in 1984, while field strains 25-29 obtained in 1999 were from Karen Post, Rollins Diagnostic Laboratory in North Carolina. Field strains 1-24, the most recently procured in 2004, were from Lorraine Hoffman of the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa. Because of unavailability of typing sera, partial serotyping with antisera to serotypes 2, 4, 5, 12, 13, and 14 of all 31 field strains was performed by Gallant Custom Laboratories, Cambridge, Ontario. Strains were grown in Frey’s mycoplasma base broth (Sigma, St. Louis, MO) containing 20% heat-inactivated horse serum (Invitrogen, Carlsbad, CA) and 0.016% β-nicotinamide adenine dinucleotide (β-NAD) (Sigma) at 37°C overnight. Strains were checked for purity on blood agar with a nurse streak of S. aureus across a lawn of the H. parasuis isolate and on Casman’s agar (Difco, Detroit, MI) containing 5% horse serum and 0.016% β-NAD. Plates were incubated at 37°C under humidified 5% CO₂. Outgroup strains were also studied in both RAPD and whole cell lysate experiments in order to include related organisms to H. parasuis, but ones that were not of the same species. Selected outgroup organisms were A. pleuropneumoniae (ATCC 27088), P. multocida (ATCC 15742), M. haemolytica (ATCC 43270, serotype A1), P. trehalosi (ATCC 29703, serotype T3), which were all members of the family Pasteurellaceae.

**RAPD analysis.** Three arbitrary 10mer primers from kit A (Operon Technologies, Alameda, CA) with sequences of 5’-TGCCGAGCTG-3’ (primer 2); 5’-GAAACGGGTG-3’ (primer 7); and 5’-TCGGCGATAG-3’ (primer 12) were used. H. parasuis isolates, grown on Casman’s agar, were suspended in distilled water, then serially diluted 10-fold. The cell dilutions used in the final PCR reaction mixture were optimized prior to the final RAPD experiments. RAPD experiments were replicated three times to ensure reproducibility of the assay. The PCR reaction mixture contained 60 mM Tris-HCl, pH 8.5, 15 mM (NH₄)₂SO₄, 2 mM MgCl₂, 0.125 mM each of dATP,
dCTP, dGTP, and dTTP, 7.5 picomoles of a single 10mer, 4 µl of cell suspension, and 0.625 units of Taq polymerase (Applied Biosystems, Foster City, CA). Controls containing no *H. parasuis* cells were also included.

DNA amplification was performed on a GeneAmp PCR System 9600 (Perkin Elmer, Boston, MA). Cells were lysed in a “hot start” step (13) at 94°C for 10 min, and then amplified for 45 cycles of 1 min at 94°C, 1.5 min at 36°C, and 2 min at 72°C, followed by an extension step for 10 min at 72°C, then a hold step at 4°C. PCR products were stored at -20°C, until they were analyzed on 1% agarose horizontal gels in Tris-Borate (TBE), pH 8.3 buffer and detected by ultraviolet light illumination after staining with ethidium bromide. The DNA standard was a 1 kb ladder (Invitrogen).

**SDS-PAGE analysis.** For whole cell lysates, bacterial cells grown in Frey’s broth for 22 hr were pelleted by centrifugation at 675 × g for 10 min. Cells were washed in 0.1 M PBS, pH 7.2, containing 1mM Pefabloc (Roche Diagnostics, Indianapolis, IN), then resuspended at a ratio of 32 mg cells per 100 µl PBS/Pefabloc. Cells were sonicated with a microprobe (Heat Systems-Ultrasonics, Farmingdale, NY) at 50% power for 60 1-second bursts to lyse them and centrifuged at 16,000 × g for 20 min to remove cell debris. Protein concentrations were determined by the Folin-Lowry method (16) with bovine serum albumin as a standard.

Protein (10 µg/well) was applied to 10-well NuPAGE precast 4-12% gradient Bis-Tris gels (Invitrogen). NuPAGE antioxidant was used in 3-(N-morpholino)-propane sulfonic acid (MOPS) running buffer. The protein prestained standard was BenchMark, 10-200 kDa (Invitrogen). Running conditions were 10 mA/gel for 15 min, then 200 V for 40 min. Gels were stained in 0.1% Coomassie Brilliant Blue R250 in 50% methanol/10% acetic acid and destained in 50% methanol/10% acetic acid.

**Mass spectrometry analysis of 50 kDa protein band.** Gel plugs containing the 50 kDa band were digested with sequence-grade trypsin (Promega, Madison, WI) in ammonium bicarbonate buffer at 37°C overnight. MALDI-TOF MS/MS and MS/ESI
mass spectrometry analysis was performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. All spectra were processed by MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches. Typical search parameters were as follows: Maximum missed cleavage setting was 1.0; the fixed modification setting was carboxyamidomethyl cysteine with a variable modification of oxidation of methionine. Peptide mass tolerances were +/- 200 ppm. Fragment mass tolerances were +/- 1 Da or 2 Da. No restrictions on protein molecular weight were applied. The significance threshold $p$ was set to less than 0.05. Protein identification was based on the probability based MOWSE (MOlecular Weight SEarch) Score and % coverage of peptide. Sequences containing bacteriophage (BP) genes were selected for further analysis.

**Electrophoresis pattern analysis.** Gels were photographed, scanned (Kodak Image Station, Rochester, NY) and the image was digitized (Kodak Molecular Imaging Software, New Haven, CT). RAPD and protein profiles were analyzed using Gel Compar II software (Applied Maths, Austin, TX). Optimal settings for band optimization and band position tolerance levels were calculated for each fingerprint. The optimal position tolerance value gives the highest group contrast: selected scores are as high as possible within groups and as low as possible between groups. Since a band matching algorithm (Dice) was used, both tolerance and optimization were calculated. Similarity matrices were obtained from single RAPD experiments and SDS-PAGE data using the Dice similarity coefficient: $F = 2n_{xy}/(n_x + n_y)$, where $n_x$ is the total number of fragments from isolate $X$, $n_y$ is the total number of fragments from isolate $Y$, and $n_{xy}$ is the number of fragments shared by the two isolates (12). Additionally, a combined RAPD dendogram analysis of all three RAPD fingerprints was derived from a composite data set of the individual experiments. Neighbor joining (NJ) dendograms were constructed with 1000 bootstrap values. Arbitrary subdivision (clusters and subclusters were derived for RAPD
and whole cell SDS-PAGE dendograms by examining the clusters as a function of percent similarity.

For maximum parsimony analysis, bands were recoded as 0 or 1 for their absence or presence in the profile. Maximum parsimony analysis was then coded using the PAUP4.0810 program. Consensus trees were generated by heuristic analysis of 100 replicates of the data.

Results

Comparison of RAPD profiles and pattern analysis. Outgroup strains that were in the same family as *H. parasuis* but from different genera were included (Fig. 2.1A). (Fig. 2.1B, containing outgroup strains’ whole cell lysates, will be discussed below.) Of the three arbitrary primers used for genotyping, primer 2 (Fig. 2.2) had an intermediate number of bands; primer 7 had the most polymorphic DNA bands; and primer 12 (Fig. 2.4) had the least number of polymorphic DNA bands (Fig. 2.3). DNA band sizes ranged between 220-3054 base pairs (bp). There were some bands that were more densely stained than others, but all bands were treated identically. With primer 12, field strains 1-23 seemed to have less heterogeneity than the remaining isolates tested with primer 12.

NJ dendograms from the three individual primers were generated to evaluate the similarities of the isolates based on the DNA band patterns (Figs. 2.5, A-C). With primer 2 (Fig. 2.5, panel A) three clusters (A, B, and C) and one unique isolate (field isolate 4) were observed with a band optimization of 1.12% and a band position tolerance of 0.87% and a similarity of 13%. At a similarity of 27%, cluster B grouped into three subclusters and two unique isolates (reference strain D and field isolate 23). Primer 7 (Fig. 2.5, panel B) showed two clusters (A and B) and two unique isolates (serovar 4 and field isolate 4) with a band optimization of 0.25% and a band position tolerance of 2.71% at 27% similarity. At 35% similarity, cluster A grouped into four subclusters and cluster B
grouped into two subclusters and one unique isolate (field isolate 23). Primer 12 (Fig. 2.5, panel C) had four clusters (A-D) and two unique isolates (field isolates 6 and 22) with a band optimization of 1.58% and a band position tolerance of 0.78% at 30% similarity. At 34% similarity, there was one unique isolate (field isolate 15) in cluster C, and two subclusters in cluster D. Duplicate cultures of *H. parasuis* isolate IA84-29755 were included as controls and were amplified identically with each primer.

A composite dendogram prepared from the data obtained from all three primers is shown in Fig. 2.6. At 48.5% similarity, this dendogram showed three clusters (A, B, and C) and two unique reference serovars, one virulent isolate from Germany which caused polyserositis and one avirulent nasal isolate from Switzerland from a healthy animal. At 52.7% similarity, cluster A had three subclusters, cluster B had four subclusters, and cluster C had five subclusters. Subcluster A1 contained three highly virulent isolates, one reference strain from a nasal isolate from a healthy animal from Japan and two 1999 field isolates. Subcluster A2 contained two highly virulent 2004 field strains. Subcluster A3 contained one low virulence 1984 field strain and two highly virulent reference strains from the United States. Subcluster B1 contained one low virulence reference strain from Japan and one low virulence 2004 field isolate. Subcluster B2 contained one avirulent reference strain from Sweden, and three highly virulent 2004 field isolates. Subcluster B3 contained one avirulent reference strain from Japan and one virulent 2004 field isolate. Subcluster B4 contained one avirulent reference strain from Switzerland, one low virulence reference strain from Japan, two highly virulent 2004 field isolates. Subcluster C1 contained three highly virulent 2004 field isolates from the same animal. Subcluster C2 contained one highly virulent reference strain from Japan and five highly virulent 2004 field isolates. Subcluster C3 contained one low virulence reference strain from the United States, one 1984 highly virulent field strain, and two outgroups (*M. haemolytica* and *P. trehalosi*). Subcluster C4 contained two highly virulent 1999 field isolates, four virulent 2004 field isolates, and outgroup strain *A. pleuropneumoniae*. Subcluster C5
contained one avirulent reference strain isolated from a pig diagnosed with pneumonia from Germany, one highly virulent reference strain from Germany, one low virulence reference strain from Sweden, one 1999 field isolate, and two 2004 field isolates, and outgroup strain *P. multocida*. Two duplicate cultures of *H. parasuis* IA84-29755 (virulent 1984/1999 field isolate); and two outgroups which are closely related phylogenetically were grouped within one branch of subcluster C3.

Using a combination of 3 primers’ RAPD data resulted in two unique isolates and seemed to cluster the isolates better than the separate dendograms did. For instance, there were fewer identical isolate banding patterns in the composite dendogram than those obtained with single primer dendograms. The duplicate *H. parasuis* IA84-29755 patterns were identical for all dendrogram analyses.

There were ten clusters of isolates and 27 unique isolates in the RAPD-PCR parsimony tree of the combined primer data (Fig. 2.7). Cluster 1 contained two highly virulent field isolates archived in 1999. Cluster 2 contained two highly virulent reference serovars and one field isolate from 1984 of low virulence that had lost its ability to be serotyped. Cluster 3 contained two low virulence isolates, one from 2004 and one reference strain. Cluster 4 contained two reference strains, a low virulence nasal isolate from Japan and an avirulent nasal isolate from Switzerland. Cluster 5 contained two 2004 field isolates from the same animal and isolate sites but with different serotypes. Cluster 6 contained two highly virulent lung isolates, one reference serovar and one 2004 field isolate. Cluster 7 contained the duplicate samples of *H. parasuis*. Cluster 8 contained three highly virulent 2004 isolates, all possible lung isolates but with unique serotypes. Cluster 9 contained two 1999 field isolates as well as two outgroups, *A. pleuropneumoniae* and *P. multocida*. Cluster 10 contained the remaining closely related outgroups, *M. haemolytica* and *P. trehalosi*.

**Comparison of SDS-PAGE protein profiles.** Numerous protein bands between 8 and 180 kilodalton (kDa) were present in all of the strains (Fig. 2.1B, Fig. 2.8), as well
as a few bands higher than 180 kDa in four of the reference strains (serovars 3, 6, 8, and 9). Each serovar showed unique band patterns, but there were also common protein bands among the serovars (lanes A-O) and field isolates (lanes 1-31). For example, serovars 3 and 6 showed a common protein with an estimated molecular mass of 253,000; and serovars 8 and 9 showed a common band with an estimated molecular mass of 217,000. All reference strains and 1999 field strains expressed prominent bands at apparent molecular masses of 140,000 and 70,000 and all strains except reference strains 2 and 8, showed prominent bands with molecular masses of approximately 40,000. Visual inspection of the protein profiles of the field strains 25-31 (Fig. 2.8) showed that these were more similar to but not identical to reference strains 11 and 12. Field strains 1-24 protein profiles were more heterogeneous than the reference strain, 1999, or 1984 field strain profiles.

Lanes marked 20 and 20b (Fig. 2.8) were whole cell lysate protein profiles of field isolate 34086b, grown under different conditions. Lane 20 had a sparse inoculum (2.5% v/v), while lane 20b received a heavy inoculum (10% v/v) of starter culture. Additionally, field strains 3, 6, 13, 20, and 1050-99 all had major protein bands at approximately 50 kDa, which were not apparent in the other protein profiles. The five former cultures also seemed to lyse during overnight growth in broth. The cultures containing the 50 kDa band were analyzed by MS/MS spectrometry in order to identify that major protein.

**Pattern analysis of SDS-PAGE protein bands.** Fig. 2.1B contained outgroup strains’ whole cell lysates profiles. The NJ dendogram showing phylogenetic analysis of whole cell lysates (Fig. 2.9) used a band optimization of 1.12% and a band position tolerance of 1.1% and had one unique isolate (2004 field strain 13 which was systemic, highly virulent, and had the 50 kDa band). Three clusters (A, B, and C) at 58.5% similarity were generated and three subclusters of Cluster A at 63% similarity were produced. Subcluster A1 contained five virulent, systemic 1984/1999 field strains and
one 2004 field strain which was highly virulent and had the 50 kDa band. Subcluster A2 contained eleven original reference strains of various virulences and isolation sites (Table 2.1). Subcluster A3 contained four original reference strains of varied virulence, two nasal isolates from Japan and two reference strains from Sweden (one virulent and one avirulent) as well as the 1984 virulent field strain *H. parasuis* IA84-29755 and all of the outgroup strains. Cluster B contained one 1999 virulent field strain and eight virulent, systemic 2004 field strains and Cluster C contained fifteen 2004 virulent, systemic field strains (Fig. 2.9).

It is noteworthy that in the supposed identical *H. parasuis* isolates, bands did not match sufficiently to obtain identity in the protein profile computer analysis. They were, however, in the same branch of subcluster A3. Clusters 2 and 3 contained all virulent, systemic isolates and subcluster A2 was entirely reference strains. The majority of 1984/1999 field isolates grouped in subcluster A1.

There were eight clusters and 12 unique isolates in the whole cell lysate parsimony tree (Fig. 2.10). The 12 unique isolates included reference strains 8, 9, and 12; 1999 field strain *H. parasuis* IA84-29755; 2004 field strains 2, 3, 12, 13, 19, and 24; and 2 outgroups. Cluster 1 contained seven 2004 and one 1999 virulent, systemic field isolates (eight of these were included in nine of the same isolates as in Cluster B of Fig. 2.9). Cluster 2 contained two 2004 virulent, systemic field isolates. Cluster 3 contained seven 2004 virulent, systemic field isolates. Cluster 4 contained three 2004 highly virulent, systemic field isolates. Cluster 5 contained ten reference serovars. Cluster 6 contained six 1984/1999 virulent field strains. Cluster 7 contained two reference nasal isolates from Japan, one avirulent and one virulent. Cluster 8 contained two closely related outgroups. As was observed in the NJ whole cell lysate dendogram, parsimony tree (Fig. 2.10) clusters 1, 2, 3, and 4 contained entirely 2004 field strains, while cluster 6 contained all 1984/1999 field strains, and clusters 5 and 7 contained the all reference strains except reference strain 12.
Mass spectrometry analysis of 50 kDa band. The MALDI-TOF MS/MS or MS/ESI analysis identified three approximately 50 kDa proteins in 1-D gels of lysing cultures of field isolates 3, 6, 13, 20, and 1050-99. The first protein was accession P32270 (DDA_BPT4), an ATP-dependent DNA helicase dda involved in bacteriophage T4’s DNA initiation of DNA replication. The theoretical molecular weight and pI were 49897 and 8.04, respectively. However, there was poor sequence coverage at 3% and the MS/ESI score was low at 1 out of 19 with an expected score of 2.5 at 200 ppm +/- 1 da. The second protein was accession Q01076 (VG20_BP22), DNA transfer protein gp20. This is an oligopeptidase required for ejection of phage P22 DNA from the phage and injection of phage DNA into the host. The theoretical molecular weight and pI were 50101 and 8.58, respectively. The MS/ESI score was 3 out of 20 with an expected score of 4.6, with 2.8% sequence coverage at 200 ppm +/- 1 da, and the MS/MS score was 24 where a score of 22 or greater indicated identity or extensive homology (p<0.05). A third protein was identified at 200 ppm +/- 2 da, namely, gp 29 of bacteriophage Mu, VG29_BPMU (Q9T1W5). Its molecular weight and pI were 56909 and 4.79, respectively. The MS/ESI score was 32 out of 20 with greater than 21 indicating identity or extensive homology, and with an expected score of 0.0054 and sequence coverage of 5%. Incidentally, even in the older field strain, 1050-99, bacteriophage proteins were identified, including the repressor protein cI (P13121), a primase/helicase from enterobacteriophage T4 (P04530), and the putative DNA transfer protein gp35 (Q9T1R3) as well as an HP1 integrase (P21442).

Discussion

This study was undertaken to compare the RAPD profiles and SDS-PAGE protein profiles of the H. parasuis reference strains and 31 of field isolates and determine if a relationship existed between a particular clustering profile and degree of virulence of the reference strains defined previously (3, 14) or if the epidemiology of H. parasuis could
be delineated by any of the profiles. The first method of analysis used included a
distance method (NJ dendogram) based on Dice coefficients of similarity. In other
words, there would have been more changes in the isolate if the branch were longer.
Neighbor joining (NJ) clustering does not assume the same rate of evolution for the
isolates.

The second analysis method was a character-based parsimony tree which
generated a minimum number of changes from the original 0 or 1 band codings,
consistent with the same number of events. In our case, 100 random replicates were built
on an initial heuristic tree and the rearrangements were made to improve the tree (11).
The parsimony method detects the presence or absence of a band and gives an underlying
DNA correlation to protein expression. The maximum parsimony analysis is based on
the original data while the NJ dendogram is based on pairwise distances calculated from
that data. Moreover, the NJ analysis could be described as biased because the dendogram
is initially built on two taxa with the shortest distance that joins neighbors and there is no
subsequent “improvement” of the tree as there is with the maximum parsimony analysis.

Outgroups were included to compare the presence or absence of bands in those
isolates to the bands in the more closely related *H. parasuis* isolates. The only NJ
analysis that formed a monophyletic ingroup with the four outgroups was the SDS-PAGE
dendogram (Fig. 2.9). The four species tested were too closely related to *H. parasuis* to
act as a true outgroup. Two outgroup species, *M. haemolytica* and *P. trehalosi*, formed
rooted parsimony trees in both the composite RAPD (Fig. 2.7) and SDS-PAGE (Fig.
2.10) analyses.

The RAPD technique is a PCR technique often used to differentiate closely
related strains. It is simpler to perform than pulsed field gel electrophoresis and
equipment and supplies are easily obtained for RAPD method. However, to be
discriminatory many laboratories have recommended the combination of data from three
optimized primers (8, 9, 29). Similarly, in the study reported here, the dendograms with
each of the three primers (Figs. 2.5, A-C) and of the composite diagram (Fig. 2.6) show the degree of relatedness among the reference strains and the 31 field strains. There was low confidence by bootstrap analysis using the RAPD technique and the clusters did not correlate with the SDS-PAGE results. The RAPD technique described here (Figs. 2.5-2.6) separated isolates from known systemic sites (lung, joint, brain, heart, or septicemia) into groups. Two clusters/subclusters occurred with only virulent strains when the least discriminatory primer was used (primer 12, Fig. 2.5C), primer 2 with the intermediate number of bands had two virulent clusters/subclusters (Fig. 2.5A) and primer 7 with the most bands had four virulent subclusters (Fig. 2.5B). In the composite RAPD dendogram (Fig. 2.6), nine of twelve clusters/subclusters containing all virulent isolates were found. The parsimony RAPD tree (Fig. 2.7) did not have as much discrimination as the Dice/NJ dendogram did. However, the parsimony tree (Fig. 2.7) showed that there was more genetic heterogeneity among the isolates than one would expect after examining the composite dendogram (Fig. 2.6). The RAPD analysis also did not discriminate between old and new isolates. These findings may be because DNA takes longer to mutate or change or that some isolates do not mutate as fast as others. It is also possible that using additional primers might resolve this issue. It is interesting to note that field strains 25 and 26 and \textit{A. pleuropneumoniae} clustered together in the composite RAPD analysis (Fig. 2.6). It may be that field strains 25 and 26 are actually \textit{A. pleuropneumoniae} strains since \textit{H. parasuis} and \textit{A. pleuropneumoniae} are both commensals of the upper respiratory tract or that there was lateral transfer of DNA between the two different species to make them appear to be more alike genetically (27).

On the other hand, Samra et al. (35) used RAPD analysis with four separate primers to show clonality of fifteen \textit{K. pneumoniae} isolates collected over a 10-month time frame. Another recent study found RAPD analysis to be more repeatable than amplified fragment length polymorphic (AFLP) analysis of avian mycobacteriosis (36). Additionally, these authors reported an association of virulence or disease with certain
RAPD clusters. They stated that their results could be affected by strain genetic diversity, host immune status and stress, and management of the host’s potential sources of mycobacteria. Kuene et al. (15) used a combination of total-soluble protein extracts and amplified fragment length polymorphism (AFLP) analysis to separate strains of lactic acid bacteria.

In this study, virulent isolates were associated with the majority of RAPD subclusters when bands from three different primers were combined (Fig. 2.6). In contrast, the RAPD parsimony tree showed more heterogeneity among the isolates (Fig. 2.7). Nonetheless, the RAPD technique and other molecular typing techniques used to analyze H. parasuis isolates including polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (6), species-specific PCR (1), enterobacterial repetitive intergenic (ERIC)-PCR (24, 27, 30), and restriction endonuclease analysis (37, 38), and multilocus enzyme electrophoresis (4, 28) have shown that considerable genetic diversity exists among strains of isolates of a particular serotype and that the genotyping techniques were more discriminating compared to conventional serotyping, especially for use in epidemiological studies.

Oliviera and Pijoan (26) reported that isolates from systemic sites are usually virulent and clustered together as shown by using a computer-based analysis of protein profiles. They found PAGE II protein profiles, considered to be systemic, in serovars 1, 2, 4, 5, 7, 12, 13, 14 and nontypeable (NT) isolates, isolated from the lung, upper respiratory tract, systemic, and unknown sites. Their results are similar to our protein profiles, serovars, and isolation sites shown in the whole cell lysate dendogram of Fig. 2.9, parsimony tree of Fig. 2.10, and Tables 2.1-2.2. This study’s field isolates were all virulent, mostly systemic, and our known serovars included 2, 4, 5, 12, and 13. In their studies, no isolates were recovered of serovars 6, 8, 9, 10, 11 and 15. It was unknown if any of this report’s isolates were from the latter serovars since serotyping reagents were unavailable to screen for those serovars.
A number of investigators have stated that a protein of approximately 36-38.5 kDa is associated with Glässer’s disease (19, 22, 26, 34). In this work, a protein band was observed at approximately 40 kDa in all of the field isolates and thirteen of fifteen of the reference strains (Fig. 2.7). However, although this protein could be associated with virulence in the field isolates, the assumption does not correlate with the assigned reference serovar virulence states described by Kielstein and Rapp-Gabrielson (14). On the other hand, this study’s whole cell lysate dendogram (Fig. 2.9) and parsimony tree (Fig. 2.10) showed that protein expression may be related to age or length of passage of the isolate *ex vivo*. Reference strains clustered together, as did the “old” field strains isolated in 1999.

PAGE type I and PAGE type II patterns of whole cell lysates were not observed (22, 26) in this study. The difference in banding patterns obtained here compared to the banding pattern reported by Oliveira and Pijoan (26) could be influenced by the type of medium used for propagation of the cultures (33), by the amount of starting inoculum in a broth culture, or by the relative length of time of propagation of the cells. In this study, Frey’s broth was supplemented with 20% horse serum and 0.016% β-NAD, whereas Oliveira and Pijoan (26) grew their cultures on chocolate agar plates. Thus, the results obtained in this study suggested that the type of medium used for growing the cultures may play a role in protein expression. If medium composition or culture conditions influenced expression of the 40 kDa protein, it would be of interest to determine the identity of 40 kDa protein and its regulation by altering growth parameters.

Ruiz et al. (34) suggested that the similarity of the OMP SDS-PAGE patterns of the systemic isolates and band patterns observed in the profiles of the repetitive element-based ERIC-PCR DNA showed a clonal relationship of field isolates despite the heterogeneity of reference strains. They concluded that the OMP profiles of field isolates could be used to establish virulence determinants. However, they also concluded that no relationship existed between protein and DNA profiles.
Based on these results, all field strains in this study have been classified as “virulent” types. In contrast to Kielstein and Rapp-Gabrielson (14) and Blackall et al. (3), the results reported in this study seem to indicate an association of virulence with “group” of strain, especially with those analyses which generated the most polymorphic bands. Interestingly, this work also found that the genome-based RAPD composite analysis method had more heterogeneity than the protein-based whole cell lysate analysis, which surprisingly, grouped isolates according to length of passage \textit{ex vivo} (Figs. 2.6-2.7 & 2.9-2.10). The underlying genome did not change as much as the protein expression did over time (6).

In a survey of \textit{H. parasuis} field isolates from Canada and the United States, Rapp-Gabrielson and Gabrielson (1992) found that serovars 5 (24.3\%) and 4 (16.1\%) were the most prevalent from 1982 to 1990. Both serovars were isolated from systemic (with polyserositis) and respiratory (without polyserositis) infections. They noted that the distribution of \textit{H. parasuis} serovars isolated from healthy animals may differ from that found in diseased animals and that more than one serovar could be isolated from the same animal or herd. Two strains (FS34086c and FS34086d) that had been isolated from the same sites in the same animal were also analyzed here (Table 2.2). In the 1992 report above, the more virulent isolates were found from swine with polyserositis in Canada, whereas in the United States, more isolates were associated with pneumonia in swine.

From 1999 through 2001, Oliveira et al. (24) reported a shift to serovar 4 (39\%) and nontypeable (27\%) isolates in United States swine herds. Two different authors (2, 5) combined serotyping and IHA methods to report 4, 5, 13, and NT isolates as the most prevalent in 2004 and 2005, with serovar 4 the most frequently isolated from the respiratory tract while NT isolates were usually systemic isolates. A fourth study done in 2004 (39) in the United States confirmed that serovar 4 had become more prevalent than serovar 5.
This work has shown for the first time the straightforward existence of bacteriophage in *H. parasuis* through protein profiles (Fig. 2.8) and mass spectrometry analysis. Others have suggested lateral transfer of genes between *A. pleuropneumoniae* and *H. parasuis* in the upper respiratory tract (27) and have reported bacteriophage genes from a microarray analysis of *H. parasuis* (18). Bacteriophage can introduce virulence determinants into an organism by transduction, which then contribute to the pathogenicity and heterogeneity of the bacteria (10, 23). A possible reason why the protein expression is becoming more homogeneous over time might be because the lysogenic phage has excised from the bacterial chromosome and thus the bacteria lack that means of diversity. It could be hypothesized that virulent bacteria harbor phage while avirulent organisms do not have genome-associated phage. A manuscript describing the characterization of this study’s *H. parasuis* bacteriophage is in preparation.

In summary, the results of this study suggested both the “combined” RAPD and SDS-PAGE methods provided reproducible profiles of *H. parasuis* reference strains and field isolates. Both the RAPD and the SDS-PAGE methods clustered high virulence strains from systemic sites. The RAPD technique pointed out the heterogeneity of the isolates, whereas the protein profiles showed for the first time that the number of passages *ex vivo* of an isolate contributes to its protein expression. Both RAPD and SDS-PAGE profiles demonstrated their usefulness for epidemiological studies. Moreover, the relatively low cost, involving time, supplies, and equipment performing either method was advantageous. The results also suggested that culture conditions can affect bacterial protein expression. Additionally, the protein studies revealed altered profiles due to the presence of bacteriophage in some virulent isolates, suggesting a role of bacteriophage in the diversity and possible virulence of *H. parasuis*.
Table 2.1. Description of *H. parasuis* Reference Strains

<table>
<thead>
<tr>
<th>#</th>
<th>Serovar</th>
<th>Strain</th>
<th>Country</th>
<th>Isolation Site</th>
<th>Diagnosis</th>
<th>Virulence&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>No. 4</td>
<td>Japan</td>
<td>Nose</td>
<td>Healthy</td>
<td>H</td>
</tr>
<tr>
<td>B</td>
<td>2</td>
<td>SW140</td>
<td>Japan</td>
<td>Nose</td>
<td>Healthy</td>
<td>L+</td>
</tr>
<tr>
<td>C</td>
<td>3</td>
<td>SW114</td>
<td>Japan</td>
<td>Nose</td>
<td>Healthy</td>
<td>A</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>SW124</td>
<td>Japan</td>
<td>Nose</td>
<td>Healthy</td>
<td>L+</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>Nagasaki</td>
<td>Japan</td>
<td>Meninges</td>
<td>Meningitis, Septicemia</td>
<td>H</td>
</tr>
<tr>
<td>F</td>
<td>6</td>
<td>131</td>
<td>Switzerland</td>
<td>Nose</td>
<td>Healthy</td>
<td>A</td>
</tr>
<tr>
<td>G</td>
<td>7</td>
<td>174</td>
<td>Switzerland</td>
<td>Nose</td>
<td>Healthy</td>
<td>A</td>
</tr>
<tr>
<td>H</td>
<td>8</td>
<td>C5</td>
<td>Sweden</td>
<td>Unknown</td>
<td>Unknown</td>
<td>L-</td>
</tr>
<tr>
<td>I</td>
<td>9</td>
<td>D74</td>
<td>Sweden</td>
<td>Unknown</td>
<td>Unknown</td>
<td>A</td>
</tr>
<tr>
<td>J</td>
<td>10</td>
<td>H367&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Germany</td>
<td>Unknown</td>
<td>Unknown</td>
<td>H</td>
</tr>
<tr>
<td>K</td>
<td>11</td>
<td>H465</td>
<td>Germany</td>
<td>Trachea</td>
<td>Pneumonia</td>
<td>A</td>
</tr>
<tr>
<td>L</td>
<td>12</td>
<td>H425</td>
<td>Germany</td>
<td>Lung</td>
<td>Polyserositis</td>
<td>H</td>
</tr>
<tr>
<td>M</td>
<td>13</td>
<td>84-17975</td>
<td>United States</td>
<td>Lung</td>
<td>Unknown</td>
<td>H</td>
</tr>
<tr>
<td>N</td>
<td>14</td>
<td>84-22113</td>
<td>United States</td>
<td>Joint</td>
<td>Unknown</td>
<td>H</td>
</tr>
<tr>
<td>O</td>
<td>15</td>
<td>84-15995</td>
<td>United States</td>
<td>Lung</td>
<td>Pneumonia</td>
<td>L+</td>
</tr>
</tbody>
</table>

<sup>a</sup>H, High; L+, Polyserositis; L -, Mild; A, Avirulent


<sup>b</sup>H367 (serovar 10) is a field strain with the same characteristics as the original H555. Reference strain H555 was lost during culture passage prior to our acquisition of the reference strains above.
<table>
<thead>
<tr>
<th></th>
<th>Serovar</th>
<th>Strain</th>
<th>Isolation Site</th>
<th>Diagnosis</th>
<th>Coinfection</th>
<th>Virulence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NT</td>
<td>FS10680</td>
<td>Lung, Heart</td>
<td>Septicemia</td>
<td><em>Staphylococcus, E. coli</em></td>
<td>H#</td>
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<tr>
<td>2</td>
<td>NT</td>
<td>FS12939</td>
<td>Lung</td>
<td>Pneum., Polyser.</td>
<td>PRRSV</td>
<td>H#</td>
</tr>
<tr>
<td>3</td>
<td>NT</td>
<td>FS15677</td>
<td>Brain, Heart</td>
<td>Pneum., Polyser.</td>
<td>PRRSV, <em>E. coli</em></td>
<td>H#</td>
</tr>
<tr>
<td>4</td>
<td>NT</td>
<td>FS17321</td>
<td>Brain, Lung</td>
<td>Pneum., Mening.</td>
<td><em>Mycoplasma hyo, E. coli</em></td>
<td>H#</td>
</tr>
<tr>
<td>5</td>
<td>NT</td>
<td>FS24054</td>
<td>Lung</td>
<td>Polyserositis</td>
<td>PRRSV</td>
<td>H#</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>FS24996</td>
<td>Lung</td>
<td>Pleuritis, Septic.</td>
<td><em>P. multocida</em></td>
<td>L</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>FS25718</td>
<td>Heart</td>
<td>Pericarditis</td>
<td><em>S. suis, PCV2</em></td>
<td>H</td>
</tr>
<tr>
<td>8</td>
<td>NT</td>
<td>FS28803</td>
<td>CSF, Lung</td>
<td>Mening., Septic.</td>
<td><em>S. suis, E. coli</em></td>
<td>H#</td>
</tr>
<tr>
<td>9</td>
<td>13</td>
<td>FS29612</td>
<td>Multiple sites</td>
<td>Pneum., Mening.</td>
<td><em>S. suis, PRRSV, E. coli</em></td>
<td>H</td>
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<tr>
<td>10</td>
<td>4</td>
<td>FS29613</td>
<td>Multiple sites</td>
<td>Septicemia</td>
<td>*S. suis, PRRSV, PCV2, <em>P. multocida</em></td>
<td>L</td>
</tr>
<tr>
<td>11</td>
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<td>FS29614</td>
<td>Multiple sites</td>
<td>Septicemia</td>
<td>PRRSV</td>
<td>H</td>
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<tr>
<td>12</td>
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<td>FS29814</td>
<td>Lung, Joint</td>
<td>Septicemia</td>
<td><em>Salmonella derby</em></td>
<td>H#</td>
</tr>
<tr>
<td>13</td>
<td>NT</td>
<td>FS29864</td>
<td>Brain, Joint</td>
<td>Mening., Septic.</td>
<td>None isolated</td>
<td>H#</td>
</tr>
<tr>
<td>14</td>
<td>5</td>
<td>FS30059</td>
<td>Lung</td>
<td>Septicemia</td>
<td><em>S. suis</em></td>
<td>H</td>
</tr>
<tr>
<td>15</td>
<td>NT</td>
<td>FS32585</td>
<td>Lung</td>
<td>Pneum., Polyser.</td>
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<td>H#</td>
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<tr>
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<td>12</td>
<td>FS33105</td>
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<td><em>S. suis, PRRSV, E. coli,</em></td>
<td>H</td>
</tr>
<tr>
<td>17</td>
<td>12</td>
<td>FS33206</td>
<td>Brain, Lung</td>
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<td>PRRSV</td>
<td>H</td>
</tr>
<tr>
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<td>Lung</td>
<td>Septicemia</td>
<td>None isolated</td>
<td>H</td>
</tr>
<tr>
<td>19</td>
<td>5</td>
<td>FS34086a</td>
<td>Lung, Joint</td>
<td>Severe serositis</td>
<td><em>S. suis</em></td>
<td>H</td>
</tr>
<tr>
<td>20</td>
<td>5</td>
<td>FS34086b</td>
<td>Lung, Joint</td>
<td>Pneum., Serositis</td>
<td>None isolated</td>
<td>H</td>
</tr>
<tr>
<td>21</td>
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<td>Lung, Joint</td>
<td>Serositis</td>
<td>None isolated</td>
<td>L</td>
</tr>
<tr>
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<td>FS34086d</td>
<td>Lung, Joint</td>
<td>Pneum., Pleuritis</td>
<td>None isolated</td>
<td>L</td>
</tr>
<tr>
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<td>Pneum., Polyser.</td>
<td>None isolated</td>
<td>L</td>
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<tr>
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<td><em>S. suis, P. multocida</em></td>
<td>H#</td>
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<td>Unknown</td>
<td>H#</td>
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<tr>
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<td>Unknown</td>
<td>Unknown</td>
<td>H#</td>
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<td>28</td>
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<td>Unknown</td>
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<td>H#</td>
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<td>29</td>
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<td>Unknown</td>
<td>H#</td>
</tr>
<tr>
<td>30</td>
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<td>**FS2170B</td>
<td>Joint</td>
<td>Polyserositis</td>
<td>Unknown</td>
<td>L</td>
</tr>
<tr>
<td>31</td>
<td>5</td>
<td>**FSIA84-29755</td>
<td>Lung</td>
<td>Pneum., Pleuritis</td>
<td>Unknown</td>
<td>H#</td>
</tr>
</tbody>
</table>

** "old" field strains obtained in 1999
* originally serotype 4
# designated highly virulent (H) because caused death of pig
L, Low virulence
NT, nontypeable, did not serotype to serovars 2, 4, 5, 12, 13, or 14
Figure 2.1. Characterization of outgroups strains (defined in Materials and Methods) by RAPD analysis and by whole cell lysate profiles. (A) RAPD analysis of four outgroups using primers 2, 7 and 12: Pasteurella multocida (PM), Manheimia haemolytica (MH), Pasteurella trehalosi (PT) and Actinobacillus pleuropneumoniae (AP); H. parasuis IA84-29755 (HP) was duplicate control; Std was 1 kb DNA ladder; (B) SDS-PAGE analysis of whole cell lysates of outgroups, samples were identical to those in panel A; Molecular weights (Std) are indicated in kilodaltons.
Figure 2.2. RAPD analysis of *H. parasuis* strains using primer 2. Forty six RAPD fingerprints separated on 1% horizontal agarose gels. Reference strains 1-15 (labeled A-O) are described in Table 2.1. Field strains 1-31 are described in Table 2.2. Std, 1 kb DNA ladder. Each lane was loaded with 10 µl of PCR reaction mixture containing an empirically determined optimal dilution of cells as described under Materials and Methods. A DNA control (no cells) was included in lanes marked “No”.
Figure 2.3. RAPD analysis of *H. parasuis* strains using primer 7. Forty six RAPD fingerprints separated on 1% horizontal agarose gels. Reference strains 1-15 (labeled A-O) are described in Table 2.1. Field strains 1-31 are described in Table 2.2. Std, 1 kb DNA ladder. Each lane was loaded with 10 µl of PCR reaction mixture containing an empirically determined optimal dilution of cells as described under Materials and Methods. A DNA control (no cells) was included in lanes marked “No”.
Figure 2.4. RAPD analysis of *H. parasuis* strains using primer 12. Forty six RAPD fingerprints separated on 1% horizontal agarose gels. Reference strains 1-15 (labeled A-O) are described in Table 2.1. Field strains 1-31 are described in Table 2.2. Std, 1 kb DNA ladder. Each lane was loaded with 20 µl of PCR reaction mixture containing an empirically determined optimal dilution of cells as described under Materials and Methods. A DNA control (no cells) was included in lanes marked “No”.
Figure 2.5. Electrophoresis pattern analysis of single-primer RAPD bands. Band patterns were analyzed by the Dice comparison algorithm and neighbor joining clustering software, with optimal position tolerance and optimization levels. Primers were primer 2 (Panel A), primer 7 (Panel B), and primer 12 (Panel C). Isolate identifications and cluster designations are shown for each dendogram. Numbers at the nodes indicate percentages of bootstrap values after 1000 replicates.
Figure 2.6. Electrophoresis pattern analysis of composite-primer RAPD bands by dendogram. Band patterns from all three single-primer experiments were combined to obtain a composite-primer RAPD dendogram. Analyses used the Dice comparison algorithm and the neighbor joining clustering software. Isolate identifications and three cluster designations are shown. Numbers at the nodes indicate percentages of bootstrap values after 1000 replicates.
Figure 2.7. Electrophoresis pattern analysis of composite-primer RAPD bands by parsimony tree. Band patterns from all three single-primer experiments were combined to obtain a composite-primer consensus tree by maximum parsimony heuristic analysis with 100 random addition replicates. Isolate identifications and ten cluster designations are shown.
Figure 2.8. SDS-PAGE profile of H. parasuis strains. Gradient SDS-PAGE gels of whole-cell lysates were stained with Coomassie Brilliant Blue R250. Reference strains 1-15 (A-O) are described in Table 2.1. Field strains 1-31 are described in Table 2.2. Each lane was loaded with 10 µg of protein. Molecular weights (MW) are indicated in kilodaltons.
Figure 2.9. Electrophoresis pattern analysis of the SDS-PAGE protein profiles by dendogram. Analyses used the Dice comparison algorithm and the neighbor joining clustering software. Isolate identifications and three cluster designations are shown for the dendogram. Numbers at the nodes indicate percentages of bootstrap values after 1000 replicates.
Figure 2.10. Electrophoresis pattern analysis of the SDS-PAGE profiles by parsimony tree. A consensus tree was obtained by maximum parsimony heuristic analysis with 100 random addition replicates. Isolate identifications and eight cluster designations are shown.
References


CHAPTER 3. ISOLATION AND CHARACTERIZATION OF A MU-LIKE BACTERIOPHAGE OF *Haemophilus parasuis*

A paper to be submitted to the *Journal of Bacteriology*

Emilie S. Zehr, Louisa B. Tabatabai

**Abstract**

*Haemophilus parasuis* is the causative agent of Glässer’s disease and is considered to be a remerging pathogen of swine in high-health status herds. This study is the first report of the isolation and characterization of a myophage from a field strain of *H. parasuis*. Electron microscopy showed that the bacteriophage had an iscosahedral head and contractile tail. Mass spectrometry of the bacteriophage proteins separated by one-dimensional (1-D) and two-dimensional sodium dodecyl polyacrylamide gel (2-D) electrophoresis (SDS-PAGE) samples identified twenty four homologues to bacteriophage proteins. Partial DNA sequencing revealed twenty open reading frames corresponding to fourteen proteins, including eight Mu-like homologues. This bacteriophage may be able to transduce virulence factors, which may affect the epidemiology of *H. parasuis* field isolates. We have named this bacteriophage SuMu.

**Introduction**

*Haemophilus parasuis* causes Glässer’s disease in pigs, with symptoms of fibrinous polyserositis, pericarditis, polyarthritis, and meningitis (41). *H. parasuis* also causes septicemia and pneumonia without polyserositis but can be isolated from healthy nasal passages of swine. Introduction of conventionally raised pigs into segregated early weaning (SEW) herds may result in infection and high economic losses because the latter lack immunity to *H. parasuis* (35, 54). Losses in 2006 were approximately $13.6 billion according to the National Animal Health Monitoring System (NAHMS) report (62) and (Rodney B. Baker, personal communication), based on the female pig population,
estimate of 3.5 piglets/sow/year loss, 262 pound weight of a market hog at $0.60 per pound sale price. Vaccines that are cross-protective are difficult to produce because of the many different serotypes of the organism.

Serotyping has been the primary method for the identification of isolates. *H. parasuis* strains are classified into 15 serovars based on immunodiffusion of heat-stable polysaccharide antigens (29, 53), and on virulence in specific pathogen free (SPF) pigs (29). However, there is a high incidence of nontypeable (NT) isolates and cross-serotype reactivity (30%) with the immunodiffusion test (61). A second serotyping method, the indirect hemagglutination (IHA) test, is based on particulate lipopolysaccharide antigen and only has less than 10% cross-reactivity. Some scientists prefer to combine both tests in order to improve interpretation of the tests and decrease the problems due to cross-reactivity (2, 8). Nonetheless, there are still many NT isolates that do not have serovar-specific reagents and cannot be characterized by serotyping.

In contrast to serotyping, the following methods have been able to differentiate NT isolates epidemiologically. *H. parasuis* identification has been done through the analysis of outer membrane protein (OMP) profiles (47, 55) and DNA profiles, including repetitive element-based PCR (55), restriction endonuclease fingerprinting (REF) analysis (58, 59), enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR) (45, 52), PCR-random fragment length polymorphism (PCR-RFLP) (14), multilocus enzyme electrophoresis (MLEE) (5, 49), and the analysis of 16S rRNA (1, 46).

Heterogenity in bacteria may be due to stress in bacteria which causes deletions and rearrangements in the bacterial genome which maintain virulence and broaden host range (3). Alternatively, this heterogeneity among *H. parasuis* isolates may be due to acquisition of diverse DNA segments through bacteriophage lysogeny into the host chromosome. For example, approximately 50% of strain-specific genomic DNA of *E. coli* O157 Sakai was attributed to prophage sequence acquisition through lateral gene transfer (64). Therefore, bacteriophages were instrumental in the emergence of a new *E.
coli strain and contributed to the genomic diversity of the species. Prior to this study, only Melnikow et al. (38) had reported the presence of bacteriophage genes in *H. parasuis* after growth under iron-limiting, oxygen-limiting, heat, and acidic stress conditions.

Historically, *H. parasuis* isolates have been evaluated by comparing protein profiles of whole cell lysates (43, 47) or outer membrane proteins (55). Nicolet et al. (43) observed two main patterns of protein profiles of whole-cell lysates. They suggested that strains obtained from systemic sites were homogeneous and different from those obtained from respiratory sites. These two patterns were later identified as PAGE type II and PAGE type I, respectively (42). Ruiz et al. (55) examined outer membrane protein (OMP) profiles and found that similar OMP profiles were found from isolates from systemic sites suggesting that some OMPs may be related to virulence. Similar conclusions concerning whole cell lysates were reported by Oliveira and Pijoan (47). In a previous study (E. S. Zehr, L. B. Tabatabai, D. V. Lavrov, submitted for publication), differences in whole cell lysate protein profiles were observed in selected field strains which had been subjected to starvation-like growth conditions in broth cultures and these differences were ascribed to bacteriophage lysis of the cultures.

This report describes a bacteriophage, named SuMu, isolated from a virulent *H. parasuis* serotype 5 field isolate. This Mu-like phage was characterized for the first time by electron microscopy, partial DNA sequencing, and proteomics including 1-D and 2-D gel electrophoresis followed by Mascot, Protein Prospector, and BLAST analysis.

**Materials and Methods**

**Strains and growth conditions.** *H. parasuis* field strains FS29814 (13), FS30059 (14), FS34086b (20 and 20b) (Chapter 2, Table 2.2) were all isolated from systemic sites. They were obtained in 2004 from Lorraine Hoffman of the Veterinary Diagnostic Laboratory, Iowa State University, Ames, Iowa. Because of the unavailability
of typing sera, partial serotyping with antisera to serotypes 2, 4, 5, 12, 13, and 14 of the field strains was performed by Gallant Custom Laboratories, Cambridge, Ontario. The serotypes of the four field strains were determined as nontypeable (NT) for strain 13 and as serotype 5 for the others. Strains were grown in Frey’s mycoplasm base broth (Sigma, St. Louis, MO) containing 20% heat-inactivated horse serum (Invitrogen, Carlsbad, CA) and 0.016% β-nicotinamide adenine dinucleotide (β-NAD) (Sigma) at 37°C overnight. Strains were checked for purity on blood agar with a nurse streak of S. aureus across a lawn of the H. parasuis isolate and on Casman’s agar (Difco, Detroit, MI) containing 5% horse serum and 0.016% β-NAD. Plates were incubated at 37°C under humidified 5% CO2.

**SDS-PAGE analysis.** For 1-D electrophoresis of whole cell lysates, bacterial cells grown in Frey’s broth for 22 hr were pelleted by centrifugation at 675 × g for 10 min. Cells were washed in 0.1 M PBS, pH 7.2, containing 1mM Pefabloc (Roche Diagnostics, Indianapolis, IN), then resuspended at a ratio of 32 mg cells per 100 µl PBS/Pefabloc. Cells were sonicated with a microprobe (Heat Systems-Ultrasonics, Farmingdale, NY) at 50% power for 60 1-second bursts to lyse them and centrifuged at 16,000 × g for 20 min to remove cell debris. Protein concentrations were determined by the Folin-Lowry method (33) or bicinechonic acid (BCA) assay (Sigma) with bovine serum albumin as a standard.

Fig. 3.1 is a composite of field strains described in Chapter 2. Ten µg protein/well was applied to a 10-well (Fig. 3.1), 30 µg protein was applied to a 15-well (Fig. 3.2a), or 10 µg protein/well was applied to a 10-well (Fig. 3.2b) NuPAGE precast 4-12% gradient Bis-Tris gels (Invitrogen). NuPAGE antioxidant was used in 3-(N-morpholino)-propane sulfonic acid (MOPS) running buffer. The protein prestained standard was BenchMark, 10-200 kDa (Invitrogen). Running conditions were 10 mA/gel for 15 min followed by 200 V for 40 min. Gels were stained in 0.1% Coomassie Brilliant
Blue R250 in 50% methanol/10% acetic acid and destained in 50% methanol/10% acetic acid.

For 2-D electrophoresis, 1000 µg of phage preparation or BHI media control as determined by the BCA assay were mixed with an equal volume of 100 mM MgCl₂, one-tenth volume DNase I (Sigma), and one-tenth volume RNase A (Sigma). The mixture was incubated on ice for 1 h; then an equal volume of freshly prepared 40% trichloacetic acid (TCA) in acetone was added and the mixture was stored at -20°C overnight (23, 24). The mixture was centrifuged at 17,900 × g for 10 min at room temperature and subsequently washed with 0.2% dithiothreitol (Sigma) in acetone two times. The precipitate was air-dried and resuspended in 200 µl IPG buffer. The solution was sonicated lightly to disperse the pellet. The 2D-Quant Assay (Amersham Biosciences) was performed to determine the concentration of the TCA-precipitated lysate. A 1-D gel was run after applying 10 µg/well as above to check the concentration and possible degradation of the sample (Fig. 3.2b).

The following modifications of operation of a Multiphor II isoelectric focusing system (Amersham Pharmacia Biotech, Upsula, Sweden) were implemented for the first dimension conditions of the 2-D electrophoresis procedure. A 200 µg sample in IPG buffer containing 2.5 mM tributylphosphine (39) and 0.1% DTT was applied to a 3-10 pH Immobiline dry strip (Amersham Pharmacia Biotech). Running conditions were: 1) rehydrate at 30°C for 12 h; 2) 500 V at 20°C for 90 min; 3) 1000 V at 20°C for 90 min; 4) 2000 V at 20°C for 60 min; 5) 4000 V at 20°C for 60 min; 6) 6000 V at 20°C for 90 min; 7) 8000 V at 20°C for 7 hr for a total of 35,700 Vhr. The standards for the first dimension were IEF mix 3.6-9.3 (Sigma). For the second dimension, the IEF strips were equilibrated in 1% dithiothreitol, then 5% iodoacetamide for 25 min to alkylate the sulfhydryl groups before being applied to a NuPAGE 4-12% gel with an IPG well (Invitrogen). Second dimension electrophoresis conditions and staining were done as described for the 1-D gels.
Growth curves and induction of phage with mitomycin C. *H. parasuis* cells (serovar 5) obtained from the field isolate (FS 34086b) were grown in brain heart infusion (BHI) broth (Difco/Becton Dickinson) supplemented with heme-HCl (Fluka) and L-histidine (Sigma), each at a final concentration of 10 µg/ml, as described by Williams et al. (65). One percent β-NAD (Sigma) was filtered sterilized and added at a final concentration of 0.16 mg/ml and 10 mM magnesium sulfate were also added. One-tenth volume overnight cultures were inoculated into 100 ml supplemented BHI; media and flasks were rotated at 75 rpm on an orbital shaker at 37°C. When cultures reached early stationary phase, cells were treated with chloroform and cooled to room temperature. DNase (1 µg/ml) and RNase (1 µg/ml) were added and the mixture was stirred for 30 min at room temperature.

Purification of phage. Cultures that had been treated with DNase and RNase were centrifuged at 20,000 × g for 15 min at 4°C. The supernatant was recentrifuged; then filtered through a 0.22 µm filter (Millipore) and 20 µl of chloroform per 100 ml of supernatant was added. The 20,000 × g supernatant was stored at 4°C until assayed for plaque formation or until concentrated for electron microscopy. For concentrated phage preparations, 10-ml capacity polycarbonate tubes (Beckman) were cold-sterilized with 10% hydrogen peroxide and then rinsed with sterile double distilled water. Filtered 20,000 × g supernatants were sedimented at 141,750 × g in a type 80 Ti rotor (Sorvall) for 3 hr at 15°C. Pellets were resuspended in a minimal amount of sterile TM broth with rocking overnight at 4°C and then gently mixed with a wide bore pipet tip. Preparations were dialyzed against TM broth overnight at 4°C using dialysis cassettes (Pierce) and then sterilized using 0.22 µm filters. Alternatively, 12.5 ml fractions were collected from sucrose (Sigma) or cesium chloride (International Biotechnologies, Inc., Newhaven CT) step gradients centrifuged in a SW41Ti rotor (Beckman) at 83,472 × g for 24 hr at 4°C. Fractions were dialyzed against TM broth, then concentrated with a Microcon YM100 centrifugal concentrator (Millipore) and filter-sterilized with 0.22 µm filters.
**Plaque assay.** Frey’s medium (Difco) containing 20% horse serum (Invitrogen) and 0.016% β-NAD was inoculated with high passage reference strain (Nagasaki) of *H. parasuis* serovar 5 as a control. The 20,000 × g supernatant from a BHI-grown FS34086b phage preparation was serially diluted in Frey’s medium containing β-NAD. One ml of each dilution was removed to sterile tubes containing 0.064 ml 1% β-NAD and 0.5 ml horse serum. One-tenth ml overnight culture of reference strain serovar 5 was added; the mixture was vortexed and incubated for 40 min at 37°C. Three ml of 0.375% SM top agarose was added, the tube was inverted to mix, and poured onto a Casman’s agar plate. The agarose was allowed to set, and an additional 3 ml SM top agarose was added. Plates were incubated upright in 5% carbon dioxide atmosphere at 37°C and formation of plaques was recorded after 15-18 h of incubation.

**Electron microscopy.** For bacterial electron microscopy, low passage *H. parasuis* field strain (FS23086b) serovar 5 bacteria were collected at 7 h or 27 h post-passage. An aliquot (1.5 ml) was removed, centrifuged at 4°C for 5 min at 400 × g. The supernatant was discarded and the pellet was washed in 100 µl of 5 mM PBS; then resuspended in 50 µl of 5 mM PBS. The phage/bacteria preparation (10 µl) was mixed 1:1 with 2% phosphotungstic acid (pH 7.0) for 3 min on a carbon-coated grid, wicked, and viewed on a Technai 12 microscope (FEI Company, Hillsboro, OR). Alternatively, phage and grid were incubated for 2 min, rinsed with water, wicked, stained with 1% uranyl acetate and immediately wicked (7). For sucrose fractions, grids were first coated with 1 µg/ml poly-L-Lysine (Sigma) for 30 s, then wicked with filter paper. Ten µl of phage sample diluted 1:10 in SM broth was applied to the grid, then wicked. Finally, 2% phosphotungstic acid, pH 7.0 was applied for 1 min, then wicked.

**Cloning and DNA sequencing.** For cloning, fifty µl of the 20,000 × g purified phage was treated with 0.5 units each of DNase and RNase for 30 min at 37°C, then 20 units of proteinase K (BRL) for 1 h at 65°C. The sample was extracted with phenol three times (36) and ethanol precipitated. The DNA was resuspended in 50 µl 10 mM Tris
HCl, 1 mM EDTA, pH 8.0. The phage DNA was amplified using a GenomiPhi DNA amplification kit (Amersham Pharmacia Biotech, Piscataway, NJ). The amplified product was heat-inactivated and ethanol-precipitated with 0.15 M sodium acetate, 25 mM EDTA. The DNA was blunt-ended with End-It DNA End-Repair kit (Epicenter, Madison, WI), phenol-extracted, and ethanol-precipitated. Transformation of chemically competent *E. coli* phage-resistant cells (One Shot Mach1-T1R) (Invitrogen) was done using TOPO XL Cloning reaction conditions (Invitrogen). Transformant colonies were picked and grown in 5-ml overnight cultures. The recombinant DNA was isolated using a QIAprep spin plasmid miniprep kit (Qiagen, Valencia, CA). The amplified DNA was restricted with enzymes that resulted in adenosine overhangs to be compatible with the thymidine arms of the TOPO vector. The restricted DNA was “polished” with Takara *Ex Taq* DNA polymerase (Panvera, Madison, WI) to ensure that the insert fragments had efficient 3’-A overhangs. After restriction with *Eco*RI and agarose electrophoresis, gel slices were excised between 2 and 23 kb. The DNA was eluted from the gel slices by using the Qiaquick gel extraction spin kit (Qiagen). This DNA was used to transform chemically competent cells and isolate recombinants as above. These recombinants were purified with Qiaprep columns and the DNA was restricted with a *Bam*HI/*Not*I double digest.

Automated dideoxy sequencing (57) was performed at the Iowa State University DNA Sequencing and Synthesis Facility. Reactions were set up using the Applied Biosystems (Foster City, CA) Prism BigDye Terminator v3.1 cycle sequencing kit with AmpliTaq DNA polymerase (FS) and electrophoresed on an Applied Biosystems 3730 DNA analyzer. The FS (fluorescent sequencing) polymerase is a Taq variant that has a point mutation in the active site region that allows better incorporation of the terminator/dye molecules and yields an error rate of 1 every 50,000-100,000 bases. After initial sequencing of the transformant clones, DNA sequencing was accomplished by walking down the amplified phage’s genome with overlapping primers to established
sequences. DNA sequences were analyzed with Expasy, Bioedit, Dialyn 2, BLASTN and ORF (Open Reading Frame) Finder (updated April, 2007).

**Mass spectrometry.** Mass spectrometry was done at the Iowa State University Protein Facility and the Iowa State University Proteomics Facility. For matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), three plugs of the band of interest were picked with a blunt-cut 20 gauge needle and deposited in a 96-well tray of an automated digester (ProGest, Genomic Solutions). Plugs were subjected to limited trypsin (Invitrogen) digestion. A C18 ZipTip (Millipore) was wetted with 10 µl of 70% acetonitrile in water. The solvent was discarded and the tip was washed three more times. The C18 ZipTip was washed with water two times and with 1.0% TFA once. The digested sample (10 µl) was aspirated and dispensed 30 times. The C18 ZipTip was rinsed with 0.1% TFA three times and then dried. Matrix solution (20 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/water/0.1% TFA) (0.5 µl) was dispensed onto a MALDI plate. Samples were mixed with the matrix solution, then analyzed on a Voyager System 6075 (PE Biosystems, Foster City, CA). Spectra were analyzed with the MSFit tool of the Protein Prospector program (11) at www.prospector.ucsf.edu/prospector.

MALDI-TOF MS/MS tandem mass spectrometry analysis was performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. Gel plugs containing selected bands or proteins were digested with sequence-grade trypsin (Promega, Madison, WI) in ammonium bicarbonate buffer at 37°C overnight. All spectra were processed by MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches. Typical search parameters were as follows: Maximum missed cleavage setting was 1.0; the fixed modification setting was carboxyamidomethyl cysteine with a variable modification of oxidation of methionine. Peptide mass tolerances were +/- 200 ppm. Fragment mass tolerances were +/- 1 Da or 2 Da. No restrictions on protein molecular weight were
applied. The significance threshold \( p \) was set to less than 0.05. Protein identification was based on the probability based MOWSE (MOlecular Weight SEarch) Score and % coverage of peptide. Sequences containing bacteriophage (BP) genes were selected for further analysis.

**Accession numbers.** The DNA sequence for the bacteriophage reported here was deposited in Genebank. The Genebank Accession No. for bacteriophage SuMu is EU268809.

**Results**

**SDS-PAGE analysis and mass spectrometry.** SDS-PAGE was used to compare field isolates grown under different conditions. Lanes marked 13, 14, 20 and 20b (Fig.3.1) were whole cell lysate protein profiles of field isolates, grown under different conditions. Lanes 13 and 20 had a sparse inoculum (2.5% v/v), while lanes 14 and 20b received a heavy inoculum (10% v/v) of starter culture. Lanes 13 and 20 had major protein bands at approximately 50 kDa, which were not apparent in the other protein profiles. Cultures 20 and 20b were actually cultures of the same original isolate. Cultures containing the 50 kDa band seemed to lyse overnight and the 50 kDa band was analyzed by MS/MS in order to identify the major protein. One-dimensional electrophoresis (Fig. 3.2A) of 30 \( \mu \)g the 145,421 \( \times \) g phage preparation in a 15-well SDS-PAGE gel gave concentrated protein bands which were analyzed by both MALDI-TOF and tandem mass spectrometry. Unfortunately, most bands had more than one “hit” for definitive identification of the proteins. Therefore, 1-D and 2-D electrophoresis (Fig. 3.2B, lane 1, Fig. 3.3B) of a TCA-treated portion of the original phage preparation was performed. A media-only control, concentrated in a similar manner as the phage preparation, was also TCA-precipitated in order to rule out contaminating proteins in the medium (Fig. 3.2B, lane 2, Fig. 3.3A). The combined methods resulted in the identification of 23 homologues of bacteriophage proteins and one homologue of IgA-
specific zinc metalloprotease of *Streptococcus pneumonia* (Table 3.1, Fig. 3.3B). Bacteriophage proteins included fifteen related to Myoviridae, three related to Siphoviridae, three related to Podoviridae, one Cystoviridae (dsRNA), and one Microviridae (ssDNA).

**Growth curves and plaque assay.** In order to maximize phage yield, growth curves were determined for *H. parasuis*. Cultures took approximately 10-12 h to reach early stationary phase when grown in 100-ml volumes. Incidentally, the formulation and source of the medium made a significant difference in the overall growth of the organism. Frey’s broth (Sigma) gave a higher cell density (0.2 absorbance units at 600 nm) than Frey’s broth (BRL). Adding heme and histidine to the BHI medium also gave the best yield of phage. Additionally, mitomycin C treatment did not induce the bacteriophage. There was a slight decrease in optical density at 5.75 h post-mitomycin C (0.027 units). However, if the culture was allowed to proceed to autolysis at 48.25 h, a decrease of 0.239 units resulted. The nontreated culture lysed at 47.25 h with a decrease of 0.375 units. Since the time of lysis was variable, the cultures were allowed to “autolyse” prior to harvesting at 26 to 48 h. The extent of lysis was monitored by the amount of absorbance decrease at 600 nm of the culture. Plaque assays determined the presence of approximately $2 \times 10^4$ to $2 \times 10^5$ PFU per ml of culture supernatant.

**Electron microscopy.** Electron microscopy was used to determine phage morphology. Electron micrographs of a bacteriophage associated with *H. parasuis* are shown in Fig. 3.4. The average icosahedral head width in a 7 h lysate of bacteria was 42.1 +/- 2.1 nm (Fig. 3.4A). A DNA-filled head (46.6 nm) (arrow) and contractile tail attached to a bacterium from an overnight lysate are shown in Fig. 3.4B. Bacteriophage from a 27 h lysate that are associated with the outer surface of a bacterium are shown in Fig. 3.4C. A group of bacteriophage heads can be seen in the field of view after sucrose gradient purification (Fig. 3.4D). On the other hand, the bacteriophage appeared to be sensitive to cesium chloride and no phages were seen after they were exposed to cesium
chloride ultracentrifugation. Possible bacteriophage-produced holes in the bacterium’s outer membrane are depicted in Figs. 3.4E-F and Fig. 3.4E shows bacteriophage (arrows) being released through the hole. Sucrose step gradients were necessary to concentrate and purify bacteriophage from large preparations because the titer of the phage was extremely low.

Grundy and Howe (25) reported on the effect of the staining method on the visualization of phage. The difference in staining between uranyl acetate (Fig. 3.5A) and phosphotungstic acid (Fig. 3.5B) is remarkable. The morphology between noncontractile (Fig. 3.5C) and contractile (Fig. 3.5D) tails and dimensions of the major structural features in nm are also shown (25). Electron micrographs of Mu-like bacteriophage of *H. parasuis* with a contractile tail and noncontractile tail are shown in Fig. 3.6a and Fig. 3.6b, respectively. Morphology of the SuMu bacteriophage were similar to those reported by Grundy and Howe (Fig. 3.5) (25). These representative phages were obtained after collection and concentration of the 30% sucrose fraction. The average head width was 48.2 +/- 3.1 nm, the average contractile tail length was 49.2 +/- 6.6 nm, and the average noncontractile tail length was 120.5 +/- 13.4 nm. Most of the phage observed had contractile tails. The measurements of the head and contractile tails are very close to those of published Mu phage sizes (Fig. 3.5) (25).

**Cloning and DNA sequencing.** From approximately thirty spin-column purified clones, only three contained inserts. The initial insert was 700-800 bp in length and was submitted for DNA sequencing. The insert’s DNA sequence was homologous to Enterobacteriophage Mu’s protease (MuI) (*E* value = 1e-89). However, the SuMu phage protease contained a significant deletion (254 amino acids) compared to the MuI phage protease.

Additional phage genomic DNA was prepared by amplification of purified phage. After chromosome walking with overlapping primers to already sequenced DNA of the SuMu genome, fourteen proteins were identified by using NCBI’s Open Reading Frame
Finder mining tool (Table 3.2). Eight of the fourteen proteins were homologues of Mu-like proteins. The relationship of SuMu proteins to those of Mu and FluMu is shown in Fig. 3.7. The solid arrows are homologues obtained from DNA sequencing data while the dashed arrows show protein homologues obtained from peptide mass fingerprinting results.

**Mass spectrometry analysis of 50 kDa band.** The 50 kDa protein in whole cell lysates of lysing cultures of field isolates 13 and 20 has been described previously (Chapter 2). Repeated mass peptide fingerprinting analyses of various field isolates showed that this protein is a homologue of DNA transfer protein gp20 (Accession no. Q01076), of bacteriophage P22, a Podoviridae. This protein has a theoretical molecular mass of 50,101 and a pI of 8.58.

**Discussion**

Bacteriophage can introduce foreign DNA into a bacterium by transduction, and thus, contribute to the pathogenicity or virulence of the bacterium. Double-stranded DNA tailed bacteriophages (Caudovirales), the Podoviridae (short tail stub), Siphoviridae (long non-contractile tail), and Myoviridae (contractile tail), as well as Inoviridae (filamentous tail with single-stranded DNA) all encode virulence factors and can change a nonpathogenic bacterium to a virulent one by lysogenic conversion of the bacterium through recombination events in vivo (6). The virulence genes are commonly located near the recombination sites, the tail genes, or the capsid protease of the bacteriophage. Through their virulence factors, the bacteriophages contribute to pathogenesis, strain differentiation, and genomic diversity of the bacterial species. A few of the virulence determinants found thus far in bacteriophage include surface-exposed bacterial immunoglobulin-binding regulators (56) and membrane proteins that influence envelope variability (37). Other virulence determinants include toxins, such as an enterohemolysin (6) and a mitogen (51); components that alter antigenicity, such as glycosylation proteins,
glucosyl transferase, and O-antigen acetylase; type III effectors involved in invasion; and those factors involved in intracellular survival such as superoxide dismutase, neuraminidase, and hyaluronidase. Additional virulence factors are OMPs involved in serum resistance and adhesins involved in colonization (6). A phage-encoded endosialidase which may form the tale plate of *E. coli* bacteriophage 63D has also been reported (34). Double-stranded DNA bacteriophages also express immunoglobulin-like domains, including hydrolases, peptidases, and endopeptidases that bind to or degrade bacterial cell polysaccharides or LPS, and which increase bacterial infectivity (18, 19).

This study was undertaken to determine the cause of autolysis of *H. parasuis* field isolates grown in broth culture. A Mu-like bacteriophage (SuMu) was eventually isolated and characterized, and is important to *H. parasuis* pathogenesis because it may contribute to virulence. It was also noted that *H. parasuis* field strain cultures in this study did not completely lyse and seemed to “regrow” if allowed to do so. These observations are in agreement to those reported for *Salmonella* phages (10) and an *E. coli* *O*157 phage (17).

It was determined that low passages of strain FS34086b gave better yields of bacteriophage (PFU/ml) than higher passages of the isolate did. There are two outcomes after infection with a bacteriophage: 1) multiplication of the phage and lysis of the bacterium or 2) lysogenization or integration of the phage DNA as a prophage into the bacterial chromosome. After repeated passages *in vitro*, the majority of bacteriophage may have entered the lytic phase and may have been lost from the lysogenic state in the bacterium, and therefore could no longer be recovered from the cell population.

The apparent size of its amplified DNA after agarose gel electrophoresis was approximately 20,000 bp. However, the putative length of SuMu may actually vary due to the electrophoresis conditions or due to the migration of the phage, depending on if it was closed-circular rather than linear during electrophoresis. The genome size of SuMu is comparable to genome sizes of 36717 bp, 34,676 bp, and 31,508 bp for bacteriophages Mu, FluMu, and HinMu, respectively (40, 65).
This study found that stressing the *H. parasuis* lysogens by mimicking starvation conditions induced the phage (Fig. 3.1). SDS-PAGE analysis confirmed the presence of a new 50 kDa protein, identified as a homologue of the DNA transfer protein gp20 (Accession no. Q01076) of bacteriophage P22. Of the 24 recent virulent field strains screened (Chapter 2), four isolates expressed the 50 kDa protein. Those virulent isolates lacking the 50 kDa protein may have lost their phages. In fact, another study (22) showed that nearly 75% of lysogenic prophages were lost from their bacterial hosts after storage at -70°C. These authors postulated that bacterial stress, including nutrient availability, acidity, osmolarity, and temperature, triggered the induction of phage and the resulting lysis of the cell.

Wagner and Waldor (63) believed that there was a “mutualism” in the host’s body, where bacteria were less virulent until the prophages were induced by environmental conditions such as exposure to antibiotics or superoxide dismutase. A phage-encoded virulence factor could then be up-regulated, which could contribute to the pathogenesis of the bacteria. Subsequently, the lytic release of virions could also amplify the number of infected bacteria carrying the phage-encoded virulence factor.

Bacteriophages can contribute to bacterial virulence through transduction and regulation of phage-encoded virulence factors, alteration of bacterial virulence factors, and function in the control of bacterial virulence through *in situ* prophage induction (63). One can sequence suspected phage-encoded virulence factors but mutational analysis should be done to verify transduction of the gene by phage. Phages can alter bacterial virulence factors at all stages of infection, including adhesion, colonization, invasion, and spread, resistance to immune defenses, exotoxin production, antibiotic sensitivity, and transmission to other hosts. Some bacteriophages carry DNA that changes the phenotype of the bacterial host (lysogenic conversion genes) and this lysogeny is a form of short-term bacterial evolution (9). When lysogenic Gram-negative bacteria were grown in infected animals, the prophage genes were the majority of expressed genes in
the bacteria (15). This study described Mu-like genes which mirrored those found in MuHi, a prophage of *H. influenzae* strain Rd (37). Corresponding Mu-like genes found in both MuHi and SuMu (reported here) included the C repressor, gp29, gp30, MuG, MuI, gp36, and gpL. The tail assembly protein (MuG) was postulated to be membrane-associated. These authors hypothesized that phage-encoded membrane-associated proteins of *H. influenzae* and *N. meningitidis* contributed to the variability of the bacterial envelope structure and may have therefore influenced the virulence and pathogenicity of the organisms. Proteins gp36 and gp37 of Mu were determined to be homologous to unique DNA sequences of *P. multocida* strains which caused hemorrhagic septicemia (40). These gene homologues are also present in *H. parasuis*, which causes septicemia in swine (Table 3.2).

Electron microscopy experiments were sometimes difficult due to the fragility of the bacteriophage. These findings correlate with those of Summer et al. (60) who found that phage 56 (BcepMu) particles were unstable in lysates, had decreasing titers with storage, and showed disintegrating particles with broken heads, and partially exposed tails in electron micrographs. Many empty capsids with contractile tails were also observed. Few intact virions were found by electron microscopy in this study (Fig. 3.6). Fig. 3.4C is intriguing and one would like to draw the conclusion that the phage particles may be attached to the cell wall or lipopolysaccharide (LPS) of the bacterium. In fact, Zorzopulos et al. (67) reported that a T4 bacteriophage inner tail tube penetrated the *E. coli* cell wall where a receptor that was associated with an endotoxin-like particle was located. Figs. 3.4E-F showed structures that can be interpreted to be holin-lysin structures caused by degradation of the peptidoglycan layer of the bacterium and allowing the release of virions. Holin structures were presented in electron micrographs described in a review by Young (66). They are caused when phage-encoded holin proteins (60-185 amino acids long), form pores in the membranes, which allow access of phage-encoded endolysins to degrade the peptidoglycan (13). Endolysins are known to
hydrolyze glycosidic linkages via muramidases and glucosaminidases or to hydrolyze amide bonds via amidases and peptidases (32).

In this study an endolysin was identified by DNA sequencing and mass spectrometry (Tables 3.1 and 3.2, Fig. 3.3B, protein 23) but a holin protein was not found by the latter two methods. However, the evidence for the presence of a holin is seen in Figs. 3.4E-F, which clearly shows the “holes” in the bacterial surface. Moreover, when comparing amino acid homologies between the SuMu sequence and a \textit{M. haemolytica} phage sequence with Dialign 2 (Bielefeld University Bioinformatics Server, Bielefeld University, Germany), the SuMu endolysin amino acid sequence was 36.2\% homologous to the \textit{M. haemolytica} phage endolysin amino acid sequence whereas the \textit{M. haemolytica} phage holin was found to be 32.4\% homologous to the SuMu sequence. This holin homology is only indirect evidence of a holin’s presence in SuMu. A potential reason for the lack of identification of holin sequences may be their short polypeptide lengths prevented a match in Genebank due to its restrictions of amino acid length on peptide submissions or that there may be an analogous protein that is functioning as a holin in SuMu.

Proteins were identified by mass spectrometry after 1-D and 2-D electrophoresis. Fig. 3.2B illustrates the effect of trichloroacetic acid treatment of the whole cell lysates prior to 2-D electrophoresis. The trichloroacetic acid presumably degrades lipoproteins in the preparation. One of the proteins identified by MALDI-TOF from a 1-D gel (Fig. 3.2, band 1a; Fig. 3.3B, spot 1a; Table 3.1, 1a) was a homologue of an IgA-specific zinc metalloprotease from \textit{S. pneumoniae}. It also showed 56.9\% amino acid homology to a bacteriophage T4 immunogenic outer membrane protein (Hoc) (Accession no. AAD42581), which was a member of the immunoglobulin superfamily because it formed Ig-like domains (4). Additionally, it was a homologue to a membrane-bound protein of a myophage from \textit{Burkholderia}. However, neither the \textit{Burkholderia} phage protein (85 kDa) (Table 3.1, 1b) nor the Hoc protein (40.3 kDa) approached the 220 kDa size of band
1a. Another homologous protein not listed in Table 3.1 was a zinc protease of \textit{H. influenzae} Rd KW20 (NP_439519). However, its mass was only 107 kDa. The size of the protein may have been influenced by the SuMu phage being associated with the bacterial cell membrane, because these phage preparations were not treated with proteinase K prior to electrophoresis. Finally, Gioia et al. 2006 (21) characterized the \textit{\phi}MhaMu1 prophage that was integrated into a pseudogene of an immunoglobulin metalloendopeptidase. A mechanism such as the latter may have occurred with protein 1a of SuMu. The SuMu protein 1a homology to an IgA metalloprotease is interesting in that IgA1 protease has been described as a virulence factor in nontypeable \textit{H. influenzae} (26) and has also been identified in iron-limited growth of \textit{H. parasuis} (Chapter 4).

Adsorption of tailed phages to bacterial cells may involve a complex system which could evolve by acquiring modules of genes rather than individual gene products (12). These attachment proteins could all be ascribed to virulence potential of a phage. A phage-related protein that contributes to virulence is gp37, long tail fiber receptor recognizing protein Fig. 3.2, band 2; Fig. 3.3B, spot 2; Table 3.1). This myophage protein allows the specific attachment of the phage to the bacteria. Additionally, a siphophage protein with the same function was found, the tail host specificity protein (Fig. 3.2, band 22; Fig. 3.3B, spot 22; Table 3.1). Besides the function of determining the length of the phage tail during particle morphogenesis and adsorption to the cell, the tape measure protein (Fig. 3.2, band 15; Fig. 3.3B, spot 15; Table 3.1) may be able to partially degrade bacterial peptidoglycan during initial infection (50). Two phage proteins, a long tail fiber protein and a base plate wedge protein (Fig. 3.2, bands 6 and 8; Fig. 3.3B, spots 6 and 8; and Table 3.1) are also involved in attachment of the phage to the bacteria.

Four proteins are related to the lysogeny of the phage genome or lysis of the phage, namely transposase, DNA transposition protein B, cI repressor protein, and antirepressor protein. The other homologues to phage proteins listed (Table 3.1) are involved in phage morphogenesis or gene control.
T4-type bacteriophages have evolved a mechanism for lateral gene transfer (LGT) that protects them from destroying their genomes (16). “Modules” carrying replication and structural genes of double-stranded DNA were not changed but noncore genes were involved in LGT and mosaism events. Phage host ranges often were not limited to one bacterial species, and phages with overlapping host ranges were able to exchange sequences with dissimilar hosts (27). On the other hand, a recent report stated that two-thirds of all phage orthologous groups (POG) were phage-specific (31). These authors indicated that illegitimate recombination and transduction of host genomes may not have had such an important impact on the phage genome. However, the latter statement does not mean that phage genomes have no effect on bacterial genomes.

The linear genome organization for Mu and FluMu bacteriophages was conserved over a wide range of phages (40). For example, the late genes encoding structural proteins are more conserved than the early or middle genes. However, even in the most conserved regions, there are substitutions of analogous genes or deletions of genes (40). Fig. 3.7 shows that SuMu more closely follows Mu in its genome organization than it does FluMu’s gene order.

The codon bias is a way to predict the length of time a bacteriophage has been associated with its host (65). If the values were more similar between the phage and the host, the prediction is that the phage was not recently introduced into the host. Examples of mol % G + C usage among the following three myophage and their respective hosts are: FluMu (50%), *H. influenzae* Rd (38%); HP2 (40%), *H. influenzae* (NTHI) (nontypeable) (38%); SuMu (43%), *H. parasuis* (41-42%) (30, 40, 65). It appears that nontypeable *H. influenzae* and HP2 as well as *H. parasuis* and SuMu have been associated longer than *H. influenzae* Rd and FluMu.

Interestingly, bacteriophage HP2 was isolated from *H. influenzae* (NTHI) and was found in strains associated with high virulence (65) but had a different host range than that of HP1, which infected *H. influenzae* Rd strains. The *H. influenzae* (NTHI) are also
genetically more diverse respiratory pathogens and the authors felt it was important to characterize the phages associated with them. Some of the pathogen’s diversity may be affected by the phage-encoded genes.

Bacteriophage genes from a microarray analysis have been identified in a H. parasuis genomic library (38). Table 3.3 compares five Melnikow et al. (38) bacteriophage-expressed proteins to those found in this study. Based on the results of others and those of this study, it is suggested that virulent bacteria harbor phage while avirulent organisms do not have genome-associated phage. Bacteriophage can introduce virulence determinants into an organism by transduction, which can then contribute to the pathogenicity and heterogeneity of the bacteria (20, 44). A possible reason why the protein expression in H. parasuis may become more homogeneous over time might be because the lysogenic phage has excised from the bacterial chromosome and thus the bacteria lack that means of diversity. Others have suggested lateral transfer of genes between A. pleuropneumoniae and H. parasuis, commensals of the upper respiratory tract where mucosal surfaces are prime surfaces for biofilm formation and intimate contact between different species (28, 48).

This work has characterized for the first time the SuMu (EN268809) bacteriophage of H. parasuis through partial DNA sequencing, electron microscopy and 2-D electrophoresis of outer membrane proteins followed by mass spectrometry analysis. It appears that this phage codes for at least one potential virulence factor, which is a homologue of an IgA-specific zinc metalloprotease (Accession No. Q54875).
<table>
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<tr>
<th>Label</th>
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<th>Source</th>
<th>Accession No.</th>
<th>Theoretical MW (kDa/pI)</th>
<th>Experimental MW (kDa/pI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>IgA specific zinc metalloprotease</td>
<td><em>Streptococcus pneumoniae</em></td>
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<td>220/5.6</td>
<td>220/6.5</td>
</tr>
<tr>
<td>1b</td>
<td>gp44 membrane protein</td>
<td><em>Burkholderia</em> phage Bcep 781 (Myoviridae)</td>
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<td>220/6.5</td>
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<td>2</td>
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<td><em>E. coli</em> enterobacteriophage T4 (Myoviridae)</td>
<td>P03744</td>
<td>109/8.5</td>
<td>102/8.5</td>
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<tr>
<td>3</td>
<td>Portal protein</td>
<td><em>S. typhimurium</em> enterobacteriophage P22 (Podoviridae)</td>
<td>P26744</td>
<td>82.7/4.8</td>
<td>90/4.3</td>
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<td>4</td>
<td>gp69 DNA polymerase A</td>
<td><em>Listeria</em> phage P100 (Myoviridae)</td>
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<td>82.5/5.1</td>
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<tr>
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<td>P1 protein, polymerase</td>
<td><em>Pseudomonas</em> phage phi 16 (Cystoviridae)</td>
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Table 3.2. Homologues from Open Reading Frames of SuMu DNA Sequence

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<sup>a</sup>Cds (SuMu) _1-(SuMu)_20 are specified by their locus tag number (Protein ID), <sup>b</sup>protein length in amino acids (AA), clusters of orthologous groups (COG), <sup>c</sup>theoretical molecular mass in kilodalton and isoelectric point (MW/pI), <sup>d</sup>similarity by ORF Finder and BLASTP search of NCBI protein database
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Figure 3.1. Composite SDS-PAGE gel illustrating whole cell lysates of stressed and nonstressed *H. parasuis* field strains. Lanes 13 and 20 were lysates that received sparse inoculum; lanes 14 and 20b a heavy inoculum of starter culture. Samples were separated with a 4-12% SDS-PAGE gel and stained with Coomassie R250 as described in the text. Molecular weights (MW) are indicated in kilodaltons.
Figure 3.2. Composite SDS-PAGE gel illustrating bacteriophage protein preparations. (A) phage preparation centrifuged at 145,421 × g; (B) phage whole cell lysate that was precipitated with trichloroacetic acid (lane 1) and media only control that was precipitated with trichloroacetic acid (lane 2). Samples were resolved with a 4-12% SDS-PAGE gel and stained with Coomassie R250 as described in the text. Numbers between panels A and B (lanes 1) correspond to protein spots labeled in Fig. 3.3B. BHI 1-4 (Panel B, lane 2) correspond to background proteins from brain heart infusion (BHI) medium and to areas in Fig. 3.3A. Molecular weights (M) are indicated in kilodaltons.
Figure 3.3. 2-Dimensional electrophoresis illustrating bacteriophage protein preparations. (A) media only control that was precipitated with trichloroacetic acid; (B) phage whole cell lysate that was precipitated with trichloroacetic acid. Samples were separated by isoelectric focusing in the first dimension. They were then separated with a 4-12% SDS-PAGE gel and stained with Coomassie R250 as described in the text. Circled protein spots were analyzed by tandem mass spectrometry and reported in Table 3.1. Molecular weights (M) are indicated in kilodaltons.
Figure 3.4. Electron micrographs of bacteriophage isolated from *H. parasuis*. (A) Icosahedral heads of phage attached to surface of bacteria; (B) DNA-filled head and contractile tail of phage (arrow) attached to bacteria; (C) bacteriophage from 27 h lysate attached to surface of bacteria; (D) group of bacteriophage heads after sucrose gradient purification; (E-F) possible bacteriophage-produced holes in bacterial membrane as well as phage being released through hole (arrows-E); bars, 100 nm.
Figure 3.5. Composite diagram of Mu bacteriophage. (A) stained with uranyl acetate; (B) stained with phosphotungstic acid; (C) phage with noncontracted tail; (D) phage with contracted tail. Reprinted from Virology 143, Grundy, F. J. and M. M. Howe, Morphogenetic structures present in lysates of amber mutants of bacteriophage Mu, p. 487, Copyright 1985, with permission from Elsevier.
Figure 3.6. Electron micrographs of SuMu bacteriophage after sucrose gradient centrifugation and staining with phosphotungstic acid. (A) phage with contractile tail; (B) phage with noncontractile tail; bar represents 100 nm.
Figure 3.7. Comparison of the genome organization of *H. parasuis* bacteriophage SuMu to the genome organizations of Enterobacteriophage Mu and *H. influenzae* bacteriophage FluMu.
References


CHAPTER 4. IDENTIFICATION AND CHARACTERIZATION OF IRON-REGULATED PROTEINS OF *Haemophilus parasuis*

A paper to be submitted to *Infection and Immunity*

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Abstract

*Haemophilus parasuis* is the causative agent of Glässer’s disease in pigs and is of major economic importance in the swine industry. To date, there is no vaccine available that provides cross-reactive protection to nonimmune piglets. Additionally, there are no diagnostic tests that can distinguish avirulent from virulent isolates of *H. parasuis*. Outer membrane proteins (OMPs) of bacteria probably play a role in the host’s immune response to the pathogens. This work describes 27 iron-regulated proteins that are homologues of other *Haemophilus* proteins expressed when *H. parasuis* was grown in stress (iron-restricted) and nonstress (iron-replete) conditions. The expressed proteins were further analyzed using mass spectrometry and Western blotting. Stable isotope labeling with amino acids in cell culture (SILAC) experiments confirmed the presence of a homologue of a hemoglobin binding protein. One-dimensional Far Western blots of OMPs probed with biotinylated porcine transferrin or porcine hemoglobin showed that the probes bound to *H. parasuis* OMPs. Some of these *H. parasuis* immunogenic, iron-regulated proteins are homologues of other known virulence factors of the Pasteurellaceae family and may be used in future virulence studies of *H. parasuis*. This study is the first to identify iron-regulated proteins that are potential virulence factors in *H. parasuis* by using 2-D electrophoresis, Western blotting, Far Western blotting, and SILAC.
Introduction

*Haemophilus parasuis* is a Gram-negative pleomorphic bacterium, varying from a single coccobacilli to long, thin, filamentous chains (43). In 1910 Glässer (18) associated this organism with polyserositis and polyarthritis in pigs. *H. parasuis* is spread by direct contact from pig to pig and first colonizes the nasal cavity (23). Symptoms of this disease include fibrinous polyserositis, pericarditis, polyarthritis, meningitis, and septicemia (38). Glässer’s disease causes high morbidity and mortality in nonimmune pigs of all ages, especially in specific pathogen free (SPF) animals. Exposing high health status herds to conventionally-raised pigs may result in high economic losses because the former lack immunity to *H. parasuis* (28, 44). Economic losses for the year 2006 in the United States were estimated at $13 billion dollars, based on 24.8 million sows losing an estimated 3.5 piglets/sow/year due to *H. parasuis*. Those piglets represented 22.7 billion pounds of market weight at $0.60/pound (Rodney B. Baker, Iowa State University, personal communication) (49).

Virulence factors potentially involved in the pig’s immune response to *H. parasuis* include exposed surfaces of the bacteria, such as adhesins, receptors, and capsules (16). Other virulence factors which can aid the pathogen’s entry into the host cell include invasins, hemolysins, proteases, toxins, OMPs, and secretion systems.

Additionally, some OMPs and receptors may change their antigenic epitopes through mechanisms such as antigenic variation, and thus enabling bacteria to avoid detection by the host’s immune system. In *H. influenzae* (14, 15) and *H. parasuis*, (30, 31) amino acid substitutions have been shown to cause changes in the antigenicity of adhesins like OMPs P2 and P5. Tandem repeated DNA sequences of surface-exposed proteins can allow for antigenic variation through intragenic homologous recombination (42). Examples of this type of antigenic variation include varying the amount of sialylation of surface glycoproteins or LPS and changes in pili, flagella, and the IgA protease of *H. influenzae* (16, 52).
Other examples of bacterial exposed surfaces which undergo antigenic variation are outer membrane receptors for hemoproteins, transferrin, and siderophores, all of which are highly immunogenic (44). These three OMPs are phase variable, meaning their expression is up- or down-regulated, depending on the environment of the bacteria (44). This phase variability also provides a way for the pathogen to escape the host’s immune system, because changes in OMP structure can result in changes in its immunogenicity, so the host’s acquired immune system will not recognize that antigen (16, 51).

A change in nutrients, such as nicotinamide adenine dinucleotide (NAD) or iron, may be growth limiting to *H. parasuis* when the organism invades tissues of the host (39). At this point, there might be production of fimbriae, altered outer membrane profiles, and enhanced adhesion. In normal tissues, iron is sequestered in iron-binding molecules such as transferrin or lactoferrin or in heme-binding molecules such as hemoglobin.

Stressed or nutrient-limited bacteria upregulate various proteins including iron-regulated proteins, such as lactoferrin-, transferrin-, hemoglobin-, and hemoglobin-haptoglobin-binding proteins, heme-hemopexin utilization proteins, and siderophores. Stress to bacteria caused by host defenses, such as antibiotics or antimicrobial agents, can also activate deletions and rearrangements in the bacterial genome which maintain virulence and broaden host range (3).

Iron-regulated proteins are antigens which may be potential virulence factors in *H. parasuis*’s colonization of the upper respiratory tract of swine. Furthermore, antibodies against these proteins may prevent the organism’s colonization or invasion of the respiratory tract. Historically, most vaccines have been made up of whole cell preparations of *H. parasuis* isolates (6, 44, 48). Combinations of *H. parasuis* and other viruses and bacteria that may be co-infecting the pig, such as porcine reproductive and respiratory syndrome virus and *Mycoplasma hyopneumoniae*, have been suggested for vaccine use (23). However, serovar diversity and the high number of nontypeable (NT)
isolates contribute to the lack of cross-protective vaccines (40). Recently, Bigas et al. (5) developed a thyA mutant of *H. parasuis* which lacks the ability to colonize but induces wild type levels of serum bactericidal activity in infected guinea pigs.

In this study, virulence-related proteins were examined after they were expressed by bacteria grown under stress related conditions induced by iron limitation. The rational for these studies are that stress responses due to lack of iron in the growth medium mimic the effect of pathogenic bacterial invasion and resulting sequestration of iron in the animal host, whose tissues are also iron-limiting. These iron-regulated responses result in expression of new bacterial proteins or other virulence factors that enable the pathogen to evade the host’s immune surveillance system and allow the pathogen to survive in the host. The assumption is made that these newly synthesized proteins may be used to develop improved vaccines and companion diagnostic tests.

The objectives of this study were to identify and characterize iron-regulated proteins in an avirulent strain (serovar 6) and a virulent strain (serovar 5) of *H. parasuis*. Similarities and differences of the proteins in each serovar were evaluated using 2-D electrophoresis, mass spectrometry, and Western blotting. After the 2-D electrophoresis experiments, Far Western blotting and SILAC methods were used to further evaluate selected iron-regulated proteins in serovar 6 and serovar 5.


**Materials and Methods**

**Strains and growth conditions.** *H. parasuis* reference strains, reference strain 5 (virulent strain Nagasaki) and reference strain 6 (avirulent strain 131) (Chapter 2, Table 2.1) were obtained from Richard Ross of the College of Veterinary Medicine, Iowa State University, Ames, Iowa. For 2-D electrophoresis experiments, strains were grown in Frey’s mycoplasma base broth (Sigma, St. Louis, MO) containing 20% heat-inactivated
horse serum (Invitrogen, Carlsbad, CA) and 0.016% β-nicotinamide adenine dinucleotide (β-NAD) (Sigma) at 37°C overnight. For iron-restricted media, 2-2′-dipyridyl (DP) was added at a level which inhibited 50% of the growth of the respective strain. The final DP concentrations for serovars 5 and 6 grown in Frey’s broth were 200 µm and 375 µm, respectively. The strains were checked for purity on blood agar with a nurse streak of *S. aureus* across a lawn of the *H. parasuis* isolate and on Casman’s agar (Difco, Detroit, MI) containing 5% horse serum and 0.016% β-NAD. Plates were incubated at 37°C under humidified 5% CO2.

For cell preparations that were used in Far Western blots, the growth medium was brain heart infusion broth (BHI) (Difco), supplemented with 0.016% NAD, 0.1% hemin-HCl (Fluka), and 0.1% L-histidine (Sigma) (54). The final concentrations of DP used in serovar 5 and serovar 6 grown in BHI broth iron-restricted cultures were 250 µM and 125 µM, respectively. Iron-replete cultures contained no DP, but a final concentration of 36 uM FeSO₄ 7H₂O was added to the BHI in addition to the supplements.

For the SILAC experiment, the defined minimal medium was Dulbecco’s Modified Eagle Medium (D-MEM) (Invitrogen) containing 4500 mg/L glucose and L-glutamine and with or without 10% (w/v) stable isotope-labeled amino acids (heavy) (Invitrogen), [U¹³C₆, ¹⁵N₄]-L-Arginine and [U¹³C₆]-L-Lysine, but no sodium pyruvate and supplemented with porcine hemoglobin (Sigma) as the sole iron source. D-MEM without heavy amino acids received 10% (w/v) unlabeled L-Arginine and L-Lysine (light) amino acids.

The optimal concentrations of DP and iron source had to be determined for use in this experiment. Porcine hemoglobin was dialyzed against 0.015 M NaCl in order to remove any free iron. Its final concentration was determined by using the bicinchoninic acid assay with bovine serum albumin as a standard (BCA) (Sigma).

Serovars 5 and 6 from freezer stock were grown on Casman’s agar (Difco) for 48-72 h. Cells were swabbed into D-MEM and their concentration was calibrated by
determining the absorbance at 600 nm (Model 250, Molecular Devices, Sunnyvale, CA). The cells in D-MEM media were transferred to microtiter plate wells. Titrations of porcine hemoglobin in D-MEM with and without 200 µM DP were performed with each serovar in order to determine the optimal growth conditions. After performing 24 h growth curves, serovar 5 was grown in 200 µM hemoglobin while serovar 6 was grown in 250 µM hemoglobin. Additionally, iron-limiting conditions were attained when 250 µm or 125 µm 2, 2’-dipyridyl (Sigma) was added to the medium of serovars 5 and 6, respectively.

Additionally, titration of cells grown in the supplemented minimal medium was performed in order to determine the approximate ratios of CFU/ml to absorbance at 600 nm in microtiter plate wells. For the actual SILAC experiment, the cells were inoculated into D-MEM containing the optimal concentration of porcine hemoglobin for each serovar. Each serovar’s medium either received its optimal concentration of DP or no DP. Likewise, the medium for each serovar contained stable isotope-labeled amino acids, [U^{13}C_6, ^{15}N_4]-L-Arginine and [U^{13}C_5]-L-Lysine, or the unlabeled forms of each amino acid.

**Determination of iron source for *H. parasuis***. Casman’s agar plates were prepared with or without 5% heat-inactivated horse serum and supplemented with 0.016% NAD and 200 µM DP. The plates were spread with 200 µl of 1:10 and 1:100 dilution of serovar 5 (3.12 x 10^7 CFU/ml) or serovar 6 (1.46 x 10^8 CFU/ml). After the plates dried, sterile Whatman #1 filter paper discs were applied. Then 30 µl of an iron source (200 µM solution) was pipetted onto the respective filter disc. The plates were incubated right side up at 37°C and 5% CO₂ atmosphere for approximately 1 h. Afterwards, the plates were inverted, incubated, and observed for growth around the discs for 5 days. Iron sources that were screened included porcine hemoglobin (Sigma), porcine transferrin (Mann Research Laboratories, New York, NY), bovine apotransferrin (Sigma), bovine holotransferrin (Sigma), bovine lactoferrin (Sigma), bovine hemoglobin
(Sigma), ferric citrate, ferrous sulfate, sheep hemoglobin (Sigma), ferric ammonium sulfate, and hemin (Sigma). Bovine albumin (Sigma) was included as a control.

**Preparation of cell lysates and OMP.** For 2-D electrophoresis, Frey’s broth-grown cells were centrifuged at 675 × g for 10 min. The pellet was resuspended in 25 mM Tris-HCl (Sigma), pH 7.6, containing 20% sucrose, 5 mM EDTA, pH 8, and 1 mM Pefabloc (Roche Applied Science, Indianapolis, IN). The suspension was incubated on ice for 5 min and then centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in ice cold water containing 1 mM Pefabloc. The cell lysates were frozen at -80°C until the next step in the procedure. The thawed lysates were treated with 0.1 M MgCl2, DNase (1 mg/ml), RNase (0.025 mg/ml), and Pefabloc for 1 h on ice (19, 20). Finally, the lysates were precipitated with 40% tricholoroacetic acid (TCA) in acetone at -20°C overnight. The precipitate was centrifuged at 17,900 × g for 10 min at room temperature. The supernatant was removed and the precipitate was incubated with 0.2% DTT in acetone for 1 h at -20°C. The mixture was centrifuged at 17,900 × g for 10 min, dried at room temperature, and 200 µl IPG buffer was added.

For Far Western analysis, cell lysates were prepared as modifications of Murphy et al. (37) and Leyh and Griffith (26). BHI broth-grown cells were harvested at stationary phase by centrifuging the cells at 10,000 × g for 15 min at 4°C. Cells were resuspended in 10 mM N-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) buffer, pH 7.4 in a proportion of 1.5 g cells/15 ml buffer. Additionally, protease degradation was reduced by the addition of 0.1 ml proteinase inhibitor cocktail II (Calbiochem)/0.4 g cells. After the cells were resuspended, they were frozen at -80°C overnight.

For OMP preparation, the cells were sonicated three times for two min each using the microtip of a Branson 250 sonifier at setting 5 and a 50% cycle. The sonicate was centrifuged at 5000 × g for 20 min at 4°C to remove unbroken cells. The clarified
supernatant, which contained the total membranes, was centrifuged at 100,000 × g for 1 h at 4°C. The membrane pellet was resuspended in the ratio of original 1.5 g cells/2 ml 1% N-lauryl sarcosine in 10 mM HEPES, pH 7.4 by rocking at room temperature. The insoluble outer membranes were centrifuged at 100,000 × g for 1 h at 4°C and reextracted with the sarcosine-HEPES buffer. After a final centrifugation at 100,000 × g for 1 h at 4°C, the pellets were resuspended in the ratio of 0.035 g membranes/100 µl sterile double distilled water with the addition of 9 µl proteinase inhibitor cocktail/100 µl water.

**SDS-PAGE analysis.** Initial protein concentrations were determined by the Folin-Lowry method (27) for the 2-D experiment or the bicinchoninic acid assay (BCA) (Sigma) for the Far Western experiment with bovine serum albumin as a standard for both analyses. Proteins were treated with RNase, DNase, and precipitated with trichloroacetic acid (19, 20), then resuspended in IPG buffer.

For 1-D electrophoresis, ten µg protein/well was applied to 10-well NuPAGE precast 4-12% gradient Bis-Tris gels (Invitrogen). NuPAGE antioxidant was used in 3-(N-morpholino)-propane sulfonic acid (MOPS) running buffer. The protein prestained standard was BenchMark, 10-200 kDa (Invitrogen). Running conditions were 10 mA/gel for 15 min, then 200 V for 40 min. Gels were stained in 0.1% Coomassie Brilliant Blue R250 in 50% methanol/10% acetic acid and destained in 50% methanol/10% acetic acid.

**2-D electrophoresis.** The 2D-Quant Assay (Amersham Biosciences) was performed to determine the concentration of the TCA-precipitated lysate with bovine serum albumin as a standard. A 1-D gel was run as above to check the concentration and for possible degradation of the TCA-treated (2-D) sample.

The following modifications of operation of a Multiphor II isoelectric focusing system (Amersham Pharmacia Biotech, Upsala, Sweden) were implemented for the first dimension conditions of 2-D electrophoresis. A 200 µg sample in IPG buffer containing 2.5 mM tributylphosphine (34) and 0.1% DTT were was applied to a 3-10 pH Immobiline
dry strip (Amersham Pharmacia Biotech). Running conditions were: 1) rehydrate at 30°C for 12 h; 2) 500 V at 20°C for 90 min; 3) 1000 V at 20°C for 90 min; 4) 2000 V at 20°C for 60 min; 5) 4000 V at 20°C for 60 min; 6) 6000 V at 20°C for 90 min; 7) 8000 V at 20°C for 7 h for a total of 35,700 Vhr. The standards for the first dimension were IEF mix 3.6-9.3 (Sigma), run at the same conditions as the samples. For the second dimension, all of the IEF strips were equilibrated in 1% dithiothreitol, then 5% iodoacetamide for 25 min to alkylate the sulfhydryl groups before being applied to a NuPAGE 4-12% gel with an IPG well (Invitrogen). Second dimension electrophoresis conditions were done as described for the 1-D gels. The 2-D gel shown with serovar 6 proteins (Fig. 4.1) was stained with Sypro Ruby (Molecular Probes), while the serovar 5 gel was stained with Coomassie Blue R250 (Fig. 4.2). Sypro Ruby staining gave low background but the gel was very difficult to handle because it was fragile. Therefore, Coomassie Blue R250 staining was used in subsequent gels. However, Coomassie Blue R250 staining resulted in uneven destaining.

Analysis of 2-D gels. The PD-Quest 7.0.1 software package (BioRad, Hercules, CA) was used to detect 1.5-fold or more differences in expressions of proteins when comparing two gels to one another. Each gel was digitally scanned and cropped. Subsequently, spots were then automatically detected, edited, and normalized. Gaussian (3-D) images were used to create match sets. The amount of corresponding protein on different filtered gel images was expressed as a relative volume (intensity × area), normalizing the intensity of each spot on a gel (2).

Mass spectrometry analysis. Mass spectrometry was done at the Iowa State University Protein Facility. For matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF), three plugs of the band of interest were picked with a blunt-cut 20 gauge needle and deposited in a mass spectrometry 96-well tray. Plugs were subjected to limited trypsin (Invitrogen) digestion. A C18 ZipTip (Millipore) was wetted with 10 µl of 70% acetonitrile in water. The solvent was discarded and the tip was washed three
more times. The C18 ZipTip was washed with water two times and with 1.0% TFA once. The digested sample (10 µl) was aspirated and dispensed 30 times. The C18 ZipTip was rinsed with 0.1% TFA three times and then dried. Matrix solution (20 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/water/0.1% TFA) (0.5 µl) was dispensed onto a MALDI plate. Samples were mixed with the matrix solution, then analyzed on a Voyager System 6075 (PE Biosystems, Foster City, CA). Spectra were analyzed with the MSFit tool of the Protein Prospector program (8) at prospector.ucsf.edu/prospector, using the NCBI nonredundant Haemophilus databases at 50 ppm mass accuracy. The highest MOWSE (MOlecular Weight SEarch) score and/or % coverage of peptide was used to identify homologues as well as matching molecular weight and pI to experimental results.

**MS/MS spectrometry analysis.** MALDI-TOF MS/MS tandem mass spectrometry analysis was performed using a QSTAR XL quadrupole TOF mass spectrometer (AB/MDS Sciex, Toronto, Canada) equipped with an oMALDI ion source. Selected bands excised from gels were digested with sequence-grade trypsin (Promega, Madison, WI) in ammonium bicarbonate buffer at 37°C overnight. All spectra were processed by MASCOT (MatrixScience, London, UK) database search. Peak lists were generated by Analyst QS (AB/MDS Sciex, Toronto, Canada) and were used for MS/MS ion searches. Typical search parameters were as follows: Maximum missed cleavage setting was 1.0; the fixed modification setting was carboxyamidomethyl cysteine with a variable modification of oxidation of methionine. Peptide mass tolerances were +/- 200 ppm. Fragment mass tolerances were +/- 1 Da or 2 Da. No restrictions on protein molecular weight were applied. The significance threshold \(p\) was set to less than 0.05. Alternatively, Protein Pilot software (Applied Biosystems, Foster City, CA) was used to analyze the MS/MS spectra.

**Western blotting and analysis.** For the 2-D electrophoresis experiments, proteins were blotted to polyvinylidene fluoride (PVDF) (Millipore). Blotting buffer was
10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer, pH 11 (Sigma) with 10% methanol. Running conditions were 500 mA/55 V/28 W for 60 min. After blotting, the membranes were blocked with 0.25% fish gelatin (Norland Products, Cranbury, NJ) in 0.1 M PBS, pH 7.2 containing 0.05% Tween 80 (Sigma) (PBST).

Western blots were probed with pools of negative and convalescent porcine antisera to \textit{H. parasuis}. Sera were obtained from Karen Post of Rollins Diagnostic Laboratory in North Carolina in 1999. The swine anti-sera were diluted 1:100 in PBST containing 0.25% fish gelatin (blocking buffer). The sera and blots were incubated with rocking at 37°C for 2 h, then overnight at 4°C. The blots were washed five times with PBST. The conjugate was rabbit anti-pig IgG horseradish peroxides (Sigma), diluted 1:500 in blocking buffer. The conjugate and blots were incubated overnight at 4°C. The blots were washed four times with PBST, then once with 0.1 M PBS, pH 7.2. The substrate consisted of 15 ml 0.1 M PBS, pH 7.2; 3 ml 0.3% (w/v) 4-choloro-naphthol in methanol; and 15 µl 30% (w/v) hydrogen peroxide.

For the Far Western blotting experiment, OMP were blotted to nitrocellulose (Protran NC BA85, pore size, 45 µm) (Midwest Scientific, St. Louis, MO). Blotting buffer was NuPAGE transfer buffer (Invitrogen) containing antioxidant (Invitrogen) and 10% methanol. Transfer conditions were 30 V/370 mA/11 W for 60 min in the cold. Ending readings were 30 V/174 mA/5 W/30 Vhr. Blots were blocked with 10 mg/ml egg white in PBST containing 0.25% fish gelatin for 30 min at room temperature and washed five times in PBST. The blots were then blocked in 50 ng/ml d-biotin (Sigma) for 15 min at room temperature and washed five times in PBST.

Far Western blots were probed with biotinylated iron sources (see below). Biotinylated transferrin was applied at a 1:25 dilution and biotinylated porcine hemoglobin was used at a 1:100 dilution in blocking buffer. The probes and blots were incubated at 37°C for 3 h with rocking. The blots were washed five times with PBST. Avidin-HRP conjugate (BioRad) was diluted 1:1000 in blocking buffer and the blots
were incubated overnight at 4°C with rocking. The substrate was the same as above.
Tone curves were adjusted in both blots probed with biotinylated iron sources (Figs. 4.3B
and 4.3C) to lessen the background. Biotinylated proteins that bound to the probes were
confirmed by mass spectrometry (Table 4.3).

**Biotinylation of iron sources.** Porcine transferrin and hemoglobin were
biotinylated using the EZ-Link NHS-iminobiotin trifluoracetimide kit (Pierce catalog no.
21117) instructions. The initial amount of iron source used was 4 mg. After dialysis
using 0.5-3.0 ml, 10K MWCO, Slide-A-Lyzer dialysis cassettes (Pierce), the final protein
concentration was 1.6 mg/ml.

**Stripping Western blots.** A solution that was a modification of Harlow and
Lane (22) was used to strip probes and substrate off blots in order to reuse them. The
formula was 50 mM Tris HCl containing 2% SDS and 2 M DTT. The solution can be
frozen and reused numerous times. After stripping the blots, they were washed three
times in 0.1 M PBS and reblocked with 0.25% fish gelatin before they were reprobed.

**SILAC procedure.** To study the effect of iron restriction on protein expression
an avirulent *H. parasuis* serovar 6 and a virulent *H. parasuis* serovar 5 using the SILAC
procedure, cells were grown in media with or without heavy arginine and lysine that was
also either iron-replete or iron-restricted. Thus, for each serovar, four fractions were
generated: 1) iron-replete (no DP), heavy amino acids; 2) iron-replete (no DP), light
amino acids; 3) iron-restricted (DP), heavy amino acids; 4) iron-restricted (DP), light
amino acids. Per kit instructions, six doublings of both isolates were calculated to be 5 h.
For the SILAC experiment, the following modifications were made the Invitrogen
instructions for membrane protein analysis: 1) instead of 2 x 10^6 cells/ml, the
concentration of cells was 8 x 10^8 cells/ml for serovar 5 and 1 x 10^8 cells/ml for serovar
6; 2) 10 mM Pefabloc was used in addition to the kit’s Benzonase nuclease. Twenty
microliters of lysate were run on 10-well gradient gels to assess the concentration of the
samples. Centrifugation at $17,000 \times g$ for 20 min prior to electrophoresis alleviated the viscosity of the samples.

Samples were prepared as follows before mixing the lysates at a 1:1 ratio of heavy (labeled) proteins to light (unlabeled) proteins, where one of the samples was grown in DP and the other was grown without DP. Each sample (300 µl) was mixed with 50 µl 4X Sample Buffer (Invitrogen), 20 µl 100 mM Pefabloc, and 5 µl proteinase inhibitor cocktail. Then equal amounts (1:1 ratio) of “heavy” and “light” cell lysate samples were mixed and resolved by 1-D electrophoresis. The samples were heavy and light lysates from *H. parasuis* serovar 6 or serovar 5 grown in the absence or presence of DP. Bands corresponding to molecular weights of approximately 60, 80, and 100 kDa were excised, treated with trypsin as previously described, and peptides were analyzed by tandem mass spectrometry. Mass spectra were processed by Protein Pilot, version 2.0 (Applied Biosystems) and MASCOT (Matrix Science, London). These programs analyze data from peptide pairs differing in mass by 6 atomic mass units (amu) and 10 amu. Those peptides containing one pair of light and heavy lysine and arginine are supposed to be analyzed. Iowa State University Proteomics Facility does not currently have the appropriate software in order to analyze pairs. However, labeled peptides were able to be detected.

**Results**

**2-D electrophoresis and Western blotting.** 2-dimensional electrophoresis followed by mass spectrometry and Western blotting was used to identify both up- and down-regulated proteins of *H. parasuis* serovar 6 or serovar 5 grown in stressed (iron-restricted) and nonstressed (iron replete) conditions. Table 4.1 lists 27 iron-regulated proteins that were immunoreactive or nonimmunoreactive from the results of serovar 6 (Fig. 4.1) or serovar 5 (Fig. 4.2) 2-D gels and blots. The list included 13 upregulated, immunoreactive; one upregulated, nonimmunoreactive; six downregulated,
immunoreactive; and one downregulated, nonimmunoreactive *H. parasuis* homologues of other *Haemophilus* proteins. Both serovars expressed all listed proteins but there was a difference in the immunogenicity of the proteins when comparing serovars 5 and 6. Convalescent sera reacted with ten proteins of serovar 6 (Figs. 4.1B, 4.1F) by Western blotting, whereas the corresponding proteins were not immunoreactive with serovar 5 proteins. These were transferrin binding protein B (E), ABC transporter ATP-binding protein (F), glucose 6 phosphate isomerase (G), ferrochetalase (L), heme hemopexin utilization protein C (O), uncharacterized protein HI 1042 (P), hypothetical protein 4 (capsulation locus) (Q), phage phi R-73 primase-like protein (S), pyridoxine biosynthesis protein (X), and serum resistance protein DsrA (Y). Serovar 5 (Figs. 4.2B, 4.2C, 4.2E, 4.2F) also had seven immunoreactive proteins that were nonimmunoreactive in serovar 6. These were adhesin (B1), heme hemopexin utilization protein B (H), lipoprotein (M), heat shock protein 70 (R), elongation factor Tu (T), OMP P1 (U), and a major OMP P2 variant (V).

**Optimal iron sources for *H. parasuis***. Preliminary studies performed on Casman’s agar plates containing 200 µM DP were done as described above in order to determine utilization of iron sources by serovars 5 and 6. These experiments revealed that the organisms preferred porcine hemoglobin, bovine hemoglobin, sheep hemoglobin, and ferrous sulfate as determined by growth around filter discs containing those iron sources. There was a slight utilization of porcine transferrin but no growth was observed from bovine apotransferrin, bovine holotransferrin, bovine lactoferrin, ferric citrate, ferric ammonium sulfate, hemin, or bovine albumin (control).

**Effect of DP on outer membrane protein expression.** The effect of DP on the expression of outer membrane proteins of serovars 5 and 6 is shown in Fig. 4.3A. In the presence of DP, ten proteins of serovar 6 were upregulated (Fig. 4.3A, lane 2 vs. lane 1) and six proteins of serovar 5 were upregulated whereas one protein of serovar 5 was downregulated (Fig. 4.3A, lane 4 vs. lane 3).
Blots of the same outer membrane preparations were probed with biotinylated porcine hemoglobin (Fig. 4.3B) and biotinylated porcine transferrin (Fig. 4.3C) in order to identify hemoglobin and transferrin binding proteins, respectively. The hemoglobin probe bound to four upregulated proteins of serovar 6 and 5 (Fig. 4.3B, lanes 2 and 4, respectively). Bands 1 and 3 (lane 2) and bands 8 and 10 (lane 4) showed faintly, whereas bands 11 (lanes 2 and 4) were barely discernable.

The apparent molecular weights for many of the OMPs were approximately 15 kDa higher (Fig. 4.3) than the actual molecular weights of the identified proteins in the Far Western blots. This slower migration through the polyacrylamide gel may be due to high molecular weight smooth lipopolysacharides being associated with the OMPs (13). Lipopolysacharides can also affect the efficiency of protein transfer during electrophoretic blotting, which may partially explain the extremely faint bands marked observed on these blots and marked with the arrows.

The actual molecular weights of the proteins were approximately 108 kDa (bands 1 and 8), 99 kDa (bands 3 and 10), 80 kDa (band arrow 3 in lanes 2 and 4), and 63 kDa (arrow 4, lane 2 and band 11). The molecular weight of the 108 kDa protein is consistent with the molecular weight of the *H. ducreyi* homologue of hemoglobin binding protein A (HgbA). The molecular weight of the 99 kDa protein is consistent with the molecular weights of two *H. influenzae* homologues, heme utilization protein A (HupA) or heme:hemopexin utilization protein A (HxuA). The molecular weights of the 80 kDa and 63 kDa proteins are consistent with the molecular weights of *H. influenzae* homologues heme:hemopexin utilization proteins C (HxuC) and B (HxuB), respectively.

The transferrin probe bound to two strongly upregulated proteins of both serovar 6 and serovar 5 (Fig. 4.3C, lanes 2 and 4, respectively, upper set of arrows) and one weakly upregulated protein of serovar 6 (Fig. 4.3C, lane 2, lower arrow). The molecular weights of the strongly upregulated proteins were 107 kDa and 103 kDa, respectively, consistent with the molecular weight for transferrin binding protein A (TbpA). Bands 2
and 9 (Fig. 4.3C) also contained *H. influenzae* homologues of lactoferrin binding protein B and adhesin (Hia) (Table 4.3). The weakly upregulated protein that reacted with the transferrin probe had a molecular weight of 60 kDa, which is consistent with the molecular weight of transferrin binding protein B (TbpB).

Fig. 4.3D served as a control blot for the biotinylated probes, because the streptavidin conjugate that is used may react with endogenous biotin-containing proteins. Several endogenous streptavidin-reacting proteins were observed in the control blots and in the sample blots.

**Identification and quantification of proteins using SILAC.** The advantages of SILAC methodology is that upregulated proteins can be identified and quantified by mass differences of the “heavy” and “light” pairs of peptides. The ratio of differences in intensity would reflect the relative degree of up- or downregulation or would reflect no change. In addition, the software allows identification of the selected peptides containing peptide pairs by peptide mass fingerprinting. However, the necessary software for quantitation of ratios was not available at the Proteomics Facility at Iowa State University. The data will be reanalyzed when the software becomes available. However, a few peptide pairs were identified for some proteins using Protein Pilot and MASCOT programs.

SILAC results were not as straightforward as the results obtained with the 2-D gels and biotinylated probes. Bands corresponding to approximately 100 kDa, 80 kDa, and 60 kDa were excised from gel lanes containing 1:1 ratios of serovar 5 cells, grown in light media with DP and heavy media without DP (Fig. 4.4, lane 1) or serovar 6 cells, grown in light media without DP and heavy media with DP (Fig. 4.4, lane 2). After tandem mass spectrometry, Protein Pilot software identified the serovar 6 proteins at 100 kDa and 60 kDa and the serovar 5 protein at 60 kDa similar to the hemoglobin subunit beta-2 chain (16.2 kDa) (Accession No. P02067) of various mammals, including the pig. The serovar 5 protein at 100 kDa was identified by Protein Pilot software as a homologue
of a putative copper-transporting P-type ATPase protein (85.8 kDa) (Accession No. NP_437558) or a heavy metal translocating P-type ATPase (86.2 kDa) (Accession No. YP_001313614).

MASCOT was used to search the same tandem mass spectra from the above samples. A 60 kDa protein of both serovars was identified as a homologue of the hemoglobin and hemoglobin-haptoglobin binding protein precursor (HgbA) (110.9 kDa) (Accession No. Q47952). MASCOT searches also identified the serovar 5 and 6 proteins at 80 kDa, the 60 kDa protein of serovar 6, and the 100 kDa protein of serovar 5 as homologues of ABC transporter-related proteins (72.5 kDa) (Accession No. YP_741462). Additionally, a 100 kDa protein of serovar 5 was also identified as a homologue of a TonB-dependent receptor (89.9 kDa) (ZP_01986586). The 100 kDa protein of serovar 6 was identified as a homologue of an H. influenzae IgA protease precursor (186 kDa) (Accession No. P44969).

**Discussion**

Invading bacteria must acquire iron in order to survive in the host. They have evolved high-affinity iron acquisition mechanisms which overcome the host’s sequestration of iron. Most of the bacterial proteins involved in iron and heme acquisition are upregulated during iron-limiting conditions. In iron-rich media, Fur negatively regulates the iron/heme-uptake genes as well as various virulence factor genes, such as receptors for lactoferrin, transferrin, and hemoglobin. During iron-limiting conditions, Fur is released from the Fur box upstream of the promoter and transcription of the target genes occurs.

Two mechanisms for iron acquisition in *H. parasuis* have been described: 1) a Fur-independent *tonB*-like constitutive siderophore receptor system for inorganic iron uptake (12, 36); and 2) a Fur-dependent siderophore-independent outer membrane receptor system for binding and uptake of protein-bound iron compounds (7, 11, 36).
Comparisons of OMPs between the avirulent and virulent strains of a pathogen may lead to the identification of virulence factors. Initially, 27 iron-regulated proteins that were homologues of *H. influenzae* proteins were identified (Table 4.1). Eight of these iron-regulated proteins have been reported to contribute to pathogenesis in nontypeable *H. influenzae* (21) and therefore, may be important in the pathogenesis of *H. parasuis*. The proteins include OMP P1, OMP P2, transferrin binding protein 1 (Tbp 1), transferrin binding protein 2 (Tbp 2), adhesins, pilus adhesion (HifE), IgA1 protease, and heat shock protein 70. Tbp 1 and Tbp 2 of *H. influenzae* are similar to TbpA and TbpB of *H. parasuis*, respectively.

The amino acid sequences of OMPs P1 and P2 vary between strains and contribute to antigenic variation. Recently, McVicker and Tabatabai (30, 31) characterized two *H. parasuis* OMPs homologous to *H. influenzae*’s P2 and P5 adhesins. Adhesins, including HifE, are most commonly found in NT isolates of *H. influenzae* and aid in the adherence to epithelial cells. Heat shock protein 70 has also been suggested to influence adherence to epithelial cells. IgA1 protease cleaves at the hinge region of secretory IgA1, releasing Fabα fragments which can coat the pathogen and allow it to avoid the host immune response by adhering to host tissues or by blocking access of intact antibody to the bacterial surface (29).

A high molecular weight immunoglobulin binding protein (IgBP) homologue of *H. somnus* was also found (Table 4.1). In *H. somnus*, it was associated with virulence, and was found to bind to a heparin-binding domain that adheres to bovine endothelial cells. Interestingly, immunization with an Fc-binding peptide of the IgBP protected calves against intrabronchial challenge (47).

Several enzymes that were homologues to *H. influenzae* enzymes and that are stress-induced by iron limitation were also upregulated. These included topoisomerase IV (subunit A), glucose 6 phosphate isomerase, IgA-specific endopeptidase, ferrochetalase, pyridoxine biosynthesis protein, and uncharacterized protein HI 1042.
(vitamin-B12 dependent methionine synthase). Similar results were also reported by Hill et al. (24) and Metcalf and MacInnes (33) who found mostly enzymes by growing cells at 40°C incubation temperature or under iron-restriction, respectively. Both used differential display PCR to evaluate their results.

Melnikow et al. (30) studied transcription of *H. parasuis* genes by microarray after iron-limitation. They described 75 genes that coded mostly for transporters of iron and sugar metabolites, metabolic enzymes, and DNA metabolism. Common genes to the work of Melnikow et al. (30) and this study included HxuA, HxuB, HxuC, and a phage phi-R73 primase-like protein.

Iron limitation also upregulated five proteins involved in protein synthesis or transport (Table 4.1): Fur, elongation factor Tu, and ribosomal protein L9 are involved in protein synthesis while transporter ATP-binding protein and signal recognition particle protein are involved in protein transport. A homologue to a surface lipoprotein of *H. influenzae*, which is a hypothetical protein 4 (Bcs4 capsulation locus) and is involved in serotype-specific polysaccharide synthesis was also identified (Table 4.1).

Finally, two bacteriophage-related proteins were identified by 2-D electrophoresis (Table 4.1). The serum resistance protein (DsrA) is a phage-encoded outer membrane protein required for serum resistance. The phage phi-R73 primase-like protein primes DNA replication. To date, there has been one report (32) on the presence of bacteriophage genes in *H. parasuis* after growth under iron-limiting, oxygen-limiting, heat, and acidic stress conditions. An *H. parasuis* bacteriophage named SuMu was also isolated and characterized and is described in Chapter 3.

Additional iron-regulated proteins found in this study included homologues of the heme-hemopexin utilization system proteins CBA (HxuCBA) of *H. influenzae*. The HxuCBA proteins have been described as virulence factors because they are upregulated during conditions of heme starvation (35). Heme utilization proteins of *H. parasuis* were tentatively identified in this study (Tables 4.1 and 4.3, Figs. 4.1-4.2, 4.3B). Proteins of
the heme-hemopexin utilization system are found only in Gram-negative bacteria and they are involved in the acquisition of heme from multiple sources and bring it back to a specific outer membrane receptor. The soluble HxuA (100 kDa) associates with a helper protein, HxuB (60 kDa), and is secreted from the cell; HxuA binds to heme-hemopexin and the resulting complex is presented to HxuC (80 kDa), the outer membrane-specific receptor (9, 10, 35, 51). HxuC has been described as a TonB-dependent protein involved in transport in H. influenzae (9, 10). H. parasuis may employ similar iron acquisition mechanisms for heme-hemopexin and hemoglobin.

Iron uptake studies indicated that hemoglobin served as an excellent source of iron. Therefore, a hemoglobin probe was used to identify a potential homologue of H. ducreyi’s hemoglobin binding protein A (HgbA). A putative hemoglobin binding protein, HgbA, was identified at approximately 108 kDa (Fig. 4.3B) in H. parasuis. The HxuCBA homologues were also identified in the 2-D electrophoresis experiment (Figs. 4.1-4.2, Table 4.1) and may have been detected with the hemoglobin probe (Fig. 4.3B, Table 4.3). HxuA was confirmed at 99 kDa, while HxuB was confirmed at 63 kDa (bands 3 and 10 and 11, respectively (Fig. 4.3) by mass spectrometry. Similarly, Tbp A and TbpB were identified by 2-D electrophoresis, mass spectrometry, and Western blotting (Figs. 4.1-4.2) and may have been putatively identified with the transferrin probe (Table 4.3, Fig. 4.3).

Detection of the transferrin binding proteins expressed by serovars 5 and 6 (Fig. 4.3C) showed that the biotinylated transferrin probe bound to proteins of 107 kDa, 103 kDa and 60 kDa of serovar 6, but bound only to the 107 kDa and 103 kDa proteins in serovar 5. Probing with convalescent sera gave similar results, in that the 60 kDa protein of serovar 5 was not detected (Fig. 4.2). Possible explanations include inefficient transfer of the 60 kDa protein of serovar 5 or that the appropriate epitopes were not available for binding the transferrin probe or the antibody probe. The ability of the OMPs to bind to biotinylated transferrin was shown in Fig. 4.3C. The probe reacted with TbpA in both
serovars but only with putative TbpB in serovar 6. The transferrin-binding proteins of *H. parasuis*, TbpA and TbpB, have been described as 106, 102, and 59.6 kDa, respectively (7, 36). The 106 kDa protein identified as TbpA may be the unprocessed protein containing the signal peptide. Lack of reaction of the probe with TbpB of serovar 5 could be due to the heterogeneity in protein structure or due to heterogeneous gene products of *tbpB* in *H. parasuis* serovars (11).

Other proteins were putatively identified by Far Western blotting (Table 4.3, Fig 4.3). Mass spectrometry of the original 1-D SDS-PAGE gel’s proteins identified two adhesins, lactoferrin binding protein B, hemin receptor, opacity associated protein, OMP Pom A, OMP P2, OMP P5, and ABC transporter ATP binding protein. Tbp 1, HxuA, HxuB, OMP P2, and ABC transporter ATP binding proteins were identified by mass spectrometry with both 1-D and 2-D electrophoresis methods (Fig. 4.1-4.3, Tables 4.1-4.3).

The conjugate-only control Far Western blot (Fig. 4.3D) shows the nonspecific reactions obtained with the probes. Blocking the blots with egg white albumin and d-biotin alleviated some of the nonspecificity but not all of it. Possibly, incorporation of additional blocking agents such as bovine serum albumin or nonfat dry milk, might have improved the results.

Direct comparisons cannot be made between the Western blot and Far Western blot results. In Western blots, protein antigens are probed with antibodies while in Far Western blots, protein receptors are probed with biotinylated antigens. The type of membrane was different in each experiment, where PVDF was used for the Western blots and nitrocellulose was used for the Far Western blots. The OMP samples were prepared differently in the two experiments. Both of these factors may have affected the binding of ligand to the probe.

The SILAC is preliminary and further information could be gained. The lack of SILAC results by Protein Pilot and MASCOT software programs may have been due to
deficiencies in the program parameters. This became evident when the same data were analyzed both programs. However, SILAC data analyzed by MASCOT confirmed the presence of a homologue of a hemoglobin binding protein, which was also identified with the hemoglobin probe. Another protein identified from the 100 kDa protein of serovar 6 was a homologue of the 183 kDa IgA protease of *H. influenzae*. Homologues of the ABC transporter proteins (72 kDa) and a TonB-dependent receptor (90 kDa) of *H. influenzae* were also identified in *H. parasuis*. Previously, HxuC (80 kDa) was shown to be theTonB-dependent receptor in *H. influenzae* (9, 10). Additionally, HgpA (120 kDa) has been described as a TonB-dependent receptor in *H. parasuis* (25). Once the *H. parasuis* annotated genome sequence is deposited in the database, identification of its proteins should be easier.

The presence of a hemoglobin binding protein is another mechanism for acquisition of iron through removal of heme as has been described for *H. influenzae* which utilizes both a HxuCBA-like iron-specific transport system (17) or binding to OMP receptors homologous to *H. ducreyi*’s hemoglobin binding protein (HgbA) (1, 25). It appears that HxuC, HgbA, or HgpA homologues may be involved in the transport of hemoglobin in *H. parasuis* (1, 9, 25). Patterson et al. (41) have developed an immunochromatography diagnostic test, based on the ability of anti-HgbA monoclonal antibodies to bind to HgbA. This test has already been used to ascertain if animals have been infected with *H. ducreyi* and might be adaptable to an *H. parasuis* assay.

Other outer membrane proteins that could also bind hemoglobin (Hgb) or hemoglobin-haptoglobin are the phase-variable HgbA, HgbB, and HgbC TonB-dependent receptors. Under iron-limiting conditions, *H. influenzae* has expressed redundant mechanisms to obtain heme and iron from host sources (53). These mechanisms may also be present in *H. parasuis*.

Bacterins or autogenous vaccines are used mainly for certain serovars that are known to be causing Glässer’s disease (46). It remains to be seen if any of the iron-
regulated proteins of this study will be efficacious if they are used in subunit vaccines.
Recombinant iron-regulated proteins in a subunit vaccine might induce cross-protection in swine.

The results of the 2-D electrophoresis experiment showed that there were differences in the types of antigenic proteins in serovar 5 and 6. However, identification of the seven antigens unique to serovar 5 or the ten antigens present only in serovar 6 probably depended on the serum antibodies present in the probes. There may have been some convalescent antibodies in the “negative” pools of antisera since the animals were all from field cases and were not from caesarean-derived colostrum-deprived (CDCD) sources. Nevertheless, these differences could be explored for use in a diagnostic test in order to distinguish virulent from avirulent isolates.

Until this study, no high molecular weight adhesins or immunoglobulin binding proteins had been reported in *H. parasuis*. Both variable molecular weight adhesins and high molecular weight immunoglobulin binding proteins were found in this work. An IgA protease and several adhesins of *H. parasuis* were also identified in this study. These proteins have been characterized as virulence proteins in *H. influenzae* because they are involved in adherence to host cells and cleavage of IgA antibody, respectively (21).

Homologues of HgbA and HxuA as well as TbpA and TbpB were putatively demonstrated to be present by Far Western analysis and confirmed by mass spectrometry. Although a tentative homologue of TbpB in serovar 6 was found at approximately 60 kDa, the probes failed to detect TbpB in serovar 5 by 2-D blots or by Far Western analysis. MASCOT analysis of the SILAC data did confirm the presence of *H. ducreyi* homologues of HgbA in both serovars 5 and 6.

The OMPs of *H. parasuis* have not been fully characterized to date. Morton and Williams (36) stated that *H. parasuis* could only sequester porcine transferrin. However, they did not test for hemoglobin-binding proteins in *H. parasuis*. Additionally, Vogel et
al. (50) described lactoferrin receptor activity in *H. influenzae* after growth in liquid medium. This study provided more information about the hemoglobin binding proteins and confirmed the molecular weights of *H. parasuis* previously described transferrin binding proteins. It also presented evidence for the presence of at least 15 virulence factors in *H. parasuis* (Table 4.1). These virulence factors were iron-regulated and most were immunogenic as determined by Western blotting using convalescent sera (Table 4.2). Many of them have been described as surface-exposed OMP in *H. influenzae*, *A. pleuropneumoniae*, and *M. haemolytica* (4, 9, 25, 35, 45).

Some of the proteins in both serovars 5 and 6 of the iron-regulated proteins described here have the potential to be used in a subunit vaccine for *H. parasuis*. These include the Hia adhesin, transferrin binding proteins A and B, the HxuCBA proteins, and the OMP1 and OMP2 proteins. However, more serovars should be screened to determine if they possess the antigen of interest or not. At the least, *H. parasuis*-specific recombinant proteins could be used in diagnostic tests, such as enzyme linked immunosorbant assays (ELISA), to ascertain if animals have been infected with *H. parasuis*. 
Table 4.1. *H. parasuis* homologues of *Haemophilus* strains identified by 2-D electrophoresis, mass spectrometry, and Western blotting

<table>
<thead>
<tr>
<th>Label</th>
<th>Homologue</th>
<th>Accession #</th>
<th>Species</th>
<th>MOWSE</th>
<th>% Fit Masses Matched</th>
<th>% Coverage AA’s of peptide</th>
<th>Actual MW(kDa)/pI</th>
<th>Experimental MW(kDa)/pI</th>
<th>Iron Regulation</th>
</tr>
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<tbody>
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<td>A</td>
<td>High molecular weight Ig binding protein</td>
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<td>1.265 e+12</td>
<td>78/1236 (6)</td>
<td>18</td>
<td>450/5.5</td>
<td>335/5.8</td>
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</tr>
<tr>
<td>AA</td>
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<td>1.429 e+04</td>
<td>23/726 (3)</td>
<td>15</td>
<td>99.0/6.4</td>
<td>105/6.2</td>
<td>Upreg^5,6</td>
</tr>
<tr>
<td>B1</td>
<td>Adhesin (Hia)</td>
<td>AAL79954</td>
<td><em>H. influenzae</em> NT Str. 3179B</td>
<td>1.253 e+05</td>
<td>567/1443 (39)</td>
<td>100</td>
<td>125/6.2</td>
<td>150/6.4</td>
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</tr>
<tr>
<td>B2</td>
<td>Adhesin (Hia)</td>
<td>AAL79950</td>
<td><em>H. influenzae</em> NT Str.3A</td>
<td>0.431</td>
<td>27/711 (4)</td>
<td>17</td>
<td>114.2/6.1</td>
<td>115/6.3</td>
<td>Upreg^5,6</td>
</tr>
<tr>
<td>C</td>
<td>Transferrin binding protein 1</td>
<td>NP_439373</td>
<td><em>H. influenzae</em> Rd KW20</td>
<td>5.296 e+05</td>
<td>33/1045 (3)</td>
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<td>102.8/9.2</td>
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<td>D</td>
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<td>18/934 (2)</td>
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<td>12/734 (2)</td>
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<td>Signal recognition particle protein</td>
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<td><em>H. influenzae</em> Str. H1 689</td>
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<td>I</td>
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<td><em>H. influenzae</em> Rd KW20</td>
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<td>364/934 (38)</td>
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<td>51.2/6.7</td>
<td>52/6.3</td>
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<td><em>H. influenzae</em> Str. HK284</td>
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<td>18/7705 (26)</td>
<td>99</td>
<td>52.6/6.0</td>
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<td>K</td>
<td>Putative pilus adhesin (Hif E)</td>
<td>AAC12711</td>
<td><em>H. influenzae</em> Str. GA2078</td>
<td>3.427 e+04</td>
<td>15/705 (2)</td>
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<td>4/275 (1)</td>
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<td>Experimental MW(kDa)/pI</td>
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<td>28/5.7</td>
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<td>185.0/6.0</td>
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<td><em>H. influenzae</em> Str. E1a</td>
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<td>80/9.0</td>
<td>Upreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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<td>Downreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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<td>64/5.3</td>
<td>Downreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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<td>S</td>
<td>Phage phi-R73 primase-like protein</td>
<td>AAF27348</td>
<td><em>H. influenzae</em> Str. Eagan</td>
<td>7.365 e+06</td>
<td>42</td>
<td>67.0/6.6</td>
<td>67/7.0</td>
<td>Downreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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<td><em>H. influenzae</em> Rd KW20</td>
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<td>42</td>
<td>43.4/5.3</td>
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<td>Downreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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<td>U</td>
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<td><em>H. influenzae</em> Str. 7416</td>
<td>19.7</td>
<td>13</td>
<td>49.4/8.3</td>
<td>45/8.3</td>
<td>Downreg&lt;sup&gt;5&lt;/sup&gt;</td>
<td></td>
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<td>V</td>
<td>Major OMP P2 variant</td>
<td>AAC06316</td>
<td><em>H. influenzae</em> Str. 32-82e</td>
<td>1.153 e+05</td>
<td>60</td>
<td>39.8/7.9</td>
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<td>Equal&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>X</td>
<td>Pyridoxine biosynthesis protein</td>
<td>NP_439789</td>
<td><em>H. influenzae</em> Rd KW20</td>
<td>45</td>
<td>23</td>
<td>31.7/5.7</td>
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<td>Upreg&lt;sup&gt;6&lt;/sup&gt;</td>
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<td>Y</td>
<td>Serum resistance protein DsrA</td>
<td>AAF37812</td>
<td><em>H. ducreyi</em></td>
<td>1259</td>
<td>47</td>
<td>29.2/9.4</td>
<td>30/9.3</td>
<td>Equal&lt;sup&gt;6&lt;/sup&gt;</td>
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<tr>
<td>Z</td>
<td>50S Ribosomal protein L9</td>
<td>NP_438702</td>
<td><em>H. influenzae</em> Rd KW20</td>
<td>67.4</td>
<td>38</td>
<td>15.6/6.4</td>
<td>15/6.2</td>
<td>Equal&lt;sup&gt;6, 6&lt;/sup&gt;</td>
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<tr>
<td>BB</td>
<td>Iron repressor protein, Fur</td>
<td>AAB71196</td>
<td><em>H. influenzae</em> Str. F3031</td>
<td>6.86</td>
<td>10</td>
<td>17/5.8</td>
<td>20/5.9</td>
<td>Downreg&lt;sup&gt;5, 6&lt;/sup&gt;</td>
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</tbody>
</table>

<sup>5</sup> *H. parasuis* serovar 5; <sup>6</sup> *H. parasuis* serovar 6

<sup>a</sup> Ureg, iron-restricted protein expression was 1.5 times higher by PD Quest quantitation than iron-replete expression; Downreg, iron-restricted protein expression was 1.5 times lower than iron-replete expression; Equal, no difference in protein expression
Table 4.2. Immunoreactive Proteins

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<tr>
<th>Label</th>
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<th>Label</th>
<th>Serovar 5 (Virulent)</th>
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<tr>
<td>*AA</td>
<td>Heme hemopexin utilization protein A</td>
<td>*AA</td>
<td>Heme hemopexin utilization protein A</td>
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<td>*B2</td>
<td>Adhesin (Hia)</td>
<td>B1/*B2</td>
<td>Adhesin (Hia)</td>
</tr>
<tr>
<td>*C</td>
<td>Transferrin binding protein 1</td>
<td>*C</td>
<td>Transferrin binding protein 1</td>
</tr>
<tr>
<td>*D</td>
<td>Topoisomerase IV</td>
<td>*D</td>
<td>Topoisomerase IV</td>
</tr>
<tr>
<td>E</td>
<td>Transferrin binding protein 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>ABC transporter ATP-binding protein**</td>
<td>H</td>
<td>Heme hemopexin utilization protein B**</td>
</tr>
<tr>
<td>G</td>
<td>Glucose 6 phosphate isomerase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*I</td>
<td>Signal recognition particle</td>
<td>*I</td>
<td>Signal recognition particle</td>
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<td>*J1/*J2</td>
<td>IgA1 protease</td>
<td>*J1/*J2</td>
<td>IgA1 protease</td>
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<td>*K</td>
<td>putative pilus adhesion (HifE)</td>
<td>*K</td>
<td>Putative pilus adhesion (HifE)</td>
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<td>L</td>
<td>Ferrochetalase</td>
<td>M</td>
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<td>*N</td>
<td>IgA1 protease</td>
<td>*N</td>
<td>IgA1 protease</td>
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<td>O</td>
<td>Heme hemopexin utilization protein C**</td>
<td>R</td>
<td>Heat shock protein 70</td>
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<td>P</td>
<td>Uncharacterized protein HI 1042</td>
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<td>Q</td>
<td>Bcs4**</td>
<td>U</td>
<td>OMP P1</td>
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<tr>
<td>S</td>
<td>Phage phi-R73 primase like protein</td>
<td>V</td>
<td>Major OMP2 variant</td>
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<td></td>
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<td>Pyridoxine biosynthesis protein</td>
<td></td>
<td></td>
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<tr>
<td>Y</td>
<td>Serum resistance protein DsrA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*Z</td>
<td>50S ribosomal protein L9</td>
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* proteins common to both serovars
** proteins with weak response to “negative sera”
<table>
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<tr>
<th>Label</th>
<th>Homologue</th>
<th>Accession #</th>
<th>Species</th>
<th>MOWSE Matched</th>
<th>% Coverage AA’s of peptide</th>
<th>Actual MW(kDa)/pI</th>
<th>Experimental MW(kDa)</th>
<th>Iron Regulation</th>
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<td>Hemoglobin binding protein A</td>
<td>AAU12584</td>
<td><em>H. ducreyi</em> Str. SSMC71</td>
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<td>Transferrin binding protein A</td>
<td>AAQ02787</td>
<td><em>H. parasuis</em> Str. H410</td>
<td>0.630</td>
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<td>Adhesin (Hia)</td>
<td>AAL79951</td>
<td><em>H. influenzae</em> NT Str. 3248A</td>
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<td>NP_439373</td>
<td><em>H. influenzae</em> Rd KW20</td>
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<td>98.8/7.2</td>
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<td>3, 10</td>
<td>Lactoferrin binding protein B</td>
<td>AAC31369</td>
<td><em>Moraxella catarrhalis</em> 4223</td>
<td>1.14</td>
<td>5/1434</td>
<td>27.2</td>
<td>85.0/9.5</td>
<td>88</td>
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<td>4</td>
<td>Heme hemopexin utilization protein A</td>
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<td><em>H. influenzae</em> Rd KW20</td>
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<td>NP_438494</td>
<td><em>H. influenzae</em> Rd KW20</td>
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<td>Opacity associated protein Adhesin (hmw2A)</td>
<td>CAI77660</td>
<td><em>H. influenzae</em> Str. H188</td>
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<td>5/2400</td>
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<tr>
<td>*6</td>
<td>OMP Pom A</td>
<td>AAD53408</td>
<td><em>M. haemolytica</em> Str. 89010807N</td>
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<td>ABC transporter ATP binding protein B</td>
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<td>AAQ10745</td>
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<td><em>H. influenzae</em> Str. 1613B</td>
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<td><em>H. influenzae</em> Str. 74P4HI</td>
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<td>4/2400</td>
<td>40.8</td>
<td>38.7/9.3</td>
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5 *H. parasuis* serovar 5; 6 *H. parasuis* serovar 6; *tandem mass spectrometry results
Figure 4.1. Characterization of whole cell lysate proteins of *H. parasuis* avirulent reference strain 6 by 2-D electrophoresis and Western blotting. Panels A-C contain control proteins obtained from *H. parasuis* grown under iron-replete conditions; Panels D-F contain proteins obtained from *H. parasuis* grown under iron-restricted conditions. Panels A and D: 2-D gels stained with Sypro Ruby; Panels B and E, Western blots probed with negative sera; Panels C and F Western blots probed with positive sera. Blots were incubated with rabbit anti-pig horse radish peroxidase-conjugated to IgG and developed with hydrogen peroxide and 4-chloronaphthol. Proteins upregulated 1.5 fold or more by PD Quest quantitation are circled in Panel D. Molecular weights (M) are indicated in kilodaltons. Proteins of interest were picked from gels as marked, digested with trypsin, and analyzed by MALDI-TOF and MS-Fit ([www.ucsf.edu/prospector](http://www.ucsf.edu/prospector)).
Figure 4.2. Characterization of whole cell lysate proteins of *H. parasuis* virulent reference strain 5 by 2-D electrophoresis and by Western blotting. Panels A-C contain control proteins obtained from *H. parasuis* grown under iron-replete conditions; Panels D-F contain proteins obtained from *H. parasuis* grown under iron-restricted conditions. Panels A and D: 2-D gels stained with Coomassie Blue R250; Panels B and E, Western blots probed with negative sera; Panels C and F Western blots probed with positive sera. Blots were incubated with rabbit anti-pig horse radish peroxidase-conjugated to IgG and developed with hydrogen peroxide and 4-chloronaphthol. Proteins upregulated 1.5 fold or more by PD Quest quantitation are circled in Panel D. Molecular weights (M) are indicated in kilodaltons. Proteins of interest were picked from gels as marked, digested with trypsin, and analyzed by MALDI-TOF and MS-Fit ([www.ucsf.edu/prospector](http://www.ucsf.edu/prospector)).
Figurer 4.3. Characterization of outer membrane proteins of *H. parasuis* avirulent serovar 6 and virulent serovar 5 by SDS-PAGE and by Far Western blotting. (A) 4-12% SDS-PAGE gel; lanes 1 and 2 illustrate serovar 6 outer membrane proteins isolated from cells grown in the absence or presence of DP, respectively; lanes 3 and 4 correspond to serovar 5 outer membranes isolated cells grown in the absence or presence of DP, respectively; (B) Far Western blot probed with biotinylated porcine hemoglobin probe, samples in lanes 1-4 are identical to those of panel A; (C) Far Western blot probed with biotinylated porcine transferrin, samples in lanes 1-4 are identical to those of panel A; (D) control Far Western blot blocked as described in the text and reacted only with avidin-HRP conjugate, samples in lanes 1-4 are identical to those in panel A. Molecular asterisk weights (M) are indicated in kilodaltons. Black arrows and dashes indicate upregulated proteins; black asterisk indicates downregulated protein. Numbers correspond to proteins identified in Table 4.3.
Figure 4.4. Identification of proteins by SILAC (stable isotope labeling with amino acids in cell culture. (Lane 1) a 1:1 ratio of *H. parasuis* serovar 5 cells grown in media containing light arginine and lysine with DP and cells grown in media containing heavy arginine and lysine without DP or (lane 2) a 1:1 ratio of *H. parasuis* serovar 6 cells grown in media containing heavy arginine and lysine without DP and cells grown in media containing light arginine and lysine with DP. Samples were resolved on a 4-12% SDS-PAGE gel and stained with Coomassie Blue R250. Bands at approximately 60, 80, and 100 kDa (arrows) were excised and submitted for tandem mass spectrometry analysis. Molecular weights (M) are indicated in kilodaltons.
References


CHAPTER 5. GENERAL CONCLUSIONS

Summary. *H. parasuis* reference strains and field isolates were compared by RAPD profiles and SDS-PAGE protein profiles (Chapter 2). Two methods of similarity analysis, the Dice algorithm/neighbor-joining dendogram and the maximum parsimony heuristic analysis/consensus tree were used. It was concluded that the genome-based method showed more heterogeneity among the isolates than the protein-based method of analysis. However, there was better discrimination when three primers were combined in the RAPD method rather than when the individual primers were employed. This finding is in agreement with other reports (1). Other genome-based analyses of *H. parasuis* include repetitive element-based PCR (20), restriction endonuclease fingerprinting (REF) analysis (22, 23), enterobacterial repetitive intergenic consensus-based PCR (ERIC-PCR) (14, 18), PCR-random fragment length polymorphism (PCR-RFLP) (5), multilocus enzyme electrophoresis (MLEE) (3, 17), and the analysis of 16S rRNA (1, 15).

The SDS-PAGE protein profiles showed that the passage number *ex vivo* of the isolate can have an effect on its protein expression. The older isolates had more homogenous banding patterns than the more recent isolates did. Both of these analyses grouped virulent strains isolated from systemic sites together. One of the first analysis of whole cell lysates of *H. parasuis* were done using SDS-PAGE by Nicolet et al (13). Others have also analyzed *H. parasuis* outer membrane proteins by SDS-PAGE (16, 20).

Supposedly, some of these methods can be used to follow epidemiological trends to be used in the detection of *H. parasuis* outbreaks so that appropriate measures could be taken in a timely manner. However, some of the techniques are too time-consuming for epidemiological studies. It would be advantageous to have a simple, inexpensive diagnostic reagent for use in the field. Similarly, a subunit vaccine made up of cross-protective components would be beneficial.
The study described in Chapter 2 also reported the identification of a major 50 kDa band in SDS-PAGE gels of whole cell lysates of selected field strains. This protein was identified as phage-related by mass spectrometry. This led to the isolation and characterization of a Mu-like bacteriophage (Chapter 3). It was very difficult to work with this bacteriophage because of its low titer in lysates and its tendency to deteriorate during storage. These characteristics are in agreement with another Mu-like phage (BcepMu phage 56) as reported by Summer et al. (24).

The partial DNA sequence of SuMu has been deposited in Genebank (Accession No. EU268809). It seems that many of the SuMu genes sequenced are colinear with Mu genes and many of the mass spectrometry-identified proteins are homologues of Mu proteins. Additionally, 23 homologues of proteins of other bacteriophage were identified. Interestingly, one homologue of an IgA-specific zinc metalloprotease was identified in both a 1-D gel and a 2-D gel of phage proteins. This may be a potential phage-encoded virulence factor (8). However, it could not be ruled out that this protein was as a bacterial membrane-associated protein that accompanied the phage during purification procedures. More studies need to be done to determine whether this phage contributes to the heterogeneity of *H. parasuis* isolates.

The comparison of iron-regulated proteins from virulent (serovar 5) and avirulent (serovar 6) reference strains in *H. parasuis* was described in Chapter 4. The bacteria were grown under iron-restricted (stressed) or iron-replete (nonstressed) conditions. To evaluate protein expression, the outer membrane proteins were separated by 2-D electrophoresis, transferred to a membrane, then probed with either pooled negative or convalescent sera to assess their immunoreactivity. There were a few differences in immunogenicity of the proteins between the two strains. Lipoprotein, heme hemopexin utilization protein B, heat shock protein 70, Elongation factor Tu, OMP P1, major OMP P2 variant were immunoreactive in serovar 5 but not in serovar 6. Transferrin binding protein B, phage phi-R73 primase-like protein, ABC transporter ATP-binding protein,
glucose 6 phosphate isomerase, ferrochetalase, heme hemopexin utilization protein C, uncharacterized protein HI 1042, hypothetical protein 4 (Bcs4 capsulation locus), serum resistance protein DsrA, and pyridoxine biosynthesis protein were immunoreactive only in serovar 6. Combinations of one or more of the corresponding serovar’s unique proteins might be useful as part of a subunit vaccine.

In this study, proteins identified in *H. parasuis* that were homologues of nontypeable *H. influenzae* proteins involved in pathogenesis were OMP P1, OMP P2 variant, Tbp 1, Tbp 2, adhesins, pilus adhesion (HifE), IgA1 protease, and heat shock protein 70 (8). Many of them have been described as surface-exposed and related to OMPs of *H. influenzae, A. pleuropneumoniae,* and *M. haemolytica* (2, 4, 9, 12, 19).

In agreement with the results of Melnikow et al. (10), this study found proteins expressed from genes for transporters of iron and sugar metabolites, metabolic enzymes, and DNA synthesis as well as bacteriophage genes. In addition, Metcalf and MacInnes (11) reported several enzymes as well as proteins involved in protein biosynthesis and protein transport which were also found in this study.

Far Western blotting and SILAC were performed in this study in order to confirm the presence of selected iron-regulated proteins in serovars 5 and 6 (Chapter 4). Far Western blotting putatively detected TbpA and TbpB as well as HgbA, HxuA, and HxuB. HxuC was shown to be upregulated under iron-restriction on 1-D gels in both serovars, but this protein was not detected when a blot was probed with biotinylated hemoglobin. HxuC was shown to be upregulated and immunoreactive in both serovars 5 and 6 by 2-D electrophoresis and Western blotting. Interestingly, TbpB was not detected by the biotinylated transferrin probe in serovar 5, and TbpB also was not immunoreactive in the Western blot of the 2-D gel. Others have reported heterogeneity in the PCR gene products of *tbpB* in *H. parasuis* serovars, but did not report the absence of the protein (6). Failure to detect TbpB may be related to the unavailability of the appropriate epitopes to the probes.
SILAC is a relatively new methodology. Most of the methods have been optimized for use in mammalian cell cultures and not for bacterial cells. The lysis procedure yields excessive DNA, which interferes with the subsequent analysis, especially the SDS-PAGE separation of proteins. The Protein Pilot analysis software for the method is still being developed by Applied Biosystems. MASCOT recently added a feature that accommodates the SILAC technology so that data analyzed by this program could be used. However, the Proteomics Facility at Iowa State University does not have Mascot Distiller, which should be used to process the raw mass spectrometry files before they are submitted to MASCOT searches. Another problem encountered with protein identification was that the complete *H. parasuis* genome is not yet in the database.

Despite these problems, several homologues of iron-regulated proteins were identified by at least two methods. These proteins are the ABC transporter-related proteins, the hemoglobin binding protein (HgbA), IgA protease, and HxuCBA receptors.

**General conclusions.** The following conclusions can be made after considering the experimental results of this dissertation: 1) RAPD analysis can separate virulent systemic isolates of *H. parasuis* into clusters by using the Dice algorithm/neighbor joining analysis; 2) SDS-PAGE analysis of whole cell lysates of *H. parasuis* clustered by passage number *ex vivo* of isolates; 3) a Mu-like bacteriophage of *H. parasuis* was isolated and characterized by electron microscopy, mass spectrometry, and 1-D and 2-D electrophoresis; 4) fifteen virulence-associated proteins of *H. parasuis* that were homologues to *H. influenzae* proteins were identified by 2-D electrophoresis and Western blotting; 5) *H. parasuis* homologues to TbpA and TbpB of *H. influenzae* were identified by Far Western blotting after probing with biotinylated transferrin; 6) *H. parasuis* homologues of HgbA, HxuA, and HxB of *H. influenzae* were identified by Far Western blotting after probing with biotinylated hemoglobin; 7) *H. parasuis* homologues to HgbA, TbpAB and HuxCBA of *H. influenzae* were identified by 1-D electrophoresis of up-regulated OMPs of serovar 5 and 6; and 8) SILAC experiments gave inconclusive
results and the mass spectra should be reanalyzed when the Matrix distiller processing software is available.

**Recommendations for future research.** There are a number of areas that could be investigated in the future. It would be beneficial to find a specific gene sequence for each serotype and then use primers to those genes as part of a PCR reaction in combination with primers of virulence genes. Possibly this novel RAPD analysis could classify the *H. parasuis* nontypeable isolates as well as the reference strains and be able to be used in epidemiological studies.

The *in vitro* virulence-related genes of a pathogen may not be the same as the *in vivo* virulence-related genes. An alternative technique to proteomics is selective capture of transcribed sequences (SCOTS) (7). It can be used to selectively capture bacterial cDNA derived from infected tissues by hybridization to biotinylated bacterial genomic DNA. This could be used to identify pathogen-specific genes expressed *in vivo*.

One of the major concerns for the swine industry is to develop a vaccine that protects against challenge by *H. parasuis*. Possible immunogens in a *H. parasuis* vaccine include TbpB, HxuC, and Hgb receptors. All are surface-exposed and involved in iron scavenging by the bacteria. Possibly, the TbpB vaccines can be made up of TbpB proteins from several different isolates in order to avoid the issue of TbpB heterogeneity among isolates. Both the HxuC and Hgb receptors were identified as virulence determinants for *H. influenzae* because mutations which disrupt either iron acquisition pathway decrease the ability of typeable and nontypeable strains to colonize or persist in the host (21).

After the *H. parasuis* genome is deposited in a database, possible virulence factors could be identified and tested *in vitro*. Deletion mutants of possible virulence factors could then be tested *in vivo* for deficiencies in colonization, spread, and persistence in infection compared to the wild type. The deletion mutants might be efficacious as vaccines, since they could confer immunity without causing infection.
As stated previously, some of the putative virulence proteins found in *H. parasuis* in this study could be utilized in subunit vaccines that might provide protection against infection. Diagnostic tests based on selected identified proteins can be developed and evaluated.

A specific antibody or DNA probe could be developed that might detect *H. parasuis* containing phage. Known proteins that are phage-encoded could be used to design a probe for phage detection in *H. parasuis* isolates. For example, the genes or specific proteins of the protease or tape measure proteins could be utilized because they were both found in SuMu. Then *H. parasuis* DNA could be probed on Southern blots by using phage DNA sequences or proteins could be probed for on dot blots of intact bacteria or Western blots of whole cell lysates by using antibodies to phage proteins.

In the future, bacteriophage may be used as vaccines in a phage display construct, which delivers DNA encoding heterologous peptides into the phage genome through transcriptional fusion to phage coat protein genes. The peptides are then displayed on the phage capsid. Another type of phage vaccine uses the entire phage as a delivery vehicle with up to 23 kb of DNA packaged inside the phage. In the future, hybrid phages that incorporate both phage display and DNA phage should be used as vaccines. Another benefit of these vaccines is that they could be inoculated orally.

**References**


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