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Proteomic and bioinformatic analyses of putative *Mannheimia haemolytica* secretome by liquid chromatography and tandem mass spectrometry

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ABSTRACT

Mannheimia haemolytica is a major bacterial contributor to bovine respiratory disease complex that costs the livestock industry a billion dollars a year in USA. Commercial vaccines are only partially efficacious under field conditions. Earlier studies found that outer membrane protein preparations and culture supernatants can induce immune responses that enhance resistance to challenge by *M. haemolytica* strains. The objective of this study was to characterize secretome of two *M. haemolytica* stains grown under two different media. Bacteria-free concentrated supernatants from *M. haemolytica* culture was subjected to LC–MS/MS. The secretome of *M. haemolytica* from both strains yielded 923 proteins. Using bioinformatic tools, 283 were identified as secreted proteins. Further breakdown of 283 proteins showed that 114 (40.2%), 184 (65.0%), 138(48.7%), 151 (53.3%) and 172 (60.7%) were characterized as secreted proteins by SignalP 4.1, SecretomeP 2.0, LipoP, Phobius, and PRED-TAT, respectively. A total of 95 (33.56%) proteins were characterized as being secreted via non-classical pathway as opposed to the majority that were secreted in signal peptide dependent pathway. The demonstrated proteins include all previously immunologically characterized *M. haemolytica* proteins. The potential of using secretome analysis in the design and development of a multivalent vaccine is discussed.

1. Introduction

Bovine respiratory disease (BRD) is the major cause of economic losses to the beef cattle industry (Miles, 2009). In feedlot cattle, *Mannheimia haemolytica* (formerly *Pasteurella haemolytica*) serotype 1 is most commonly associated with bovine pneumonia found in feedlot cattle known as Shipping Fever (Frank, 1986, 1989). Much effort has been expended toward development and improvement of vaccines for prevention of Shipping Fever (Confer, 1993). Current vaccines are largely ineffective or of limited efficacy under field conditions in a feedlot (Larson and Step, 2012; Perino and Hunsaker, 1997). The development of more efficacious vaccines will require a thorough understanding of virulence factors, the outer membrane proteins (OMPs), and secreted proteins of *M. haemolytica* as well as the host immune response to these factors. Such a vaccine will most likely contain antigenic components derived through recombinant DNA technology.

Shewen and Wilkie (1988) demonstrated that immunity against *M. haemolytica* requires leukotoxin (LKT) neutralizing antibodies as well as antibodies against bacterial cell surface antigens. Studies in our and others' laboratories demonstrated that high antibody responses to OMPs, ranging from 16 to 86 kDa, correlated with resistance to challenge with virulent *M. haemolytica* S1, and vaccination of cattle with OMP-enriched cellular fractions from *M. haemolytica* S1 enhanced resistance of cattle against experimental challenge (Ayalew et al., 2010, 2011a,b; Confer et al., 2003, 2006; Morton et al., 1995; Mosier et al., 1989; Orouji et al., 2012). In this regard, we used immunoproteomics, which uses a combination of 2D-gel electrophoresis (2-DE) and Western blotting with bovine convalescent sera, and identified 132 immunoreactive proteins in outer membrane preparations of *M. haemolytica* using LC–MS/MS. Of those, 55 are putative vaccine candidates (Ayalew et al., 2010).

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Demonstration of the importance of neutralizing antibodies to the 105 kDa LKT led to the development of a commercial culture supernatant-based vaccine, and preliminary examination of secreted *M. haemolytica* immunogens that are of potential importance (Gentry et al., 1985; Mosier et al., 1994; Shewen and Wilkie, 1988). Mosier et al. (1994) demonstrated that antibodies to five proteins in culture supernatant ranging from 16 to 100 kDa correlated with resistance to experimental challenge. In addition, a 32.5 kDa sialoglycoprotease was identified in *M. haemolytica* supernatants and antibodies to that protein correlated with resistance (Lee et al., 1994).

Because LKT is secreted by *M. haemolytica* into the culture supernatant as well as various bacterial enzymes and proteins of unknown origin, we undertook to examine the proteins within the culture supernatant (secretome) and determine which are potentially secreted proteins. Gram-negative bacteria have developed several pathways for secreting cellular components into their immediate environments for several purposes (Costa et al., 2015; Green and Mecsas, 2016; Lycklama and Driessen, 2012; Palmer and Berks, 2012). These highly sophisticated and dedicated protein secretion systems fall into at least eight characterized secretion systems (Sec, Tat, T1SS, T2SS, T3SS, T4SS, T5SS and T6SS) that either work in tandem or individually to effect the transport of substrates across inner and outer membrane into the external environment (Costa et al., 2015; Green and Mecsas, 2016; Lycklama and Driessen, 2012; Palmer and Berks, 2012). In addition to these dedicated secretion systems, Gram-negative bacteria also utilize outer membrane vesicles to export cellular components (Kuehn and Kesty, 2005). Such vesicles have been shown to carry proteins, DNA, RNA, lipids, etc., originating from the cells from which vesicles slough off. With the exception of vesicles, whose mechanism of formation has not been clearly established, dedicated secretion systems identify which protein is secreted by what mechanism by the nature of secretion signals, also referred to as signal peptides (Green and Mecsas, 2016). Bioinformatic protein prediction tools use the presence or absence of signal peptides, their locations (N or C termini), amino acid composition, and other criteria to predict if and how bacteria secrete proteins. We present here that in many cases none of the bioinformatic tools used can conclusively predict or completely agree if a protein is a secreted protein. The objective of this research was to study the secretomes of two virulent *M. haemolytica* strains, characterize the proteins as to whether they are potentially secreted proteins, and identify if proteins that have been shown to be highly immunogenic are present in the secretome. To accomplish this objective, bacteria-free concentrated supernatants from *M. haemolytica* cultures were subjected to trypsinolysis and LC–MS/MS.

2. Materials and methods

2.1. M. haemolytica *strains and culture*

Two virulent strains of *M. haemolytica* were used in this study. While both belong to serotype 1 (S1), strain 89010807N was isolated in 1989 and Oklahoma (OKL) was isolated in 1978. Both were isolated from lungs of feedlot calves with pneumonia (Pandher et al., 1999). We used these two strains with consistent disease causing profile in our hands to show the reproducibility of secretome production and if passage in our laboratories have in anyway impacted the same. Supernatants from three biological replicates (same strain grown in three flasks with the same medium) of each strain grown in two media, a total of 12 biological replicates, were prepared for studying secretome profiles. Two growth media viz., Brain Heart Infusion (BHI), a complex and inexpensive medium routinely used in culturing this and other fastidious bacteria and RPMI-1640, a synthetic medium, routinely used to grow this organism albeit expensive were used in this study. To achieve this, well isolated colonies of *M. haemolytica* strains from BHI agar plates supplemented with 5% defibrinated sheep blood were transferred into triplicates of culture tubes containing BHI broth and incubated overnight in a shaker incubator. The overnight starter cultures were transferred (1/100th volume) into 3 Erlenmeyer flasks each containing 250 ml of RPMI-1640 medium and an additional 3 flasks containing 250 ml of BHI. Both sets of cultures were incubated in an orbital shaker (150 rpm) at 37 °C. Growth was stopped at late log phase by centrifugation of each culture at 12,000×*g* for 20 minutes and pellets were discarded. Supernatants from each were filtered through 0.2 μM Nalgene filters (Thermo Scientific, Waltham, MA) to remove any bacterial cells and concentrated with Centricon Plus-70 Centrifugal filter devices with 5000 Da MWCO (Millipore, Billerica, MA). Protein concentration in each preparation was determined by Bicinchoninic acid (BCA) assay. The preparations were submitted to the Recombinant DNA/Protein Resource Facility, Oklahoma State University, for identification using LC–MS/MS as described below. Three technical replicates of concentrated BHI was analyzed in the same manner to identify proteins contributed by the medium.

2.2. LC–MS/MS methodology

Proteomes from 12 biological and 3 technical samples were analyzed as described previously (Voruganti et al., 2013), but using chromatography columns packed with 40 cm of 3-micron Magic C18 AQ particles (Bruker) and eluted using a 2.8–44% acetonitrile gradient over a period of 262 min. LC–MS/MS RAW files were analyzed with MaxQuant v1.5.3.8 (Cox and Mann, 2008), using 1% protein and peptide FDR thresholds to search a database of 15,043 *Mannheimia* sequences downloaded from Uniprot on 07/16/15 (see Supplementary Table for the full description of the MaxQuant settings used). Relative abundance of proteins was determined on the basis of their normalized peptide intensities via the Label Free Quantification (LFQ) algorithm in MaxQuant v1.5.3.8 (Cox et al., 2014; Cox and Mann, 2008). Within the MaxQuant software output, this parameter is referred to as LFQ intensity.

2.3. Bioinformatic analysis

To maximize the accuracy of peptide identification through mass spectrometry, multiple bioinformatics algorithms (Romine, 2011) for prediction of protein secretion were leveraged which make use of different prediction methodology and account for non-canonical secretion. The prediction softwares included and brief descriptions are as follows. Amino acid sequences of identified proteins in FASTA formats were analyzed using bioinformatic tools shown here. PSORTb (http://www.psort.org/psortb), predicts subcellular locales of bacterial proteins (Yu et al., 2010). SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP) predicts the presence and location of signal peptide cleavage sites (Petersen et al., 2011). SecP (http://www.cbs.dtu.dk/services/SecretomeP) is a bacterial non-classical secretion and a prediction method for identification of proteins following signal peptide independent secretion pathways (Bendtsen et al., 2005a). LipoP (http://www.cbs.dtu.dk/services/LipoP/) generates predictions of lipoproteins and discriminates between lipoprotein signal peptides, other signal peptides and n-terminal membrane helices in Gram-negative bacteria (Juncker et al., 2003), TatP (http://www.cbs.dtu.dk/services/TatP) predicts the presence and location of Twin-arginine signal peptide cleavage sites in bacteria (Bendtsen et al., 2005b), and PRED-TAT (http://www.compgen.org/tools/PRED-TAT), a fairly recent addition to the toolbox of bioinformatics,