Immunoproteomics to identify *Staphylococcus aureus* antigens expressed in bovine milk during mastitis

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ABSTRACT

Staphylococcus aureus is an opportunistic pathogen affecting both human and animal species. An effective vaccine to prevent *S. aureus* bovine disease and transmission would have positive effects on animal well-being, food production, and human health. The objective of this study was to identify multiple antigens that are immunoreactive during udder colonization and disease for exploration as vaccine antigens to prevent bovine mastitis. *Staphylococcus aureus* produces several cell wall-anchored and surface-associated virulence factors that play key roles in the pathogenesis of mastitis. Many of these proteins are conserved between different strains of *S. aureus* and represent promising vaccine candidates. We used an immunoproteomics approach to identify antigenic proteins from the surface of *S. aureus*. The expression of cell wall and surface proteins from *S. aureus* was induced under low iron conditions, followed by trypsin extraction and separation by 2-dimensional electrophoresis. The separated proteins were blotted with antibodies from mastitic bovine milk and identified by liquid chromatography-mass spectrometry. Thirty-eight unique proteins were identified, of which 8 were predicted to be surface exposed and involved in *S. aureus* virulence. Two surface proteins, iron-regulated surface determinant protein C (IsdC) and ESAT-6 secretion system extracellular protein (EsxA), were cloned, expressed, and purified from *Escherichia coli* for confirmation of immune reactivity by ELISA. A PCR of 37 bovine *S. aureus* isolates indicated that the presence of *esxA* and *isdC* is conserved, and amino acid alignments revealed that IsdC and EsxA sequences are highly conserved. The immunoproteomics technique used in this study generated reproducible results and identified surface exposed and reactive antigens for further characterization.

Key words: *Staphylococcus*, mastitis, vaccine, immunoproteomics

INTRODUCTION

Bovine mastitis is an important and economically relevant disease that affects the dairy industry worldwide (Bar et al., 2011; Gomes and Henriques, 2016). This disease causes large economic losses every year; estimated to be as high as \$2 billion/year in the United States alone (Blosser, 1979; Petrovski et al., 2006). These losses are mainly due to reduced milk yield, antibiotic therapy, veterinary services, and the reduced price of culled animals (Blosser, 1979; Kiku et al., 2017). Mastitis is most often an acute or chronic inflammation of the mammary glands caused by bacterial infection and commonly leads to increased SCC, cytokine production, antibody secretion, and bacterial load in milk (De Vliegher et al., 2012; Thompson-Crispi et al., 2014). Multiple pathogens are known to cause bovine mastitis; however, *Staphylococcus aureus* is one of the most commonly isolated pathogens in milk, with an estimated 3% of dairy cows worldwide being infected (Schukken et al., 2009). *Staphylococcus aureus* infection often results in chronic, subclinical disease that is highly contagious and difficult or impossible to treat, with cure rates lower than 25% (Rainard, 2005). Despite decades of research, an effective vaccine that can prevent *S. aureus* bovine disease is not yet available, likely due to significant strain to strain variability and redundancy of *S. aureus* virulence factors. Thus, the strategy to design an effective vaccine for *S. aureus* mastitis must include multiple conserved and immunogenic virulence factors that can provide cross-protection.

Staphylococcus aureus expresses a broad range of virulence factors that include surface proteins covalently attached to the cell wall and secreted proteins expressed during infection (Foster et al., 2014). These exposed proteins are essential for the survival and proliferation of *S. aureus*, and the presence of many of them is conserved (Foster and McDevitt, 1994; Ni Eldhin et

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al., 1998). Depending upon the stage of infection and physiological conditions inside the host, virulence factor expression will promote binding to the extracellular matrix, colonization, invasion, and avoidance of the immune response (Sheldon and Heinrichs, 2012). Several these factors have been emphasized in both human and veterinary vaccines as important targets for vaccine development (Pereira et al., 2011). The iron-regulated surface determinant A (**IsdA**) is a *S. aureus* surface adhesin that is also involved in iron sequestration and found to be highly expressed, conserved, and immunogenic during bovine mastitis (Misra et al., 2017). Other adhesins, such as IsdB, ClfA and Hla, also have antigenic properties in models for human *S. aureus* infection; however, their role in bovine disease requires further exploration (Maira-Litrán et al., 2012; Adhikari et al., 2016).

Proteomics is an important tool to identify potential vaccine antigens, and is especially useful for pathogens such as *S. aureus* that express numerous surface exposed proteins (Collado et al., 2016; Dwivedi et al., 2016). Studies have reported the use of 2-dimensional electrophoresis (**2DE**) for whole or subcellular proteome analysis of *Staphylococcus* for human vaccine development (Sellman et al., 2005; Brady et al., 2006; Solis et al., 2010). Couto et al. (2016) recently described the use of immunoproteomics, or specifically the serome proteome analysis technique, to explore and characterize novel vaccine and therapeutic targets for *Staphylococcus pseudointermedius* infections in dogs. Immunoproteomics uses a combination of proteomics and immunoblotting from infected hosts to identify antigenic proteins and factors. Mastitic cow milk contains neutrophils, macrophages, lymphocytes, and antibodies as a result of infection and subsequent inflammation (Eisenberg et al., 2016). Secreted antibodies, including IgG and IgM, are present in milk during *S. aureus* acute or chronic infection and can be used as tools to identify bacterial antigens that are expressed and immunoreactive during infection. In addition, high concentrations of IgG1 antibody in milk has been found to negatively associate with *S. aureus* colony-forming units in experimental infections, indicating these antibodies are important for control of colonization (Boerhout et al., 2016). Serome proteome analysis has been employed to identify antigenic proteins using serum from infected cows with subclinical mastitis (Tedeschi et al., 2009; Xia et al., 2012). The combination of immunoproteomics with bioinformatics tools, such as reverse vaccinology and other in silico approaches, has also been explored to analyze the surface proteome of *S. aureus* with a focus on vaccine antigenic targets (Hashmi et al., 2010; Argondizzo et al., 2015; Atshan et al., 2015). Important contributions have been made using these

techniques, but more immunologically extensive and disease-specific, as well as host-specific, approaches are needed to develop an effective *S. aureus* vaccine against bovine mastitis.

Our goal was to use mastitic milk antibodies to promote the identification of relevant antigens for the prevention of bovine mastitis. Here we report that 2DE coupled with immunoblotting and mass spectrometry promoted the identification of immunoreactive candidates from *S. aureus*. We characterized and prioritized these candidates in silico and cloned and purified the antigens, ESAT-6 secretion system extracellular protein (**EsxA**) and IsdC. Both EsxA and IsdC were confirmed by ELISA to be immunoreactive during *S. aureus* infection of the udder. In addition, sequence analysis and PCR on genomic *S. aureus* DNA indicated that EsxA and IsdC are conserved at the AA level and that the presence of these antigens is found in the majority of bovine isolates. This is the first known report of the use of immunoproteomics to identify *S. aureus* antigens using milk from cows with mastitis.

MATERIALS AND METHODS

Bacterial Strains, Culture Conditions, and Milk Samples

The *S. aureus* strains used in 2DE were Newbould 305 (Prasad and Newbould, 1968; Bouchard et al., 2012) and an isolate from clinical mastitis (C1, provided by M. McGuire; Table 1). Thirty-seven additional bovine *S. aureus* isolates from at least 5 different, mostly northwestern, US states and 16 different farms (kindly supplied by Udder Health Systems Inc., Meridian, ID, and Larry Fox, Washington State University, Pullman) were used for PCR and sequence analysis. *Staphylococcus aureus* was characterized and confirmed by growth and hemolysis on MP2 agar (Udder Health Systems Inc.), coagulase production, and 16S rRNA sequencing. For genomic DNA isolation, *S. aureus* was grown in Luria broth with shaking at 37°C. For cell wall protein and surface adhesin expression for 2DE, *S. aureus* was grown in low iron media (**LIM**; Baratéla et al., 2001) with shaking at 37°C. Pooled quarter milk from 4 different acutely clinical mastitic cows (SCC >200,000 cells/mL) was obtained from a diagnostic laboratory for 2DE after confirmation of the presence of *S. aureus* by culture and mass spectrometry (MALDI, Udder Health Systems Inc.). Ten and 100 μ L of each sample was plated on MP2 agar for colony-forming units determination (3.2×10^3 , 1.1×10^5 , 1.6×10^5 , and 1.7×10^5 cfu/mL, respectively), *S. aureus* isolation, and confirmation by 16S rRNA sequencing. Pooled quarter milk obtained from 5 *S. aureus* culture-

negative and clinically healthy cows (SCC <200,000), as well commercially available pasteurized milk, was used for spot comparison in 2DE. For ELISA, pooled quarter milk from 5 different *S. aureus* acutely infected culture-positive cows (SCC >200,000) and 5 *S. aureus* culture-negative cows (SCC <200,000) was used. With the exception of the 4 acutely infected *S. aureus* milk samples for use in 2DE, all other milk samples came from the University of Idaho dairy herd, and collection was approved by the University of Idaho Institutional Animal Care and Use Committee as has been described previously (Misra et al., 2017).

Isolation of *S. aureus* and *Escherichia coli* Cell Wall Associated Proteins

Cell wall-associated proteins were isolated using trypsinization, as reported by Rodríguez-Ortega et al. (2006). Briefly, *S. aureus* Newbould 305, *S. aureus* C1, and *Escherichia coli* (DH10-β Top10, Thermo Fisher Scientific, Waltham, MA) were grown in LIM overnight

to an optical density (OD) of 0.75 to 1.2 and harvested by centrifugation ($6,000 \times g$ for 10 min at 4°C), before washing 3 times with 1× PBS. Cells were then resuspended in 1/50 volume of the original culture in 1× PBS containing 40% sucrose (pH 7.4) for 5 min. Digestion was carried out with the addition of 50 µg of trypsin plus 2 mM dithiothreitol for 1 h at 37°C. The digested mixture was centrifuged at $3,500 \times g$ for 10 min at room temperature, and the supernatant with peptides was filtered with a 0.22-µm filter. Protease reactions were stopped with protease inhibitor solution (Halt Protease Inhibitor Cocktail 100X, Thermo Fisher Scientific). Total protein was analyzed by SDS-PAGE and measured for concentration using bicinchoninic acid assay (Pierce BCA protein assay kit, Thermo Fisher Scientific).

2DE and Western Blotting

Trypsinized protein extracts were cleaned using the ReadyPrep 2-D Cleanup kit (Bio-Rad, Hercules,

Table 1. Bacterial strains, plasmids and primers used in this study

Item ¹	Genotype/ relevant information	Gene	Vector	Amplicon (bp)	Source
Bacterial strains					
<i>Staphylococcus aureus</i> Newbould 305	Bovine clinical isolate				Prasad and Newbould, 1968
<i>S. aureus</i> C1	Bovine clinical isolate				This study
<i>Escherichia coli</i> Top10	DH10β				Invitrogen (Thermo Fisher)
Plasmid					
pNM002		<i>esxA</i> (Newbould 305)	pTRCHisA		This study
pNM003		<i>isdC</i> (Newbould 305)	pTRCHisA		This study
Primer					
PCR					
F: GGCAATGATTAAGATGAGTCC		<i>esxA</i>		287	This study
R: GCAAACCGAAATTATTAG					
F: GAGTATCGAAGGACATAAAG		<i>isdC</i>		384	This study
R: GCTAAGGATGCAACTGG					
Clone					
pNM002		<i>esxA</i>		297	This study
F: GCTTCCGGGATCCGCAATGATTAAGATGAGTC					
CAG					
R:					
CAGTGCGGAATTCTTATTGCAAACCGAAATTATTAG					
pNM003		<i>isdC</i>		606	This study
F:					
GCTTCCGGGATCCGCGAGATAGCGGTACTTTGAATTAT					
R: CAGTGCGAAGCTTTTATTCCACATTGCCTTTAG					
pCK001		<i>isdA</i>		852	Arlian and Tinker, 2011
F: GCTACTGGATCCGCGGCAACAGAAGCTACGAAC					
R:					
GTGCATAAGCTTTTCAAGTTTTTGGTAATTCTTTAGC					

¹F = forward, R = reverse. Clone restriction sites underlined.

CA) following manufacturer's instructions (<http://www.bio-rad.com/webroot/web/pdf/lsr/literature/MS4110143A.pdf>), and resuspended in ReadyPrep rehydration buffer. A total of 150 μ L of 165 μ g/mL of protein was used to rehydrate a 7-cm immobilized pH gradient (IPG) strip (Bio-Rad), pH 4 to 7 for 24 h. Rehydrated IPG strips were then isoelectric focused (IEF) on the first dimension using the Protean IEF system (Bio-Rad) at cycles of 250 V for 20 min linear, followed by 4,000 V for 120 min and 4,000 V at 10,000 voltage-hours ratio on a rapid slope. After IEF, strips were equilibrated using equilibration solution I and II (2-D Starter kit, Bio-Rad) for 15 min each. For second-dimension separation, the equilibrated strips were run on 12% SDS-PAGE and stained with Coomassie blue. The 2-dimension gels were electro-transferred to a polyvinylidene difluoride membrane and blocked using blocking buffer (0.05% Tween 20 + 5% skim milk + 1 $\times$ PBS). Membranes were washed and blotted with pooled mastitic milk or *S. aureus*-negative milk (1:5,000 dilution) and a secondary anti-bovine IgG (H⁺L) horseradish peroxidase conjugate (1:10,000 dilution, A10-102P, Bethyl Laboratories Inc., Montgomery, TX). The membrane was developed using the ECL Western blotting substrate (Pierce, Thermo Fisher Scientific) and exposed to film for 24 h to detect chemiluminescence. Reported data represent 7 independent blots performed using pooled mastitic milk on separated proteins from *S. aureus* Newbould 305 and *S. aureus* C1. Additional blots for comparison were performed each at least twice, using (1) pooled milk from 5 *S. aureus* culture-negative cows on proteins from Newbould 305, (2) uninfected pasteurized milk on proteins from Newbould 305, and (3) pooled mastitic milk on trypsinized proteins from *E. coli*. Spots identified on the Western blot film were compared and aligned to the Coomassie blue-stained 2D gel using ImageJ software (<https://imagej.nih.gov/ij/>) to enable the selection of proteins with the brightest spot. Proteins showing prominent bright spots on *S. aureus* Newbould 305 or C1 immunoblots using *S. aureus*-infected milk were picked for identification by mass spectrometry. Prominent spots from *E. coli* and culture-negative milk blots were also identified, and these antigens eliminated from further consideration.

Mass Spectrometry and In Silico Protein Analysis

The proteins aligned with the brightest spots on the western were picked using a spot picker and digested with trypsin as described with modifications (Couto et al., 2016). Briefly, gel spots were destained in buffer (50 mM ammonium bicarbonate/50% acetonitrile, reduced in 10 mM dithiothreitol) for 60 min at 56°C, and alkylated in 55 mM iodoacetamide for 60 min at room

temperature in the dark. Proteins were then digested with 20 μ g/mL of trypsin overnight at 30°C. Peptides were extracted from the gel, dried under vacuum, and reconstituted in buffer (5% acetonitrile, 0.1% formic acid in water) for LC-electrospray ionization (ESI)-MS/MS analysis. Five microliters of peptide mixture was injected onto a C18 reverse-phase column (10 cm $\times$ 75 μ m, 3 μ m, 120 Å). A linear gradient with 2 mobile phases at a flow rate of 300 nL/min was used to separate peptide mixtures. Full-scan MS spectra were acquired from m/z 300 to 2,000. Collision-induced dissociation was used to fragment the precursor ions. Tandem MS spectra were acquired in the data-dependent acquisition mode for the 10 most abundant precursor ions in the preceding full MS scan. Peptide spectral matching and protein identification were achieved by database search using Sequest HT and Mascot algorithms in Proteome Discoverer 1.4 (Thermo Fisher Scientific). Raw spectrum data were searched against the UniProtKB/Swiss-Prot protein database for *S. aureus* (www.uniprot.org/; obtained on June 14, 2016). Immunogenic proteins were ranked on the basis of location and function using the PSORTb v3.0.2 (www.psort.org/psortb/) and UniProt (www.uniprot.org/), and adhesion probability and transmembrane helices determined using Vaxign (www.violinet.org/vaxign/; Table 2 and Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14040>).

PCR, Cloning, and Purification of Recombinant Proteins

Gene presence or absence was determined by electrophoresis of PCR products performed 3 times under optimized conditions. Primer and amplicon sizes are as indicated (Table 1). The *isdC* and *esxA* PCR products from 8 bovine *S. aureus* were sequenced (Idaho State Molecular Research Core, Pocatello, ID) and aligned on Clone Manager software (Sci-Ed, Denver, CO). The plasmids pNM002 and pNM003 were constructed for the expression of His-EsxA and His-IsdC. Corresponding primers for *isdC* and *esxA* (Table 1; restriction sites underlined) were used to PCR amplify genomic DNA from *S. aureus* Newbould 305. The resulting product was cloned into pTrisHisA (Invitrogen, Carlsbad, CA) and plasmids were transformed into *E. coli* Top10 (Thermo Fisher Scientific). Plasmids were confirmed by sequencing through the junctions. Transformed cells were induced and proteins were isolated from the bacterial cytosol (B-PER Bacterial Protein Extraction Reagent, Thermo Fisher Scientific) for purification by cobalt affinity chromatography (Talon Metal Affinity Resin; Clontech Laboratories, Mountain View, CA) under denaturing conditions, according to manufacturer instructions (<http://www.clontech.com/>

Table 2. Selected antigens on the basis of coverage by mass spectrometry, function, adhesion probability and location

Gene	Protein name	Coverage (%)	Function ¹	Location ²	Adhesion probability ³ (%)
<i>isdC</i>	Iron-regulated surface determinant protein C	29.07	Hemin binding, and haem transfer	Cell wall	57
<i>esxA</i>	ESAT-6 secretion system extracellular protein A	89.6	Virulence factor	Extracellular	43
<i>isaA</i>	Immunodominant staphylococcal antigen A	15.02	Hydrolase activity, acts on glycosyl bonds	Extracellular	71.9
<i>lukL1</i>	Uncharacterized leucocidin like protein 1	2.66	Pathogenesis	Extracellular	59.4
<i>fntA</i>	Methicillin-resistance factor	4.28	Antibiotic resistance	Cell membrane	39.8
<i>spA</i>	Immunoglobulin G-binding protein A	18.8	IgG binding, immune evasion	Cell wall	53
<i>feoB</i>	Ferrous iron transport protein B	3.01	Ferrous iron transmembrane transporter activity	Cell membrane	34.1
<i>map</i>	MHC class II analog protein	1.89	Modulating host immunity	Cell membrane	51.7

¹UniProt (www.uniprot.org).

²PSORTb v3.0.2 (www.psort.org/psortb/).

³Vaxign (www.violinet.org/vaxign/).

US/Support/xxclt_ibeCCtpSctDspRte.jsp?section=16300&beginIndex=0). Purified proteins were dialyzed against phosphate-buffered saline (1× PBS) with 5% glycerol, analyzed by SDS-PAGE, and confirmed by tryptic digestion and tandem mass spectrometry (Boise State Biomolecular Research Center). Protein concentrations were determined by bicinchoninic acid assay as described above.

ELISA

An ELISA was used to detect antigen-specific antibody responses in milk from 5 independent *S. aureus* culture-positive cows (SCC >200,000) and compared with 5 *S. aureus* culture-negative cows (SCC <200,000). Microtiter plates (Nunc, Rochester, NY) were coated with 10 µg of purified protein (EsxA and IsdC, described above) per well in 1× PBS, blocked with buffer (1% skim milk + 1× PBS), and incubated with 2-fold dilutions of bovine milk, starting at a 1:10 dilution, for 12 h at 4°C. Plates were then washed with PBS-T (1× PBS + 0.05% Tween 20), incubated with horseradish peroxidase-conjugated anti-bovine IgG (1:10,000 dilution, A10–102P, as above) in blocking buffer for 1 h at 37°C, washed again, and developed with tetramethylbenzidine (TMB One, Promega, Madison, WI). Antibody titers were calculated after background values (protocol minus samples) were subtracted. Endpoint titers were defined as the reciprocal of the dilution giving an OD of 0.2. For the ovalbumin control, 10 µg of purified chicken ovalbumin (Thermo Fisher Scientific) was coated onto microtiter plates and assays performed as above.

Ovalbumin ELISA endpoint titers were defined as the reciprocal of the dilution, giving an OD of 0.02, and adjusted by a factor of 10 for comparison. The ELISA results are representative of the assay performed independently 3 times.

Data and Statistical Analysis

Graphing and statistical analysis was performed with JMP SAS software (ver. 13, SAS Institute Inc., Cary, NC). Significance for ELISA was assessed using 2-tailed Student's *t*-test for unequal variance. Significance was reported as $P \leq 0.05$. Decoy database search was performed to calculate false discovery rate of MS data. Proteins containing 1 or more peptides with false discovery rate ≤ 0.05 were considered positively identified and reported.

RESULTS

Extraction of Cell Wall-Associated Proteins

The *S. aureus* surface proteome was used as the target for immunoproteomics in our study. Figure 1 illustrates the method of preparation of protein samples used to promote reliable identification of immunogenic surface or cell wall-bound proteins. *Staphylococcus aureus* was cultured in LIM for the induction of virulence-related surface- or cell wall-anchored proteins and collected during late exponential and early stationary phase. Cells were trypsinized to release the cleaved surface fraction and separated in the first dimension by IEF.

After second-dimension separation, immunoproteomic analysis was conducted by performing a Western blot on the 2DE gel using *S. aureus*-infected pooled milk. The goal of these methods was to detect surface exposed *S. aureus* proteins that induced humoral responses in the mastitic milk of infected cows.

Identification of Immunoreactive Antigens

Staphylococcus aureus Newbould 305 and *E. coli* (DH10- β) surface proteins were trypsinized and extracted and separated in one dimension on SDS-PAGE (Figure 2A, lanes 1 and 2). The isolated proteins were then rehydrated and separated on a 2-dimensional gel, as shown in Figure 2B and D. To identify reactive *S. aureus* proteins, Western blots were performed with pooled milk from 4 acutely infected cows. *Escherichia coli* surface proteins were also subjected to Western blot with the same milk to eliminate cross-reactive cytosolic and housekeeping proteins. As expected, mastitic milk recognized more proteins from the *S. aureus* extract (Figure 2C). Several highly immunogenic areas (containing multiple spots) were detected only in *S. aureus* blots and not in the *E. coli* blots (Figure 2C and E). Very few immunogenic proteins were detected in Western blots using *S. aureus* culture negative milk; however, these spots were analyzed and resulting proteins eliminated from consideration (Supplemental Figure S1; <https://doi.org/10.3168/jds.2017-14040>). The anti-staphylococcal antibody repertoires from mastitic cow milk were deemed to be unique and a relevant tool to identify reactive antigens.

LC-MS/MS and In Silico Analysis

The 2DE was performed a total of 7 times using trypsinized proteins from Newbould 305 and *S. aureus* C1. The collected 2DE spots corresponding to immunogenic proteins on Western blots were picked and identified by MS/MS. An uninterpreted database search approach using Sequest and Mascot algorithms was employed to identify peptides and proteins. The complete set of identified proteins using this approach is listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2017-14040>) and ranked by coverage. The most promising immunoreactive proteins were further ranked on the basis of location, function, and potential to be cell wall-associated adhesins using PSORTb v3.0.2, UniProt, and Vaxign online tools (Table 2). Two specific surface-exposed proteins and virulence factors, IsdC and EsxA, were identified independently 2 and 3 times, respectively, on separate blots and were targeted for further characterization. Figure 3 shows a represen-

tative peptide spectral match of the isolated IsdC and EsxA 2DE peptides.

Conservation and Prevalence of Antigens

To determine if IsdC and EsxA are conserved at the AA level, phylogenetic analysis was completed using 50 published sequences (www.ncbi.nlm.nih.gov), as shown in Figure 4. Results include 5 known cow isolates and percent identity is compared with *S. aureus* Newbould 305. Sequences of both proteins are highly conserved, with EsxA 100% conserved among *S. aureus*. The PCR was performed to estimate the genetic prevalence of *isdC* and *esxA* in bovine isolates of *S. aureus*. Gene-specific primers (Table 1) were used to amplify the genes from 37 clinical bovine *S. aureus* isolates and compared with previously reported analysis of *isdA* (Misra et al., 2017). As shown in Table 3, results indicate that the presence of *isdC* and *esxA* is 92% conserved in bovine *S. aureus* isolates. The PCR products from 8 bovine isolates were sequenced and alignments support the high AA conservation of these proteins (Supplemental Figure S2; <https://doi.org/10.3168/jds.2017-14040>).

Cloning, Expression and Immunogenicity of Antigens

Both *isdC* and *esxA* were cloned into a 6 $\times$ histidine vector to construct the expression plasmids pNM003 and pNM002 (Figure 5A and C). The IsdC (~25.5 kD) and EsxA (~14.5 kD) proteins were expressed and analyzed on SDS-PAGE (Figure 5B and D). Purified proteins were then used to determine the immunoreactivity of IsdC (Figure 6B) and EsxA (Figure 6C) by ELISA using *S. aureus* culture-positive or -negative milk from 5 individual cows. Antibody responses to the previously characterized IsdA (Figure 6A) and to the nonspecific chicken ovalbumin antigen (Figure 6D) were also determined for comparison. Antibodies to IsdC and EsxA were detected at significantly higher amounts in mastitic milk as compared with healthy milk with undetectable counts of *S. aureus*. Results support 2DE evidence that IsdC and EsxA are expressed during infection and trigger humoral responses in vivo in the bovine udder.

DISCUSSION

Staphylococcus aureus surface proteins are critical immunodominant antigens that are often the first molecules to interact with host cells and tissues (Foster et al., 2014). These proteins are especially important targets for *S. aureus* vaccine development, as they

Figure 1. Diagram of the immunoproteomics techniques used for detection of immunogenic surface proteins from *Staphylococcus aureus*. The experimental workflow, including 2-dimensional electrophoresis (2DE), immunoblots, and mass spectrometry analysis, for identifying bovine disease relevant antigens is shown. LC = liquid chromatography; IPG = immobilized pH gradient; IEF = isoelectric focusing; MW = molecular weight.

Figure 2. Representative SDS-PAGE, 2-dimensional electrophoresis (2DE), and Western blots showing the workflow for selection of immunoreactive spots. (A) The 1-dimensional SDS-PAGE image of *Staphylococcus aureus* Newbould 305 trypsinized protein (lane 1) and *Escherichia coli* trypsinized protein (lane 2). (B) 2DE gel conducted on 7 cm, pH 4 to 7, immobilized pH gradient (IPG) strips of *S. aureus* Newbould 305 trypsinized protein. Mr = molecular weight. (C) Immunoblot of *S. aureus* Newbould 305 trypsinized protein using pooled bovine mastitic milk, with immunoreactive regions highlighted in red circles. (D) 2DE gel conducted on 7 cm, pH 4 to 7, IPG strips of *Escherichia coli* DH10-β trypsinized protein. (E) Immunoblot of *E. coli* DH10-β trypsinized protein using bovine mastitic milk, with the immunoreactive regions highlighted in red circles. Color version available online.

are also key for adhesion and nutrient acquisition and their presence and structure are more conserved across strains than the polysaccharide capsule (Le Maréchal et al., 2011; Cheung et al., 2014; Foster, 2016; Lacey et al., 2016; Josse et al., 2017). Our approach was to identify immunoreactive cell wall-associated and surface pro-

teins for characterization as vaccine antigens to prevent *S. aureus* bovine mastitis. The presence of anti-staphylococcal IgG antibodies in milk during subclinical and clinical bovine mastitis has been reported (Eisenberg et al., 2016). Thus, we used a unique immunoproteomics strategy to detect antigenic staphylococcal proteins

using antibodies present in milk. A total of 38 immunoreactive *S. aureus* proteins were identified, out of which 17 proteins were determined to be cell wall associated, or extracellular, and previously reported as involved in virulence. Eight proteins were further ranked based upon their subcellular location, coverage, and function. Two proteins, IsdC and EsxA, had the highest coverage by mass spectrometry and were isolated independently more than once. These proteins were purified for confirmation of immune reactivity in vitro, and will be used to test in vivo in future studies, as described using other proteomics approaches (Rodríguez-Ortega et al., 2006).

Despite taking precautions to minimize cell lysis, the approach we used also identified intracellular or cytosolic proteins, including elongation factors and ribosomal proteins (Supplemental Table S1; <https://doi.org/10.3168/jds.2017-14040>). Adjustment of the trypsinization time and digest conditions did not eliminate the presence of these proteins, and the detection of cytosolic

proteins is a recognized limitation of immunoproteomics (Broker and van Belkum, 2011). Whereas immunogenic cytosolic proteins can be potential vaccine antigens, they may also be detected due to abundance; thus, we did not pursue their characterization (Elsholz et al., 2017). Phase of growth is also a key consideration for immunoproteomics. We targeted the late exponential phase and early stationary phases of growth (0.75–1.2 OD₆₀₀ in LIM), as previous reports have indicated this range is optimal for *isdA* and other virulence factor expression (Cheung et al., 2004; Ythier et al., 2012). The IsdA is a well characterized immunogenic surface adhesin from *S. aureus*, and we, unexpectedly, did not identify this protein using these techniques (Clarke et al., 2004). Lack of identification of IsdA could be due to trypsinization methods that result in aberrant IsdA cleavage, lack of release of IsdA due to surface hydrophobicity, or low peptide concentration that was not detected, or not fully resolved, from other spots or proteins (Dutoit-Lefèvre et al., 2015). We attempted

Figure 3. Representative MS/MS spectra of peptides identified by Mascot (Matrix Science, Boston, MA). (A) MS/MS fragmentation of FNGPTDVGANAPGKDDK found in the iron-regulated surface determinant protein C (IsdC); (B) MS/MS fragmentation of AQGEIAANWEGQAFSR found in the ESAT-6 secretion system extracellular protein (EsxA). Color version available online.

Table 3. Presence of *isdC* and *esxA* in bovine *Staphylococcus aureus*

Item	<i>isdC</i>	<i>esxA</i>	<i>isdA</i> ¹
No. of positive isolates	34	34	36
No. of strains tested	37	37	37
Percent	92	92	97

¹Results reported previously (Misra et al., 2017).

to isolate surface proteins using alternate techniques, including proteinase K, lysostaphin, and silica beads (data not shown), but these efforts resulted in the identification of many fewer surface proteins, or more cytosolic proteins. Trypsinization enables extraction of surface proteins effectively, but is limited to exposed lysine and arginine AA residues on the surface. Despite

these limitations, several immunoreactive *S. aureus* virulence factors for further study were identified. These results support the utility of milk antibodies for the identification of antigens that are exposed and reactive during udder colonization and mastitis.

Out of the top 8 identified proteins, IsdC and EsxA were selected for further analysis on the basis of coverage by mass spectrometry, cell wall location, and function in relation to virulence and pathogenesis. Purified IsdC and EsxA were tested for immune reactivity to bovine milk from *S. aureus*-infected cows as an indicator of expression and exposure in vivo. In comparison to the IsdA antigen, which has been reported to induce opsonizing antibody production during *S. aureus* mastitis, EsxA and IsdC were also immunoreactive in this

Figure 4. Dendrogram of pairwise AA alignments of (A) iron-regulated surface determinant protein C (IsdC) and (B) ESAT-6 secretion system extracellular protein (EsxA) from 50 published sequences. Evolutionary analysis was performed with the maximum likelihood method based on the JTT matrix-based model (MEGA, Version 7, Mega Software, Tempe, AZ). Percent identity to the sequence from the bovine Newbould 305 *Staphylococcus aureus* strain is shown. Isolates from cow (*) are indicated.

assay (Misra et al., 2017). Whereas this assay does not indicate protective efficacy, it is supportive of 2DE outcomes. Both EsxA and IsdC have been studied and described as virulence factors for *S. aureus* pathogenesis. The EsxA is a surface-exposed *S. aureus* protein, as determined by trypsinization and mass spectrometry, that is likely secreted through a unique Type VII pathway (WXG, Supplemental Figure S2; <https://doi.org/10.3168/jds.2017-14040>; Solis et al., 2010); this protein is also biofilm-associated and an important factor for pathogenesis and abscess formation in mice (Burts et al., 2005; Cinvarova et al., 2016). A recent report identified anti-EsxA antibodies in patients infected with antibiotic-resistant strains of *S. aureus* (Zhou et al., 2013); EsxA has also been evaluated as a human vaccine candidate, and induces protective Th1 and Th17 immune responses against invasive *S. aureus* in murine models (Bagnoli et al., 2015; Zhang et al., 2015). The IsdC is a surface adhesin from the Isd (iron-regulated

Figure 5. Expression and purification of iron-regulated surface determinant protein C (IsdC) and ESAT-6 secretion system extracellular protein (EsxA) proteins. (A) Structure and operon organization of pNM003 for His-IsdC expression and (B) SDS-PAGE of purified IsdC (~25.5 kDa). (C) Structure and operon organization of pNM002 for His-EsxA expression and (D) SDS-PAGE of purified EsxA (~14.5 kDa). E1 = elution 1, E2 = elution 2. HIS = 6× histidine; BamHI, HindIII, EcoRI = restriction enzyme sites; trc = tryptophan/lactose promoter; lacIq = lactose repressor; Amp = ampicillin resistance gene (β -lactamase); ori = origin of replication. Color version available online.

Figure 6. Antigen-specific antibody responses from *Staphylococcus aureus* culture-positive and *S. aureus* culture-negative bovine milk, showing (A) anti-iron-regulated surface determinant protein A (IsdA), (B) anti-IsdC, (C) anti-ESAT-6 secretion system extracellular protein (EsxA), and (D) anti-ovalbumin responses. Data are presented as the log-transformed geometric mean $\pm$ SE of independent titers from 5 cows. Significance of anti-IsdA ($P = 0.014^*$), anti-IsdC ($P = 0.035^*$), EsxA ($P = 0.019^*$), and ovalbumin ($P = 0.0517$) was determined using a 2-tailed Student's *t*-test for unequal variance.

surface determinant) family that is required for iron sequestration and extracellular matrix binding. The IsdC is involved in heme transfer from the membrane to the cytosol and important, but not essential, for *S. aureus* iron uptake (Hurd et al., 2012). The IsdC contains 1 near-iron-transporter (Supplemental Figure S2) domain similar to IsdA, and may contribute to biofilm formation through dimerization (Missineo et al., 2014). To date, IsdC has not been assessed for protective efficacy in animal models; however, the related IsdA and IsdB have been found to be protective in mice (Kim et al., 2010). We found both IsdC and EsxA to be highly conserved at the AA level using published *S. aureus* sequences and sequences from 8 bovine isolates. Genes *isdC* and *esxA* were also present in 92% of tested bovine *S. aureus* isolates.

Other proteins identified using this technique also represent interesting vaccine candidates, including FeoB, Map, SpA, and IsaA (Lee et al., 2002; Glowalla et al., 2009; Hashmi et al., 2010; Lorenz et al., 2011; Falugi et al., 2013; Oprea and Antohe, 2013). These

proteins were not currently selected for analysis due to lower coverage by MS; however, several of them have been pursued as human vaccine candidates and may also represent bovine candidates. In addition, 4 proteins with unknown function were detected, including 1 with high coverage, and these may represent novel candidates. We acknowledge that the methods used here do not distinguish proteins that are immunoreactive but poorly protective. Additional studies are required to assess the specific immunogenicity and efficacy of these proteins, and may include in vivo protection and the use of milk from convalescent or chronically infected cows. However, the use of immunoproteomics with milk antibodies was found to be a useful method to aid in the selection of *S. aureus* antigens expressed during mastitis for their assessment as vaccine candidates.

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REFERENCES

- Adhikari, R. P., C. D. Thompson, M. J. Aman, and J. C. Lee. 2016. Protective efficacy of a novel alpha hemolysin subunit vaccine (AT62) against *Staphylococcus aureus* skin and soft tissue infections. *Vaccine* 34:6402–6407. <https://doi.org/10.1016/j.vaccine.2016.09.061>.
- Argondizzo, A. P. C., F. F. da Mota, C. P. Pestana, J. N. Reis, A. B. de Miranda, R. Galler, and M. A. Medeiros. 2015. Identification of proteins in *Streptococcus pneumoniae* by reverse vaccinology and genetic diversity of these proteins in clinical isolates. *Appl. Biochem. Biotechnol.* 175:2124–2165. <https://doi.org/10.1007/s12010-014-1375-3>.
- Arlian, B. M., and J. K. Tinker. 2011. Mucosal immunization with a *Staphylococcus aureus* IsdA-cholera toxin A2/B chimera induces antigen-specific Th2-type responses in mice. *Clin. Vaccine Immunol.* 18:1543–1551. <https://doi.org/10.1128/CVI.05146-11>.
- Atshan, S. S., M. N. Shamsudin, Z. Sekawi, L. T. T. Lung, F. Barantalab, Y. K. Liew, M. A. Alreshidi, S. A. Abduljaleel, and R. A. Hamat. 2015. Comparative proteomic analysis of extracellular proteins expressed by various clonal types of *Staphylococcus aureus* and during planktonic growth and biofilm development. *Front. Microbiol.* 6:524. <https://doi.org/10.3389/fmicb.2015.00524>.
- Bagnoli, F., M. R. Fontana, E. Soldaini, R. P. N. Mishra, L. Fiaschi, E. Cartocci, V. Nardi-Dei, P. Ruggiero, S. Nosari, M. G. De Falco, G. Lofano, S. Marchi, B. Galletti, P. Mariotti, M. Bacconi, A. Torre, S. Maccari, M. Scarselli, C. D. Rinaudo, N. Inoshima, S. Savino, E. Mori, S. Rossi-Paccani, B. Baudner, M. Pallaoro, E. Swennen, R. Petracca, C. Brettoni, S. Liberatori, N. Norais, E. Monaci, J. B. Wardenburg, O. Schneewind, D. T. O'Hagan, N. M. Valiante, G. Bensi, S. Bertholet, E. De Gregorio, R. Rappuoli, and G. Grandi. 2015. Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. USA* 112:3680–3685. <https://doi.org/10.1073/pnas.1424924112>.
- Bar, D., J. Hertl, L. W. Tauer, G. Bennett, Y. H. Schukken, and F. L. Welcome. 2011. The cost and management of different types of clinical mastitis in dairy cows estimated by dynamic programming. 94:4476–4487. <https://doi.org/10.3168/jds.2010-4123>.
- Baratela, K. C., H. O. Saridakis, L. C. J. Gaziri, and J. S. Pelayo. 2001. Effects of medium composition, calcium, iron and oxygen on haemolysin production by *Plesiomonas shigelloides* isolated from water. *J. Appl. Microbiol.* 90:482–487. <https://doi.org/10.1046/j.1365-2672.2001.01270.x>.
- Blosser, T. H. 1979. Economic losses from and the national research program on mastitis in the United States. *J. Dairy Sci.* 62:119–127. [https://doi.org/10.3168/jds.S0022-0302\(79\)83213-0](https://doi.org/10.3168/jds.S0022-0302(79)83213-0).
- Boerhout, E. M., A. P. Koets, J. C. Vernooij, T. G. Mols-Vorstermans, P. J. Nuijten, V. P. Rutten, J. J. Bijlsma, and S. W. Eisenberg. 2016. Reisolation of *Staphylococcus aureus* from bovine milk following experimental inoculation is influenced by fat percentage and specific immunoglobulin G1 titer in milk. *J. Dairy Sci.* 99:4259–4269. <https://doi.org/10.3168/jds.2015-10648>.
- Bouchard, D., V. Peton, S. Almeida, C. Le Maréchal, A. Miyoshi, V. Azevedo, N. Berkova, L. Rault, P. François, J. Schrenzel, S. Even, D. Hernandez, and Y. Le Loir. 2012. Genome sequence of *Staphylococcus aureus* newbould 305, a strain associated with mild bovine mastitis. *J. Bacteriol.* <https://doi.org/10.1128/JB.01188-12>.
- Brady, R. A., J. G. Leid, A. K. Camper, J. W. Costerton, and M. E. Shirliff. 2006. Identification of *Staphylococcus aureus* proteins recognized by the antibody-mediated immune response to a biofilm infection. *Infect. Immun.* 74:3415–3426. <https://doi.org/10.1128/IAI.00392-06>.
- Broker, B. M., and A. van Belkum. 2011. Immune proteomics of *Staphylococcus aureus*. *Proteomics* 11:3221–3231. <https://doi.org/10.1002/pmic.201100010>.
- Burts, M. L., W. A. Williams, K. DeBord, and D. M. Missiakas. 2005. EsxA and EsxB are secreted by an ESAT-6-like system that is required for the pathogenesis of *Staphylococcus aureus* infections. *Proc. Natl. Acad. Sci. USA* 102:1169–1174. <https://doi.org/10.1073/pnas.0405620102>.
- Cheung, A. L., A. S. Bayer, G. Zhang, H. Gresham, and Y. Q. Xiong. 2004. Regulation of virulence determinants in vitro and in vivo in *Staphylococcus aureus*. *FEMS Immunol. Med. Microbiol.* 40:1–9. [https://doi.org/10.1016/S0928-8244\(03\)00309-2](https://doi.org/10.1016/S0928-8244(03)00309-2).
- Cheung, G. Y. C., H.-S. Joo, S. S. Chatterjee, and M. Otto. 2014. Phenol-soluble modulins—critical determinants of staphylococcal virulence. *FEMS Microbiol. Rev.* 38:698–719. <https://doi.org/10.1111/1574-6976.12057>.
- Cincarova, L., O. Polansky, V. Babak, P. Kulich, and P. Kralik. 2016. Changes in the expression of biofilm-associated surface proteins in *Staphylococcus aureus* food-environmental isolates subjected to sublethal concentrations of disinfectants. *BioMed Res. Int.* 2016. <https://doi.org/10.1155/2016/4034517>.
- Clarke, S. R., M. D. Wiltshire, and S. J. Foster. 2004. IsdA of *Staphylococcus aureus* is a broad spectrum, iron-regulated adhesin. *Mol. Microbiol.* 51:1509–1519.

- Collado, R., A. Prenafeta, L. González-González, J. A. Pérez-Pons, and M. Sitjà. 2016. Probing vaccine antigens against bovine mastitis caused by *Streptococcus uberis*. *Vaccine* 34:3848–3854. <https://doi.org/10.1016/j.vaccine.2016.05.044>.
- Couto, N., J. Martins, A. M. Lourenco, C. Pomba, and A. Varela Coelho. 2016. Identification of vaccine candidate antigens of *Staphylococcus pseudintermedius* by whole proteome characterization and serological proteomic analyses. *J. Proteomics* 133:113–124. <https://doi.org/10.1016/j.jprot.2015.12.017>.
- De Vlieghe, S., L. K. Fox, S. Piepers, S. McDougall, and H. W. Barkema. 2012. Invited review: Mastitis in dairy heifers: Nature of the disease, potential impact, prevention, and control. *J. Dairy Sci.* 95:1025–1040. <https://doi.org/10.3168/jds.2010.4074>.
- Dutoit-Lefèvre, V., S. Dubucquoi, D. Launay, V. Sobanski, P. Dussart, P. Chafey, C. Broussard, S. Duban-Deweier, P. Vermersch, L. Prin, and D. Lefranc. 2015. An optimized fluorescence-based bidimensional immunoproteomic approach for accurate screening of autoantibodies. *PLoS One* 10:e0132142. <https://doi.org/10.1371/journal.pone.0132142>.
- Dwivedi, P., S. I. Alam, and R. S. Tomar. 2016. Secretome, surfome and immunome: emerging approaches for the discovery of new vaccine candidates against bacterial infections. *World J. Microbiol. Biotechnol.* 32:155. <https://doi.org/10.1007/s11274-016-2107-3>.
- Eisenberg, S. W. F., E. M. Boerhout, L. Ravesloot, A. J. J. M. Daemen, L. Benedictus, V. P. M. G. Rutten, and A. P. Koets. 2016. Diurnal differences in milk composition and its influence on in vitro growth of *Staphylococcus aureus* and *Escherichia coli* in bovine quarter milk. *J. Dairy Sci.* 99:5690–5700. <https://doi.org/10.3168/jds.2015-10757>.
- Elsholz, A. K. W., S. Alicia, R. Losick, T. Extracellular, and C. Link. 2017. The extracellular matrix of *Staphylococcus aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. *MBio* 5:e01667-14. <https://doi.org/10.1128/mBio.01667-14>.
- Falugi, F., H. K. Kim, and D. M. Missiakas. 2013. Role of protein A in the evasion of host adaptive immune responses. *MBio* 4:e00575. <https://doi.org/10.1128/mBio.00575-13>.
- Foster, T. J. 2016. The remarkably multifunctional fibronectin binding proteins of *Staphylococcus aureus*. *Eur. J. Clin. Microbiol. Infect.* Dis. 35:1923–1931. <https://doi.org/10.1007/s10096-016-2763-0>.
- Foster, T. J., J. A. Geoghegan, V. K. Ganesh, and M. Höök. 2014. Adhesion, invasion and evasion: The many functions of the surface proteins of *Staphylococcus aureus*. *Nat. Rev. Microbiol.* 12:49–62. <https://doi.org/10.1038/nrmicro3161>.
- Foster, T. J., and D. McDevitt. 1994. Surface-associated proteins of *Staphylococcus aureus*: Their possible roles in virulence. *FEMS Microbiol. Lett.* 118:199–205. <https://doi.org/10.1111/j.1574-6968.1994.tb06828.x>.
- Glowalla, E., B. Tosetti, M. Krönke, and O. Krut. 2009. Proteomics-based identification of anchorless cell wall proteins as vaccine candidates against *Staphylococcus aureus*. *Infect. Immun.* 77:2719–2729. <https://doi.org/10.1128/IAI.00617-08>.
- Gomes, F., and M. Henriques. 2016. Control of bovine mastitis: Old and recent therapeutic approaches. *Curr. Microbiol.* 72:377–382. <https://doi.org/10.1007/s00284-015-0958-8>.
- Hashmi, T., S. Khan, S. Zafar, and H. Bokhari. 2010. In silico identification of vaccine candidates against enteric pathogens by a comparative genome sequence approach. *Asia Pac. J. Mol. Biol. Biotechnol.* 18:327–331.
- Hurd, A. F., J. Garcia-Lara, Y. Rauter, M. Cartron, R. Mohamed, and S. J. Foster. 2012. The iron-regulated surface proteins IsdA, IsdB, and IsdH are not required for heme iron utilization in *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 329:93–100. <https://doi.org/10.1111/j.1574-6968.2012.02502.x>.
- Josse, J., F. Laurent, and A. Diot. 2017. Staphylococcal adhesion and host cell invasion: Fibronectin-binding and other mechanisms. *Front. Microbiol.* 8:2433. <https://doi.org/10.3389/fmicb.2017.02433>.
- Kiku, Y., T. Ozawa, H. Takahashi, S. Kushibiki, S. Inumaru, H. Shingu, Y. Nagasawa, A. Watanabe, E. Hata, and T. Hayashi. 2017. Effect of intramammary infusion of recombinant bovine GM-CSF and IL-8 on CMT score, somatic cell count, and milk mononuclear cell populations in Holstein cows with *Staphylococcus aureus* sub-clinical mastitis. *Vet. Res. Commun.* 41:175–182. <https://doi.org/10.1007/s11259-017-9684-y>.
- Kim, H. K., A. DeDent, A. G. Cheng, M. McAdow, F. Bagnoli, D. M. Missiakas, and O. Schneewind. 2010. IsdA and IsdB antibodies protect mice against *Staphylococcus aureus* abscess formation and lethal challenge. *Vaccine* 28:6382–6392. <https://doi.org/10.1016/j.vaccine.2010.02.097>.
- Lacey, K. A., J. Geoghegan, and R. McLoughlin. 2016. The Role of *Staphylococcus aureus* Virulence Factors in Skin Infection and Their Potential as Vaccine Antigens. *Pathogens* 5:22. <https://doi.org/10.3390/pathogens5010022>.
- Le Maréchal, C., N. Seyffert, J. Jardin, D. Hernandez, G. Jan, L. Rault, V. Azevedo, P. François, J. Schrenzel, M. van de Guchte, S. Even, N. Berkova, R. Thiéry, J. R. Fitzgerald, E. Vautou, and Y. Le Loir. 2011. Molecular basis of virulence in *Staphylococcus aureus* mastitis. *PLoS One*. <https://doi.org/10.1371/journal.pone.0027354>.
- Lee, L. Y., Y. J. Miyamoto, B. W. McIntyre, M. Höök, K. W. McCrea, D. McDevitt, and E. L. Brown. 2002. The *Staphylococcus aureus* Map protein is an immunomodulator that interferes with T cell-mediated responses. *J. Clin. Invest.* 110:1461–1471. <https://doi.org/10.1172/JCI16318>.
- Lippolis, J. D., B. W. Brunelle, T. A. Reinhardt, R. E. Sacco, B. J. Nonnecke, B. Dogan, K. Simpson, and Y. H. Schukken. 2014. Proteomic analysis reveals protein expression differences in *Escherichia coli* strains associated with persistent versus transient mastitis. *J. Proteomics*. <https://doi.org/10.1016/j.jprot.2014.06.008>.
- Lorenz, U., B. Lorenz, T. Schmitter, K. Streker, C. Erck, J. Wehland, J. Nicke, B. Zimmermann, and K. Ohlsen. 2011. Functional antibodies targeting IsaA of *Staphylococcus aureus* augment host immune response and open new perspectives for antibacterial therapy. *Antimicrob. Agents Chemother.* 55:165–173. <https://doi.org/10.1128/AAC.01144-10>.
- Maira-Litrán, T., L. V. Bentancor, C. Bozkurt-Guzel, J. M. O'Malley, C. Cywes-Bentley, and G. B. Pier. 2012. Synthesis and evaluation of a conjugate vaccine composed of *Staphylococcus aureus* poly-N-acetyl-glucosamine and clumping factor A. *PLoS One* 7:e43813. <https://doi.org/10.1371/journal.pone.0043813>.
- Misra, N., T. Wines, C. Knopp, M. McGuire, and J. K. Tinker. 2017. Expression, immunogenicity and variation of iron-regulated surface protein A from bovine isolates of *Staphylococcus aureus*. *FEMS Microbiol. Lett.* 364:fnx082. <https://doi.org/10.1093/femsle/fnx082>.
- Missineo, A., A. Di Poto, J. A. Geoghegan, S. Rindi, S. Heilbronner, V. Gianotti, C. R. Arciola, T. J. Foster, P. Speziale, and G. Pietrocola. 2014. IsdC from *Staphylococcus lugdunensis* induces biofilm formation under low-iron growth conditions. *Infect. Immun.* 82:2448–2459. <https://doi.org/10.1128/IAI.01542-14>.
- Ni Eldhin, D. N., S. Perkins, P. Francois, P. Vaudaux, M. Höök, and T. J. Foster. 1998. Clumping factor B (ClfB), a new surface-located fibrinogen-binding adhesin of *Staphylococcus aureus*. *Mol. Microbiol.* 30:245–257. <https://doi.org/10.1046/j.1365-2958.1998.01050.x>.
- Oprea, M., and F. Antohe. 2013. Reverse-vaccinology strategy for designing T-cell epitope candidates for *Staphylococcus aureus* endocarditis vaccine. *Biologicals* 41:148–153. <https://doi.org/10.1016/j.biologicals.2013.03.001>.
- Pereira, U. P., D. G. S. Oliveira, L. R. Mesquita, G. M. Costa, and L. J. Pereira. 2011. Efficacy of *Staphylococcus aureus* vaccines for bovine mastitis: A systematic review. *Vet. Microbiol.* <https://doi.org/10.1016/j.vetmic.2010.10.003>.
- Petrovski, K. R., M. Trajcev, and G. Buneski. 2006. A review of the factors affecting the costs of bovine mastitis. *J. S. Afr. Vet. Assoc.* 77:52–60.
- Prasad, L. B., and F. H. Newbould. 1968. Inoculation of the bovine teat duct with *S. aureus*: the relationship of teat duct length, milk yield and milking rate to development of intramammary infection. *Can. Vet. J.* 9:107–115.
- Rainard, P. 2005. Tackling mastitis in dairy cows. *Nat. Biotechnol.* 23:430–432. <https://doi.org/10.1038/nbt0405-430>.

- Rodríguez-Ortega, M. J., N. Norais, G. Bensi, S. Liberatori, S. Capo, M. Mora, M. Scarselli, F. Doro, G. Ferrari, I. Garaguso, T. Maggi, A. Neumann, A. Covre, J. L. Telford, and G. Grandi. 2006. Characterization and identification of vaccine candidate proteins through analysis of the group A *Streptococcus* surface proteome. *Nat. Biotechnol.* 24:191–197. <https://doi.org/10.1038/nbt1179>.
- Schukken, Y. H., R. N. González, L. L. Tikofsky, H. F. Schulte, C. G. Santisteban, F. L. Welcome, G. J. Bennett, M. J. Zurakowski, and R. N. Zadoks. 2009. CNS mastitis: Nothing to worry about? *Vet. Microbiol.* 134:9–14. <https://doi.org/10.1016/j.vetmic.2008.09.014>.
- Sellman, B. R., A. P. Howell, C. Kelly-Boyd, and S. M. Baker. 2005. Identification of immunogenic and serum binding proteins of *Staphylococcus epidermidis*. *Infect. Immun.* 73:6591–6600. <https://doi.org/10.1128/IAI.73.10.6591-6600.2005>.
- Sheldon, J. R., and D. E. Heinrichs. 2012. The iron-regulated staphylococcal lipoproteins. *Front. Cell. Infect. Microbiol.* 2:41. <https://doi.org/10.3389/fcimb.2012.00041>.
- Solis, N., M. R. Larsen, and S. J. Cordwell. 2010. Improved accuracy of cell surface shaving proteomics in *Staphylococcus aureus* using a false-positive control. *Proteomics* 10:2037–2049. <https://doi.org/10.1002/pmic.200900564>.
- Tedeschi, G., F. Taverna, A. Negri, R. Piccinini, S. Nonnis, S. Ronchi, and A. Zeconi. 2009. Serological proteome analysis of *Staphylococcus aureus* isolated from sub-clinical mastitis. *Vet. Microbiol.* 134:388–391. <https://doi.org/10.1016/j.vetmic.2008.08.019>.
- Thompson-Crispi, K., H. Atalla, F. Miglior, and B. A. Mallard. 2014. Bovine mastitis: Frontiers in immunogenetics. *Front. Immunol.* 5:493. <https://doi.org/10.3389/fimmu.2014.00493>.
- Xia, C., H. Y. Zhang, L. Wu, C. Xu, J. S. Zheng, Y. J. Yan, L. J. Yang, and S. Shu. 2012. Proteomic analysis of plasma from cows affected with milk fever using two-dimensional differential in-gel electrophoresis and mass spectrometry. *Res. Vet. Sci.* 93:857–861. <https://doi.org/10.1016/j.rvsc.2011.10.025>.
- Ythier, M., G. Resch, P. Waridel, A. Panchaud, A. Gfeller, P. Majcher-czyk, M. Quadroni, and P. Moreillon. 2012. Proteomic and transcriptomic profiling of *Staphylococcus aureus* surface LPXTG-proteins: correlation with agr genotypes and adherence phenotypes. *Mol. Cell. Proteomics* 11:1123–1139. <https://doi.org/10.1074/mcp.M111.014191>.
- Zhang, B. Z., Y. H. Hua, B. Yu, C. C. Y. Lau, J. P. Cai, S. Y. Zheng, W. C. Yam, R. Y. T. Kao, K. H. Sze, B. J. Zheng, K. Y. Yuen, and J. D. Huang. 2015. Recombinant ESAT-6-like proteins provoke protective immune responses against invasive *Staphylococcus aureus* disease in a murine model. *Infect. Immun.* 83:339–345. <https://doi.org/10.1128/IAI.02498-14>.
- Zhou, H., H. Du, H. Zhang, H. Shen, R. Yan, Y. He, M. Wang, and X. Zhu. 2013. EsxA might as a virulence factor induce antibodies in patients with *Staphylococcus aureus* infection. *Braz. J. Microbiol.* 44:267–271. <https://doi.org/10.1590/S1517-83822013005000019>.