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Initial Proteomics Analysis of Differentially Expressed Proteins from *Mycoplasma gallisepticum* Vaccine Strains ts-11 and F Detected by Western Blotting

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Abstract: *Mycoplasma gallisepticum* (MG) is the causative agent of chronic respiratory disease in layer chickens. The live MG vaccine strains that are available for use in layer chickens include F, ts-11 and 6/85. The MG vaccine strains ts-11 and 6/85 are safer than F and they have little or no potential of spreading from bird to bird. However, ts-11 and 6/85 appear to be less efficacious than F-strain. Results from studies suggest that the use of MG vaccine strain F in replacement flocks over a period of time results in the displacement of the original field strain. Also, reports of MG breaks in layer flocks previously vaccinated with ts-11 or 6/85 have resulted in revaccination of these flocks with F. The continued use of F-strain in displacement and revaccination regimens necessitates the development of more rapid and sensitive field tests that will differentiate between wild-type and vaccine strains of MG. In the present study, ts-11 and F-strain whole cell extracts were analyzed by Western blotting and proteomic methodologies. Differentially expressed protein bands were excised, in-gel digested with trypsin, and analyzed by mass spectrometry. The proteins were identified as internal proteins and were predicted to be involved in such cellular processes as carbohydrate transport and metabolism, energy production and conversion, posttranslational modification, protein turnover, chaperone activity, transcription, and translation. The results of this study suggest that proteomics may aid in the characterization of proteins that could contribute to the development and improvement of current MG diagnostic tests.

Key words: Western blotting, *Mycoplasma gallisepticum*, ts-11, F-strain, mass spectrometry, proteomics

Introduction

Mycoplasma gallisepticum (MG) results in more economic losses in chickens via morbidity and mortality than other poultry mycoplasmas (May *et al*., 1994). The ensuing chronic respiratory infection reduces egg production and contributes to condemnation of meat at poultry processing plants (Branton *et al*., 1988; Whithear, 1996). This chronic respiratory infection is characterized by exfoliation of ciliated epithelial cells, accumulation of inflammatory exudates in the trachea, and a severe inflammatory response in the pulmonary air-sacs (Yoder, 1991).

While antibiotics have proven ineffective at clearing MG infections (Ley *et al*., 1997), current control practices against MG infection include intense biosecurity and testing by the serological monitoring of flocks, MG isolation techniques, and/or DNA-based detection methods (Ley *et al*., 1997; Liu *et al*., 2001). Within the broiler and turkey industries, regulatory measures have been largely successful at minimizing MG outbreaks that occur only in a sporadic nature. Success within these sectors of the poultry industry is primarily due to their "all in-all out" nature that allow for complete eradication of infected flocks. However, within the table egg sector eradication of infected flocks is not feasible due to the extreme size (>1 million birds) and multi-age nature of the complexes. Once egg laying facilities become infected with MG, they are considered infected for life and may serve as the point source of contamination, thereby endangering other poultry facilities of close proximity.

Because of the unique structure of the layer industry, an alternative approach has been developed to control MG infections by the use of live attenuated MG vaccines (Whithear, 1996). There are currently three available live MG vaccines approved for use in the United States. Theses include FVAX-MG® (Schering-Plough Animal Health, Omaha, NE), which is commonly referred to as F-strain MG; MYCOVAC-L® (Intervet Inc., Millsboro, DE), which is referred to as 6/85 strain MG; and ts-11 MG vaccine® (Select Laboratories, Gainesville, GA), which is referred to as ts-11 strain MG. The F-strain was the first developed (Gingerich, 2002) and today is the most commonly used vaccine strain in the United States (Abd-el-Motelib and Kleven, 1993). However, as a result of the

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pathology associated with F-strain towards turkeys and young chickens, restrictions have been placed on the use of F-strain in certain areas (Levisohn and Kleven, 1981). Subsequently, because these restrictions limit the applicability of F-strain towards the control of MG, ts-11 and 6/85 vaccine strains were developed.

MG vaccine strains ts-11 and 6/85 are non-pathogenic towards turkeys and young chickens and they have little or no potential of spreading from bird to bird (Ley *et al.*, 1997). Therefore, their use has been promoted in the current control of MG infections. Nevertheless, ts-11 and 6/85 appear to be less efficacious than F-strain (Ley *et al.*, 1997). Results from previous studies suggest that the use of F-strain in replacement flocks over a period of time results in the displacement of the original field strain (Kleven *et al.*, 1990). More recently, Kleven *et al.* (1998) showed that in pen trail studies the F-strain displaced the virulent R-strain, but ts-11 and 6/85 strains did not. Also reports of MG breaks in layer flocks previously vaccinated with ts-11 or 6/85 have resulted in revaccination of these flocks with F-strain (Gingerich, 2002). The continuous use of F-strain to control field strain MG via displacement and revaccination regimens necessitates the development of a more rapid and sensitive field test that will differentiate between wild-type and vaccine strains of MG.

Recently, a model was established to determine the impact on egg production and selected egg quality parameters of administering F-strain vaccine to layer hens during lay after having been previously vaccinated as pullets with ts-11 at 10 wk of age (Vance *et al.,* in preparation). The model was also used to identify and characterize, via Western blotting and proteomics methodologies, ts-11 and F-strain proteins. These proteins may aid in current intervention strategies to control and diagnose MG infections in poultry.

Materials and Methods

Chickens: Two hundred day-old Hyline W36 chicks originating from flocks monitored for MG and *Mycoplasma synoviae* (MS) and certified free (National Poultry Improvement Plan and Auxiliary Provisions, 1995) were obtained from a commercial source and reared to 10 wk of age under conditions previously described (Branton *et al.*, 2002). Chicks were vaccinated at 10 d of age for infectious bursal disease and at 12 d and 4 wk of age for Newcastle disease and infectious bronchitis via drinking water. At 5 wk of age, pullets were randomly selected and bled from the wing vein and tested for antibodies to MG and MS via serum plate agglutination and hemagglutination inhibition tests (Yoder, 1975). At 10 wk of age, 176 randomly selected pullets were moved to a poultry disease isolation facility containing 16 negative pressure fiberglass biological isolation units (11 birds per unit). Environmental conditions for the experiment were as previously described (Branton *et al.*, 2002). The chicks were divided into 4 treatment groups consisting of control (1); ts-11 administered at 10 wk of age (2); ts-11 administered at 10 wk of age and F-strain administered at 22 wk of age (3); and ts-11 administered at 10 wk of age and F-strain administered at 45 wk of age (4). The ts-11 strain of MG was obtained from Select Laboratories, Gainesville, GA and was used according to the manufacturer's instructions. The F-strain was provided by Dr. S. H. Kleven, University of Georgia and was given by eye drop inoculation with 0.04 ml of a 24 h broth culture. Bird numbers were reduced to 10 birds/isolation unit when control birds achieved 10% hen day egg production (21 wk of age). Serum was collected at 24 and 47 wk of age from 3 birds per treatment. Because similar banding patterns were observed between treatment groups 3 and 4 (data not shown), reported in the present study are data from serum collected from birds administered ts-11 at 10 wk of age and F-strain at 45 wk of age, treatment group 4.

Mycoplasma whole cell lysates: The ts-11 and F-strain of MG were propagated in Frey's medium supplemented with 10% swine serum (Frey *et al.*, 1968) at 37°C in anaerobic chambers. Mycoplasma cells were collected from 24 h cultures by centrifugation, washed 2 times with phosphate buffered saline (PBS) and incubated in lysis buffer at 37°C for 30 min as described by Avakian and Kleven (1990). Whole cell extracts (300 µl per tube) were transferred to 1.5 ml microcentrifuge tubes and stored at -80°C. Protein concentration was determined using the microplate version of the bicinchoninic acid protein assay (Pierce, Rockford ILL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): Protein samples were mixed with sample buffer (1:1) containing 5% 2-mercaptoethanol and heated at 95°C for 5 min. Samples were then resolved on 10% SDS-PAGE gels at 100 volts for 2 h using the Laemmli buffer system (Laemmli, 1970).

Western blotting: Proteins were transferred (transfer buffer, 25 mM Tris, 192 mM glycine, 20% V/V methanol) to nitrocellulose membranes using an XCell II™ Blot Module (Invitrogen) at 25 volts for 2 h. Membranes were then blocked overnight at 4°C in Tris buffered saline (TBS) containing 4% BSA. Membranes were washed 4 times with TBS containing 0.05% Tween-20 and then cut into strips. Test sera were diluted 1:1000 in TBS containing 1% porcine serum and 1% BSA and then incubated at room temperature for 15 min (Avakian and Kleven, 1990). Diluted sera were then incubated with nitrocellulose membrane strips for 1 hour at room temperature. Nitrocellulose membrane strips were washed 4 times with TBS containing 0.05% Tween-20.

Anti-chicken IgG (whole molecule) alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) was diluted 1:10000 in TBS containing 1% porcine serum and 1% BSA and incubated with nitrocellulose membrane strips for 1 hour at room temperature. Strips were washed as described above and then developed with Sigma Fast NBT/BCIP (Sigma Chemical Co., St. Louis, MO) in water for 5 min.

In-gel trypsin disgestion: Stained protein bands were excised, cut into gel pieces and then placed in appropriately labeled 1.5 ml microcentrifuge tubes. Coomassie destaining solution [10% ammonium bicarbonate (0.1 M), 50% acetonitrile; at 100 µl] was added to each microcentrifuge tube and incubated at 37°C for 10 min. The solution was aspirated and discarded and the above procedure was repeated until the gel pieces were destained. The gel pieces were dehydrated via incubation with 50 µl of acetonitrile (ACN) at 37°C for 5 min. The solution was aspirated and the gel pieces were incubated for 10 min at 37°C. Proteins were reduced by incubating gel pieces at 37°C (30 min) with 50 µl of dithiothreitol (50 mM) containing 10% ammonium bicarbonate (1 M). The solution was aspirated and discarded. Proteins were alkylated by incubating gel pieces at 37°C (20 min) with 50 µl of iodoacetamide (0.1 M) containing 10% ammonium bicarbonate (1 M). Gel pieces were then incubated (37°C) with ACN (100 µl) for 5 min. The solution was aspirated and discarded. The gel pieces were dehydrated via incubation with 50 µl of ACN at 37°C for 5 min. The solution was aspirated, discarded and ACN dehydration was repeated. Microcentrifuge tubes were incubated at 37°C for 5 min, centrifuged, and residual ACN aspirated. Gel pieces were incubated at 37°C for 12 h with trypsin (25 µl). Gel pieces were then incubated at 37°C (30 min) with extraction solution (30 µl) containing 1% trifluoroacetic acid, and 2% ACN to extract tryptic peptides from the gel pieces. The solution was aspirated and transferred to a new tube. After extraction and dehydration solutions (12 µl/each) were added to each tube, each tube was incubated at 37°C (30 min), then the solutions were aspirated and transferred to the appropriate tube. The samples were subsequently desalted, vacuum dried, and resuspended in 0.1% formic acid prior to mass spectrometry analysis. The amino acid sequence of the tryptic peptides were determined by mass spectrometry analysis as described by Corzo *et al.* (2004). Briefly, tryptic peptides were fractionated on a 218MS C18 reverse-phase liquid chromatography column with a salt step gradient. Peptides were then eluted from the reverse-phase column connected in-line to an electrospray ion trap mass spectrometer. The turboSEQUEST™ software (ThermoElectron Corporation, San Jose, USA) was used to match the amino acid sequence data with translated sequences from the *Mycoplasma gallisepticum* strain R_{low} genome sequence (Papazisi *et al.*, 2003). Clusters of Orthologous Groups of proteins were used to evaluate the function of the identified proteins (Tatusov *et al.*, 2003).

Results and Discussion

Western blot analysis of serum samples collected from control birds did not show an antibody response against MG vaccines strains ts-11 or F-strain (data not shown). Chickens infected with ts-11 and F-strain produce antibodies which can be detected in serum (Kleven *et al.*, 1998; Noormohammadi *et al.*, 2002a; Noormohammadi *et al.*, 2002b). The model of mycoplasma vaccination was designed to evaluate changes in egg production parameters when applying F-strain to hens previously vaccinated with ts-11 (Vance *et al.*, in preparation). Serum samples from these animals were also evaluated for reactivity with the vaccine strains by Western blotting. Western blot results with antisera from animals immunized with both vaccine strains recognized proteins at the 26 kDa range (Band 1 in Fig. 1A) and the 18 kDa range (Band 2 in Fig. 1A) in ts-11, but absent in F-strain. This result suggested that the differentially expressed proteins could be used in a serological test to detect ts-11 specific antibodies. Differentially expressed protein bands 1 and 2 were then excised from SDS-PAGE gels (Fig. 1B and C) and in-gel digested with trypsin and characterized with mass spectrometry. The proteins identified by mass spectrometry analysis are presented in Table 1. Fructose-bisphosphate aldolase and ribosomal protein L3 were found in band 1 and MsrA peptide methionine sulfoxide was found in band 2 of both F-strain and ts-11 (Table 1). Rp1A ribosomal protein L1 and thymidine kinase were found in band 1 of F-strain, and AceF dihydrolipoamide acyltransferases was found in band 1 of ts-11 (Table 1). RpoE RNA polymerase delta subunit was found in band 2 of ts-11 (Table 1). In the present study, bands 1 and 2 were not detected by Western blotting in F-strain, however mass spectrometry analysis did reveal proteins in bands 1 and 2 that were shared between F-strain and ts-11. These results are not surprising considering that the F-strain and ts-11 are attenuated vaccine strains of MG, and some proteins might be expressed at levels that are not detectable by Western blotting.

There are a number of well known mycoplasma surface membrane proteins that have been characterized, such as GapA, CrmA, MGC2, VlhA, and PvpA (Boguslavsky *et al.*, 2000; Hnatow *et al.*, 1998; Liu *et al.*, 2002; Liu *et al.*, 2001; Mudahi-Orenstein *et al.*, 2003; Papazisi *et al.*, 2002; Papazisi *et al.*, 2000; Winner *et al.*, 2003). GapA, CrmA, and MGC2 proteins have been shown to play a major role in the colonization and pathogenesis of MG (Hnatow *et al.*, 1998; Mudahi-Orenstein *et al.*, 2003;

Table 1: Proteins identified from whole cell lysate, resolved on a 10% SDS-PAGE gel

	Protein	Accession number	Function
A. F-Strain			
Band 1			
	Fructose-bisphosphate aldolase	AAL91129	Carbohydrate transport and metabolism
	Hypothetical protein	NP_853110	
	Ribosomal protein L3	AAB95387	Translation
	Rp1A ribosomal protein L1	AAP57015	Translation
	Thymidine kinase	AAL91131	Nucleotide transport and metabolism
Band 2			
	Hypothetical protein	NP_853049	
	MsrA peptide methionine sulfoxide	AAP57051	Posttranslational modification, protein turnover, chaperones
B. ts-11			
Band 1			
	AceF dihydrolipoamide acyltransferases	AAP56833	Energy production and conversion
	Fructose-bisphosphate aldolase	AAL91129	Carbohydrate transport and metabolism
	Ribosomal protein L3	AAB95387	Translation
Band 2			
	MsrA peptide methionine sulfoxide	AAP57051	Posttranslational modification, protein turnover, chaperones
	RpoE RNA polymerase delta subunit	AAP57010	Transcription

Papazisi *et al.*, 2002; Papazisi *et al.*, 2000). VlhA and PvpA proteins undergo phase variation, which is thought to be one mechanism by which mycoplasma evades the host immune system and is able to persist for the life of the bird (Boguslavsky *et al.*, 2000; Liu *et al.*, 2002; Liu *et al.*, 2001; Winner *et al.*, 2003). In the present study, the proteins extracted from bands 1 and 2 were characterized as internal proteins. The proteins were predicted to be involved in such cellular processes as carbohydrate transport and metabolism, energy production and conversion, posttranslational modification, protein turnover, chaperone activity, and transcription and translation (Table 1) (Tatusov *et al.*, 2003).

There are various serologically based MG detection methods available for use in the United States (Feberwee *et al.*, 2005). In the past, maintaining MG free flocks was dependent on serologic tests which include serum plate agglutination and hemagglutination inhibition (Feberwee *et al.*, 2005). Recently, enzyme-linked immunosorbent assays have been developed for use in serologic monitoring of flocks (Feberwee *et al.*,

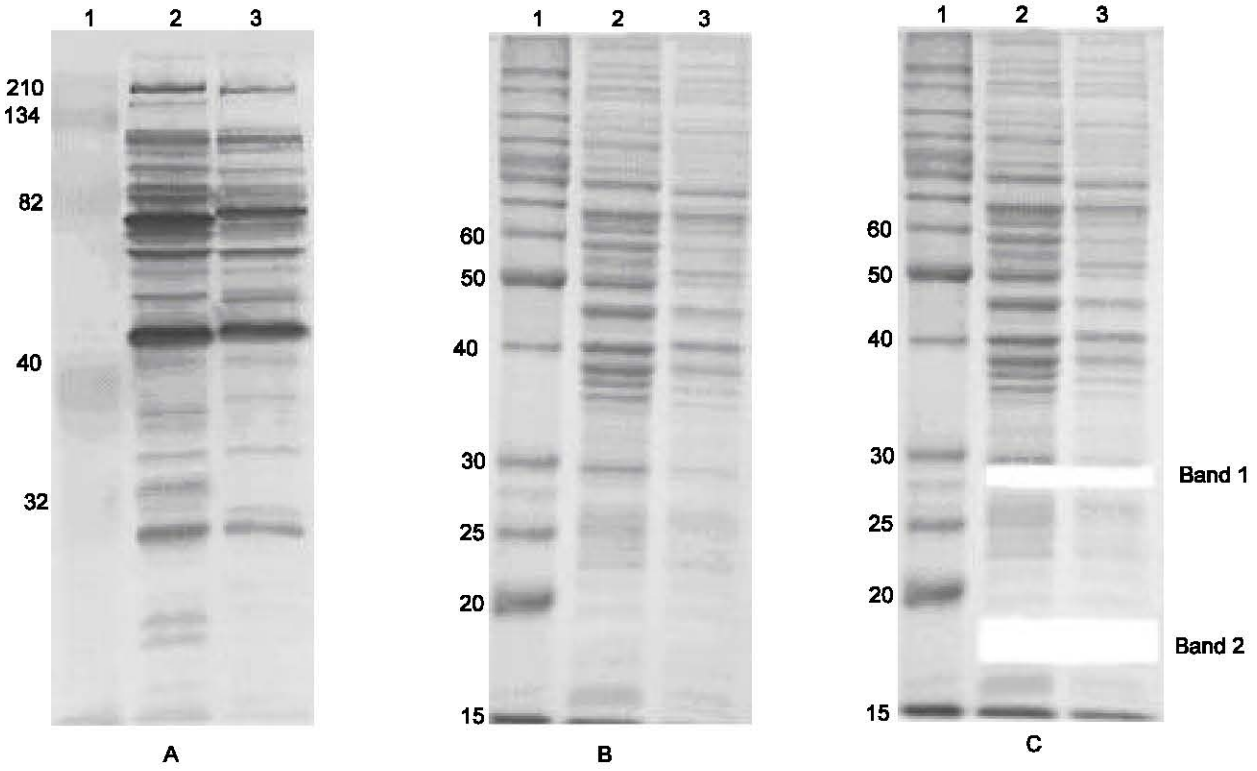

Fig. 1: Western blot analysis of serum samples taken from birds administered ts-11 and F-strain at 10 wk and 45 wk of age, respectively (A). SDS-PAGE profile of ts-11 and F-strain whole cell lysates (B) and (C). Rectangles indicate the excised bands.
Lane 1: Molecular weight standards in kDa; Lane 2: ts-11; Lane 3: F-strain.

2005). However, with the increased use of MG vaccines and the ability of mycoplasma to alter the expression of major surface membrane proteins, more sensitive and rapid DNA-based detection methods have been developed (Feberwee *et al.*, 2005; Ferguson *et al.*, 2005; Garcia *et al.*, 2005; Hong *et al.*, 2005; Mardassi *et al.*, 2005).

In conclusion, this study identified cytosolic proteins that are differentially expressed between ts-11 and F-strain MG. The development of antibody reagents specific for these proteins could be useful in differentiating the two vaccine strains in the laboratory with Western blotting. However, for monitoring large flocks it would be more desirable to identify immunodominant surface proteins for the development of rapid serologic-based tests. The complete genome of *Mycoplasma gallisepticum* strain R_{low} has been sequenced (Papazisi *et al.*, 2003), and this will facilitate further studies involving a more complete proteomic analysis of F-strain and ts-11. Future studies will be conducted to identify and characterize surface expressed proteins that can be used in the development of serologic-based tests for evaluating poultry flocks in the field.

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