Genetic and phenotypic characterization of livestock associated methicillin resistant *Staphylococcus aureus* sequence type (ST) 5 in comparison with clinical ST5 isolates from humans

by

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DEDICATION

For L.B. Your enthusiasm and sense of adventure make life more interesting.

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NOMENCLATURE

AAC	Aminoglycoside Acetyltransferase
ACME	Arginine Catabolic Mobile Element
AMR	Antimicrobial Resistance
AMDUCA	Animal Medicinal Drug Use Clarification Act
ANT	Aminoglycoside Nucleotidyltransferase
APH	Aminoglycoside Phosphotransferase
BLAST	Basic Local Alignment Search Tool
CA-MRSA	Community Acquired Methicillin Resistant *Staphylococcus aureus*
CARD	Comprehensive Antibiotic Resistance Database
CC	Clonal Complex
CFU	Colony Forming Units
CLSI	Clinical Laboratory Standards Institute
DCBM	Dermal Cell Basal Medium
DNA	Deoxyribonucleic Acid
dNTP	Deoxyneucleoside Triphosphates
ECM	Extracellular Matrix
HA-MRSA	Hospital Acquired Methicillin Resistant *Staphylococcus aureus*
HEK	Human Epidermal Keratinocyte
HGT	Horizontal Gene Transfer
Human - LT	Human with Long Term Swine Contact
Human - NSC	Human with No Swine Contact

Human - ST	Human with Short Term Swine Contact
IEC	Immune Evasion Cluster
INT	Intergenic Mutation
ISU	Iowa State University
LA-MRSA	Livestock Associated Methicillin Resistant *Staphylococcus aureus*
MGE	Mobile Genetic Element
MIC	Minimum Inhibitory Concentration
MLS	Macrolide, Lincosamide, Streptogramin Antibiotics
MLST	Multi-Locus Sequence Typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
MSCRAMM	Microbial Surface Component Recognizing Adhesive Matrix Molecules
MSSA	Methicillin Susceptible *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NEAT motif	Near Iron Transporter Motif
NET	Neutrophil Extracellular Trap
NSYN	Non-synonymous Mutation
ORISE	Oak Ridge Institute for Science and Education
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PVL	Panton Valentine Leukocidin
QRDR	Quinolone Resistance Determining Region

RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
SCC*mec*	Staphylococcal Cassette Chromosome *mec*
SMRT	Single Molecule Real-Time
SNP	Single Nucleotide Polymorphism
SYN	Synonymous Mutation
Spa	*S. aureus* Protein A
SSSS	Staphylococcal Scalded Skin Syndrome
ST	Sequence Type
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
TSS	Toxic Shock Syndrome
TSST-1	Toxic Shock Syndrome Toxin 1
UCI	University of California Irvine
UCSF	University of California San Francisco
UMN	University of Minnesota
USDA	United States Department of Agriculture
UV	Ultraviolet
VISA	Vancomycin Intermediate *Staphylococcus aureus*
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WGS	Whole Genome Sequencing

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ABSTRACT

Staphylococcus aureus is a commensal and pathogen of humans and other animals. Disease with *S. aureus* is complicated by dissemination of antimicrobial resistance, including methicillin resistance. Methicillin resistant *S. aureus* (MRSA) is a significant burden on the health care industry (HA-MRSA); however, in recent years, public health concern has arisen from isolates harbored in the community (CA-MRSA) and livestock species (LA-MRSA). Concerns with LA-MRSA isolates are the direct impact of infection with livestock isolates and indirect impacts of genetic transfer of virulence or antimicrobial resistance genes from LA-MRSA isolates. The prototypical LA-MRSA strain, sequence type (ST) 398, is considered less virulent than HA- and CA-MRSA isolates. Reduced virulence of LA-MRSA ST398 isolates is attributed to loss of human specific virulence factors and reduced colonization and transmission in humans. While LA-MRSA ST398 isolates are common in European swine, LA-MRSA isolates in the United States are diverse including ST398, ST9, and ST5. LA-MRSA ST5 elevated public health concerns, because, unlike MRSA ST398 and ST9, MRSA ST5 is a globally disseminated and highly pathogenic lineage.

To better understand direct and indirect impact of swine associated LA-MRSA ST5, this thesis investigated the genetics of swine associated and clinical MRSA ST5 isolates. Phylogenetic analysis revealed LA-MRSA ST5 isolates are genetically distinct from clinical MRSA ST5 isolates, which was confirmed by differences in virulence and antimicrobial resistance genes harbored on mobile genetic elements. LA-MRSA ST5 isolates lacked immune evasion genes harbored by the β-hemolysin converting

bacteriophage and resistance genes differed between swine associated and clinical MRSA ST5 isolates, which indicated genetic exchange was unlikely between the screened populations. Resistance genes were consistent with selective pressures from antimicrobial use in the swine industry and hospital environment. Finally, swine associated and clinical MRSA ST5 isolates adhered equivalently to human keratinocytes, although LA-MRSA ST5 isolates lacked virulence factors contributing to colonization. These results indicate the isolates screened were distinct with no evidence of mobile genetic element transfer between subsets. The virulence of LA-MRSA ST5 isolates is expected to be reduced as compared to clinical MRSA ST5 isolates due to the absence of genes that contribute to disease in humans.

CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

General Biology of *Staphylococcus aureus*

Staphylococcus aureus is a Gram positive, facultative anaerobe that grows in clusters of coccoid shaped cells. It has been characteristically thought of as non-motile; however, a form of spreading motility was recently reported that involves production of dendrites that enable movement of cells from the primary colony [1]. Though *S. aureus* is non-spore forming, it is highly resistant to various environmental conditions. It is able to grow at a wide range of temperatures, from 7.0-48.5°C with an optimum growth temperature of 30-37°C [2]. *S. aureus* is also tolerant of osmotic stress and highly salt resistant, as it can withstand salt at concentrations of 15% NaCl and readily grows at concentrations up to 10% NaCl [3]. Additionally, it is tolerant to a pH range of 4.2-9.3, with an optimum pH of 7.0-7.5 [4]. These characteristics allow *S. aureus* to grow in a variety of substrates, including various food products and epidermal tissue.

Isolates grow readily on standard laboratory media such as trypticase soy agar and blood agar plates. The colonies are smooth, raised, and translucent [5]. They have a cream to golden color due to the production of staphyloxanthin, a carotenoid pigment that functions in the detoxification of reactive oxygen species [6]. Isolates typically show beta-hemolysis on blood agar plates, which is associated with the production of one of four hemolysins: alpha (α), beta (β), delta (δ), or gamma (γ) [7]. In addition to physiological traits, *S. aureus* is identified using biochemical tests. *Staphylococcus* species are differentiated from *Streptococcus* and *Micrococcus* species using catalase and oxidase testing, in which *Staphylococcus* species are positive and negative, respectively

[8]. The coagulase test is further used to differentiate *S. aureus* from other *Staphylococcus* species, also termed coagulase-negative Staphylococci [5]; however, not all *S. aureus* isolates are coagulase positive [8].

S. aureus Classification and Genetics

Isolates of *S. aureus* are classified in a variety of ways including restriction fragment length polymorphisms (RFLP), multi-locus sequence typing (MLST), and *spa* typing. RFLP uses digestion with a selected restriction enzyme, typically *Sma*I in *S. aureus*, and pulse field gel electrophoresis (PFGE) to resolve a RFLP pattern [9]. This pattern can be compared between isolates to determine similarity and understand the epidemiology of a disease outbreak; however, it is not as sensitive or specific in determining isolate relatedness as genome sequencing techniques.

MLST and *spa* typing are genetic mechanisms of classification. MLST is used to classify *S. aureus* into sequence types and clonal complexes. Sequence types (STs) are defined by sequencing and denoting the specific allele for seven housekeeping genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*) [10]. The specific combination of alleles is compared to the MLST database and used to assign a ST to the isolate. Clonal complexes (CCs) are composed of isolates with a specific ST along with STs that harbor at least 5 of 7 matching alleles and are used to group similar STs [11]. Additionally, lineages defined by a ST or CC generally possess similar characteristics, some of which contribute to the success of these lineages as commensals and pathogens [12].

Another common method to classify *S. aureus* isolates is *spa* typing, which involves sequencing the short sequence repeat region (SSR or X region) of the protein A gene (*spa*) [13]. This region contains a variable number of 24 bp repeats that accumulate diversity through duplication, deletion, and point mutations [14]. This diversity can be utilized to define the *spa* type for the isolate through sequencing of the X region and comparison to the database of identified *spa* types. The *spa* type of an isolate often correlates with a specific ST; however, defining MLST based on *spa* types can be difficult [15-17]. The classifications described here are often used in epidemiologic investigations to determine the relatedness of isolates and detect an isolate's source.

S. aureus is taxonomically classified within the phylum *Firmicutes*, the class *Cocci*, the order *Bacillales*, and the family *Staphylococcaceae*. Similar to other members of the *Firmicutes* phylum, *S. aureus* contains a genome with a low G+C content, which ranged from 32.7-32.9% in 16 evaluated *S. aureus* isolates with closed genomes [18]. The *S. aureus* genome size in the evaluated isolates ranged from 2,742,531 bp to 2,937,129 bp [18]; however, many *S. aureus* isolates carry a diverse array of plasmids that harbor virulence factors and/or antimicrobial resistance genes that impact genome size [19].

The genome of *S. aureus* can be divided into three segments: the core genome, core variable genes, and mobile genetic elements (MGEs) [20-22]. The core genome is highly conserved among all isolates; however, minor variability occurs as single nucleotide polymorphisms (SNPs) that arise from mutations. The core variable genes are lineage associated and will vary between different STs and CCs. Core variable genes with known functions include primarily surface proteins and their associated regulatory genes

[21]. MGEs are the most variable portion of the *S. aureus* genome and comprise around 25% of all genetic material in *S. aureus* isolates [23, 24]. MGEs enable the transfer of fragments of DNA between cells or from the surrounding environment through horizontal gene transfer (HGT).

HGT mechanisms employed by *S. aureus* include transformation, transduction, and conjugation. Transformation occurs in naturally competent bacteria that possess the machinery involved in the uptake of free DNA from the surrounding environment [25]. Transformation is a less common form of HGT in *S. aureus* due to a low level of natural competency that requires induction [26, 27]. Transduction involves bacteriophage mediated transfer of DNA between cells [28]. Bacteriophages can encode virulence genes, such as the immune evasion genes harbored on the β-hemolysin converting bacteriophage [29], and become integrated into the bacterial chromosome as a component of the prophage during the lysogenic cycle [28]. They can also transmit bacterial genetic material from the host cell through generalized transduction, where host genetic material is packaged into the phage capsid rather than the phage genome [28]. Finally, conjugation is the transfer of genetic material from one bacterial cell to another through cellular contact and is the predominant mechanism by which plasmids are transferred [28]. Plasmids are common in *S. aureus* and are known to harbor antimicrobial resistance genes, genes involved in metabolic pathways, and virulence genes including toxins [30-32]

A multitude of MGEs are found in *S. aureus*. These include plasmids, insertion sequences, transposons, pathogenicity islands, genomic islands, chromosomal cassettes, and bacteriophages. These elements often encode virulence genes or antimicrobial

resistance genes that contribute to the success of *S. aureus* in specific environments [24, 30]; however, they are not without cost and isolates tend to exhibit a reduced fitness when selection pressures for MGE maintenance are not present [33-35]. While MGEs are generally widespread in *S. aureus*, it seems some MGEs have strain specificity and are restricted to specific STs or CCs, which is thought to contribute to the success of the lineage [20, 24, 36, 37]. The acquisition of MGEs plays an important role in the evolution of bacteria through large and rapid changes in genomic content that provide a greater degree of genome plasticity and a better chance of improved fitness and organism survival than chromosomal mutation alone [38, 39]. More specifically, for *S. aureus* they have enabled adaptation to a variety of host species, encode virulence factors that contribute to the wide range of disease associated with *S. aureus*, and harbor antimicrobial resistance genes that complicate treatment of clinical infections with *S. aureus* (see below) [24, 30].

S. aureus Colonization and Disease

S. aureus is a commensal bacterium in a wide range of animal species, particularly mammals, although it has been isolated from birds and reptiles [40]. Humans most commonly carry *S. aureus* in the nasal cavity and on the skin, especially that of the hands and perineum [41]. In the United States, the colonization rate in humans during cross sectional studies is around 30% [42, 43]; however, colonization rate varies based on age, socioeconomic status, health status, and geographic location [44, 45].

Colonization of *S. aureus* in humans is categorized into non-carriers, intermittent carriers, and persistent carriers [46, 47]. Non-carriers are resistant to colonization with *S. aureus* and make up around 50% of the population [41]. Humans defined as intermittent

carriers make up about 30% of the population [41]. Intermittent carriers are transiently colonized by *S. aureus* strains they are exposed to [48]. They also tend to have a lower *S. aureus* burden than persistent carriers and consequently shed the bacteria at a lower rate, reducing the risk of infection as compared to persistent carriers [49, 50]. Persistent carriers comprise around 20% of the population [41].They are consistently colonized with the same *S. aureus* strain and shed the bacteria at a much higher rate [48, 50]. To some extent, an individual's propensity for colonization is intrinsic, with the majority of experimentally *S. aureus* exposed humans returning to their original carrier state or the original strain in the case of persistent carriers [51].

Although *S. aureus* is a commensal member of the human nasal microbiome, it has become evident that colonization is a major risk factor for clinical infection with *S. aureus* [52, 53]. This has been seen with both community acquired infections and hospital acquired infections [54-56]. The diseases associated with *S. aureus* range from mild, localized disease to severe, systemic infections. The majority of *S. aureus* infections are mild skin and soft tissue infections that include styes, impetigo, boils, and furunculosis [57, 58]. More severe infections include septicemia, osteomyelitis, endocarditis, meningitis, and necrotizing pneumonia [8]. *S. aureus* also causes toxin mediated diseases including toxic shock syndrome, staphylococcal scalded skin syndrome, and enterotoxicosis [59-61]. Disease caused by *S. aureus* tends to be more severe in persons with comorbidities, such as chronic infections, traumatic injuries, immunosuppression, or indwelling medical devices [8]. This has contributed to the prevalence and prominence of *S. aureus* as a cause of nosocomial infections [62, 63]. The estimated burden of *S. aureus* infections in 2005 was over 400,000 infections requiring

hospitalization in the United States which equated to 1.38% of hospitalized patients receiving a *S. aureus* related diagnosis [64].

S. aureus Virulence Factors and Pathogenesis

Diseases associated with *S. aureus* are caused by a complex and coordinated series of events mediated by virulence factors encoded within the *S. aureus* genome or obtained through HGT of MGE. These virulence factors are associated with adherence to host tissues, biofilm formation, nutrient acquisition, tissue invasion and destruction, toxin production, and evasion of the host immune response. Many virulence factors possess multiple functions and could be placed into several of the above categories, such as the *isd* proteins, which contribute to adherence to host tissues but also play a major role in iron acquisition [65-68]. The following section outlines major virulence genes that contribute to the pathogenesis of *S. aureus* disease.

Adhesion to host tissues

Infection with *S. aureus* requires bacterial-host interaction that begins with adherence. Adherence genes are cell wall anchored proteins that, as a group, are essential for interaction with host tissues and function in the early stages of infection [69]. Proteins involved in adherence are produced in a coordinated manner and this production is influenced by growth conditions and cell density [65, 70]. The regulation of adherence genes by cell density is controlled by the *agr* autoinducer system, such that production of adherence genes occurs under low cell densities and is downregulated to allow for dispersion at higher cell densities [71-74]. The large degree of functional redundancy in adherence genes means the contribution of individual genes to adherence is difficult to

discern; however, the individual contribution of genes such as *isdA*, *clfB*, *sdrC*, and *sdrD* to colonization has been elucidated [75-78].

The genes associated with adherence in *S. aureus* can be grouped into four main categories: microbial surface component recognizing adhesive matrix molecules (MSCRAMM), near iron transporter motif (NEAT motif), three-helical bundle, and G5-E repeat family (Table 1.1). MSCRAMMs of *S. aureus* are defined by a structure containing immunoglobulin-like subdomains and domains used in ligand binding [75, 79]. The primary function of MSCRAMMs is for attachment to components of the extracellular matrix (ECM) including fibrinogen, fibronectin, and bone sialoprotein [80]; however, many of these genes have additional functions including immune evasion, biofilm production, and others [81-84]. The NEAT motif family of proteins bind to fibrinogen, fibronectin, cytokeratin 10, GPIIb/IIIa receptor, and haptoglobin [67, 68, 85, 86]. Outside of their role of adherence, the NEAT motif proteins also function in iron uptake through heme transfer and aid in survival in iron depleted environments within the host [65, 66]. The adherence factors classified as three-helical bundle proteins are named for their structure and contain modules formed by three helical bundles, which function in ligand binding [87]. Protein A, the major member of this group, is ubiquitous in *S. aureus* and functions in binding to von Willebrand factor [88]. Protein A also binds to the conserved region of the immunoglobulin IgG, which contributes to immune evasion through inhibition of phagocytosis and complement fixation [87]. G5-E repeats, the final group of *S. aureus* adhesion genes, is characterized by domains of five glycine residues separated by E regions and the main member is *sasG* [89]. The *sasG* gene has been identified to function in adherence to nasal epithelium and biofilm formation [90, 91]

Many additional adherence genes are structurally uncharacterized (Table 1.1). These proteins have been associated with a variety of functions including binding to ECM components (*emp*, *aaa*, *eap*), platelet interaction (*sraP*) and biofilm formation (*bap*, *isaB*) [84, 92-96].

Table 1.1: Adherence genes in *S. aureus*.

Adherence gene	Ligand	Reference
Microbial surface component recognizing adhesive matrix molecules (MSCRAMM)		
clfA and *clfB*	Fibrinogen	[97]
sdrC	β-neurexin	[98]
sdrD	Not yet determined	[99]
sdrE	Factor H	[81]
bbp	Bone sialoprotein	[100]
fnbpA and *fnbpB*	Fibronectin	[101]
cna	Collagen	[102]
Near iron transport motif (NEAT motif)		
isdA	Fibrinogen, fibronectin, cytokeratin 10	[67, 85]
isdB	GPIIb/IIIa (platelet receptor)	[86]
isdH	Haptoglobin	[68]
Three-helical bundle		
spa	Von Willebrand factor, immunoglobulin	[87, 88]
G5-E repeat		
sasG	Not yet determined	[91, 103]
Structurally uncharacterized adherence genes		
sasX	Not yet determined	[104]
sraP	N-acetylneuramic acid	[95, 105]
sasC	Not yet determined	[82]
sasB, *sasD*, *sasF*, *sasJ*, *sasK*, *sasL*	Not yet determined	[91]
bap	GP96	[84, 96]
vwb	von willebrand factor	[106]
emp	Fibrinogen, fibronectin, collagen, vitronectin	[93]
isaB	Nucleic acid	[107]
aaa	Fibrinogen, fibronectin	[92]
eap	ECM and plasma protein (broad specificity)	[94]
ebps	Elastin	[108]
ebh	Fibronectin	[109]
efb	Fibrinogen	[110]
eno	Laminin	[111]

Biofilm formation

Biofilm formation is an additional, important factor contributing to adherence of *S. aureus* to host tissues and fomites, including medical devices [112]. Biofilm formation is a coordinated series of events involving initial attachment, maturation, and later dispersal [113]. *S. aureus* attaches using the protein adhesion molecules described above [114, 115]. Maturation of the biofilm occurs through cell aggregation followed by structural changes that lead to the formation of a three-dimensional structure [112]. Aggregation of *S. aureus* is predominantly mediated by the *ica* operon, which encodes production of polysaccharide intercellular adhesin (PIA) [116]. Biofilms function in adherence and persistence of *S. aureus* in many environments and have a protective role through resistance to desiccation, phagocytosis, antimicrobials, and disinfectants [112].

Nutrient acquisition

Within the host, nutrients such as iron are not readily available and therefore limit bacterial growth. Because of the necessity of iron in bacterial proliferation, *S. aureus* has evolved two mechanisms to increase iron availability within the host. The first mechanism of iron acquisition is the production of siderophores. *S. aureus* produces staphyloferrin A and B siderophores that remove iron from host transferrin molecules [117]. *S. aureus* also obtains iron using the *isd* proteins to transport hemoglobin and heme across the cell wall [65]. These proteins promote iron acquisition within hosts, which allows *S. aureus* to proliferate and survive within host tissues.

Invasion and tissue destruction

Invasion and tissue destruction follow the proliferation of *S. aureus* during infection. The production of exoproteins involved in this stage of infection is regulated by

growth phase and cell density, which limits their production until *S. aureus* has reached high cell densities found in late exponential phase [71-74]. Exoproteins produced by *S. aureus* enable invasion of deeper tissues and results in destruction of surrounding tissue [118-121]. The enzymes involved in this process are proteases, lipases, and nucleases [122-124]. Protease activity is conferred by serine proteases, cysteine proteases, and metalloproteases [122], which play a role in tissue damage and dissemination of *S. aureus* [122]. Lipases contribute to abscess formation and the survival and proliferation of *S. aureus* in organs, such as the liver and kidney [123]. Finally, nucleases are important in surviving within abscesses and evading neutrophil extracellular traps (NETs) [124]. *S. aureus* is also capable of cellular invasion, which is directed by the adhesion genes *isdB, fnbpA*, and *fnbpB* [125-128].

Toxin mediated disease

S. aureus produces many toxins that are an important component of the disease process. These toxins function in cellular cytotoxicity or directly cause clinical signs through cell activation or protease activity. The cytolytic toxins cause the release of nutrients from host cells, such as iron, and function in immune evasion through the lysis of immune cells [7, 129]. The mechanism of action and cell specificity of the cytolytic toxins can be found in Table 1.2.

Table 1.2: Toxins produced by *S. aureus* and their function.

Toxin gene	Mechanism of action	Cell type affected	Reference
Cytolytic toxins			
α-hemolysin	Pore formation through heptamer integration in membrane	Erythrocytes, macrophages, lymphocytes	[130-133]
β-hemolysin	Membrane disruption through sphingomyelinase activity	Erythrocytes	[132]
γ-hemolysin	Bi-component leukocidin – pore formation by four slow and four fast proteins	Leukocytes	[134, 135]
δ-hemolysin	Peptides cause membrane disruption at high density	Non-specific – erythrocytes, leukocytes	[136, 137]
Phenol soluble modulin	Peptides cause membrane disruption at high density	Non-specific – erythrocyte, leukocytes	[138, 139]
Panton Valentine leukocidin	Bi-component leukocidin – pore formation by four slow and four fast proteins	Leukocytes	[134, 140]
LukED, LukGH	Bi-component leukocidin – pore formation by four slow and four fast proteins	Leukocytes	[141-143]
Non-cytolytic toxins			
Toxic shock syndrome toxin (TSST-1)	Non-specific binding to the major histocompatibility complex molecule	CD4 T cells	[144]
Enterotoxin	Induction of emesis and diarrhea, gastrointestinal inflammation, non-specific binding to the major histocompatibility complex molecule	Epithelium of the gastrointestinal tract, CD4 T cells	[145-147]
Enterotoxin-like proteins	Non-specific binding to the major histocompatibility complex molecule	CD4 T cells	[148-150]
Exfoliative toxins	Serine protease targeting the desmosomal proteins involved in cell-cell adhesion within the skin	Keratinocyte	[120, 151, 152]

Non-cytolytic toxins produced by *S. aureus* include toxic shock syndrome toxin 1 (TSST-1), the enterotoxins and enterotoxin-like proteins, and exfoliative toxins (Table 1.2). TSST-1 is the primary mediator of toxic shock syndrome (TSS), a disease characterized by fever, rash, organ failure, shock, and death [153]. TSST-1 is a superantigen that acts through a non-antigen-specific mechanism to activate of 5-30% of CD4 T cells within the host [144, 154]. The activation of CD4 T cells causes the release of cytokines, which stimulate vasoactivation and lead to shock [144, 155]. Enterotoxins, another group of non-cytolytic toxins, cause vomiting and diarrhea associated with foodborne intoxication due to *S. aureus* [156]. Enterotoxins and enterotoxin-like proteins, similar to TSST-1, are superantigens and also activate T cells in a non-antigen-specific manner [157, 158]; however, enterotoxins are also capable of inducing emesis and gastrointestinal inflammation [145-147], while enterotoxin-like proteins either lack the ability to induce vomiting or are untested for this ability [159]. Finally, exfoliative toxins are the cause of staphylococcal scalded skin syndrome (SSSS). Clinical signs of SSSS include fever, lethargy, and skin exfoliation that begins with large blisters that rupture, removing the epidermal layer [160]. The exfoliative toxins, *eta* and *etb*, are serine proteases that degrade desmosomal proteins in the skin with a high degree of specificity [120, 151, 152]. Desmosome degradation causes a loss of cell-cell adhesion within the skin leading to the formation of blisters [161, 162]. Toxin production in *S. aureus*, similar to exoproteins, is regulated by cell density and they are produced in late exponential phase when cell densities peak [71-74].

Immune evasion

S. aureus subverts the host immune response through the production of many immune evasion factors. These act through cell lysis, promoting dysfunction of the cellular immune response, inhibition of complement and antibody mediated actions, and detoxification of lethal compounds (Table 1.3). As described, *S. aureus* produces a variety of toxins that disrupt the cellular immune response through the lysis of innate and adaptive immune cells (Table 1.2). Additionally, the adaptive cellular immune response is subverted by *S. aureus* through the superantigen toxins described above [144, 155, 158], which cause aberrant T cell activation and prevent a coordinated response to *S. aureus*. Immune evasion proteins from *S. aureus* are also able to act on the cells of the innate immune system to prevent leukocyte migration (*eap, chp*) and reduce uptake and killing by phagocytes (capsule, *asdA*) [163-167]. The humoral immune response is inhibited by *S. aureus* through genes that disrupt the action of complement (*sak, scn, ecb*) or bind IgG to the cell surface, preventing complement activation and receptor mediated phagocytosis (opsonization) (*sbi, spa*) [168-175]. Finally, *S. aureus* possess mechanisms to detoxify lethal compounds produced by the host including reactive oxygen species (*katA, aphC, sod*), defensins (*aur*), and polyamines (*speG*) [176-179]. Immune evasion genes of *S. aureus* act to evade both the innate and adaptive arms of the immune system and ensure *S. aureus* is able to survive and proliferate within a host.

Table 1.3: Immune evasion genes in *S. aureus*.

Immune evasion gene	Function	Reference
eap (extracellular adhesion protein)	Prevents neutrophil migration by binding receptors on endothelial cells	[163, 164]
chp (chemotaxis inhibitory protein)	Prevents neutrophil migration through blockade of the C5a receptor and formyl receptor	[165]
fll (FPR-like1 inhibitory protein)	Prevents neutrophil migration through blockade of the formyl receptor	[180]
asdA (adenosine synthetase A)	Adenosine synthesis – prevents killing by neutrophils	[167, 181]
Capsule	Polysaccharide capsule production – prevents deposition of complement and antibody, antiopsonic	[166]
flr (FPR-like1 inhibitor protein-like)	Interrupts of the complement cascade	[172]
ecb (extracellular complement-binding protein)	Interrupts of the complement cascade	[171, 172]
efb (extracellular fibrinogen-binding protein)	Interrupts of the complement cascade	[173]
scn (staphylococcal complement inhibitor)	Interrupts of the complement cascade	[168]
sak (staphylokinase)	Binds defensins preventing cell membrane disruption, promotes degradation of the opsonins IgG and C3b reducing phagocytosis	[169, 170]
sbi (*S. aureus* binder of IgG)	Sequesters IgG through binding to the constant region, disrupts the complement cascade	[173, 174]
spa (protein A)	Sequesters IgG through binding to the constant region	[175]
ACME – *arc* gene cluster	Arginine deaminase – raises pH enabling survival on the skin, depletes arginine available for nitric oxide production in phagocytes	[182, 183]
ACME – *speG*	Polyamine degradation – enables survival on the skin	[176]
aur (auerolysin)	Zinc metalloprotease – degrades defensins	[177]
katA (catalase), *aphC* (alkyl hydroperoxide reductase	Detoxification of hydrogen peroxide	[178]
sod (superoxide dismutase)	Detoxification of superoxide radicals	[179]
Staphyloxanthin	Carotenoid pigment – resistance to reactive oxygen species	[6]

Drug Resistance in *S. aureus*

Prior to the introduction of penicillin, invasive *S. aureus* infections had a mortality rate exceeding 80% [184]. The discovery and application of antibiotics has significantly reduced the mortality rate associated with invasive infections; however, mortality for *S. aureus* septicemia currently ranges from 15-50% depending on the original location of the infection [185, 186]. The burden of *S. aureus* is complicated by inappropriate antimicrobial selection and treatment failures due to antimicrobial resistance (AMR) [187, 188]. Treatment failures worsen clinical prognosis and cause increased health care costs [187, 188].

Antibiotics are considered bacteriostatic, which inhibit bacterial replication, or bactericidal, which cause bacterial cell death [189]. They act by disrupting critical cell functions including: cell wall synthesis, protein synthesis, nucleic acid synthesis (DNA or RNA), folic acid synthesis, and cell membrane integrity [190]. AMR mechanisms prevent or inhibit the effects of antibiotics and can be intrinsic to the organism or acquired through mutation of chromosomal genes or acquisition of MGEs through HGT [191]. Intrinsic or innate resistance provides resistance to an entire class of antibiotics [191]. An example of this form of resistance is the ineffectiveness of metronidazole as a treatment for infections with facultative anaerobes, such as *S. aureus* [192]. Metronidazole causes cellular damage through toxic intermediates that are generated during reduction of the drug; however, this process requires metabolic pathways that are present predominantly in anaerobic bacteria and absent in *S. aureus* [193]. Alternatively, acquired resistance develops through the use of antibiotics. Antimicrobial exposure applies selective pressures that encourage the development of AMR through mutation or acquisition of

AMR genes harbored on MGE [191]. As a subpopulation of resistant organisms develops, continued use of the antibiotic allows only resistant organisms to proliferate, generating a dominant population of resistant organisms. Acquired AMR mechanisms can be divided into four categories: reduced antibiotic uptake, increased antibiotic efflux, antibiotic modification, and target replacement or modification [191]. Examples of these categories follow below and a more complete collection of genes conferring AMR in *S. aureus* listed in Table 1.4.

AMR associated with reduced antibiotic uptake is more common in Gram negative organisms due to their cell envelope structure and is commonly associated with changes in porin structure or selectivity [194, 195]. An example of this resistance mechanism in *S. aureus* is involved in intermediate vancomycin resistance. Vancomycin intermediate *S. aureus* (VISA) produce a peptidoglycan layer with reduced cross linking that generates an irregular and thickened cell wall with increased exposure of the vancomycin target, D-alanine-D-alanine [196]. Vancomycin binds the exposed residues that reduces the concentration of antibiotic reaching the cell membrane and contributes to survival of VISA with vancomycin exposure [196].

A second mechanism for resistance works through active removal of antibiotics from the cell. Efflux pumps are employed to increase antibiotic extrusion from the cell and protect intracellular targets. Acquired mechanisms of increased antibiotic efflux are due to mutation of chromosomally encoded efflux pumps or acquisition of novel efflux pumps through HGT. In *S. aureus*, low level fluoroquinolone resistance is associated with induction of the chromosomally encoded efflux pump *norA* [197, 198]. Mutations in the promoter or operator region found upstream of *norA* increases protein expression,

which increases efflux of fluoroquinolones from the cell [197, 199-201]. Alternatively, antibiotic efflux genes can be acquired through HGT of MGE encoding efflux pumps, such as the tetracycline resistance genes *tetK* and *tetL* [202].

The third mechanism of resistance involves altering the antibiotic to reduce its effect on the cell. Enzymes modifying or degrading antibiotics are widespread in *S. aureus* isolates. Prominent examples of such enzymes are the beta-lactamase, *blaZ*, and aminoglycoside modifying enzymes. The *bla* operon is found in 90% of *S. aureus* isolates [203], which explains the widespread prevalence of penicillin resistance. It inactivates beta-lactam antibiotics through hydrolysis of the beta-lactam ring [204]. This gene and its regulatory elements are often found on plasmids that also encode resistance to other compounds, such as disinfectants, dyes, heavy metals, or other antibiotics [205-207]. The beta-lactamase operon has also been identified on a transposon integrated into the chromosome [208]. Another example of antibiotic alteration is the aminoglycoside modifying enzymes, which change antibiotic structure and therefore prevent the antibiotic-target interaction. These enzymes act through acetylation, adenylation, or phosphorylation and are deemed aminoglycoside acetyltransferases (AAC), aminoglycoside nucleotidyltransferases (ANT), and aminoglycoside phosphotransferases (APH), respectively [209]. Nomenclature for aminoglycoside modifying enzymes is based on their mechanism of action with alphanumeric modifiers that indicate the location of modification and resistance profile, for example APH(3')-IIIa is a phosphorylase acting on the hydroxyl group present on the carbon at position 3' of the antibiotic [210, 211].

Table 1.4: AMR genes of interest identified in *S. aureus* and their mechanism of action

Antibiotic Class	Resistance Gene	Mechanism of Action	Type of Resistance	Transmission	Reference
Glycopeptide	*vanA*	Catalyzes formation of D-alanine-D-lactate bridges	Target replacement	Plasmid	[212, 213]
	Unknown VISA induction	Alteration in cell wall structure – binding of vancomycin	Reduced uptake	Chromosomal	[196]
Fluoroquinolone	*norA*	Induction of chromosomal efflux pump	Antibiotic efflux	Chromosomal	[197, 199, 201]
	parC, *gyrA*, *parE*, *gyrB* mutation	Alteration in the fluoroquinolone binding site	Target modification	Chromosomal	[214]
Beta-lactam	*blaZ*	Beta-lactam hydrolysis	Antibiotic degradation	Chromosomal or plasmid	[204, 205, 208]
	mecA, *mecC*	Alternative penicillin binding protein	Target replacement	Chromosomal	[215, 216]
Tetracycline	Ribosomal protection – *tetM*, *tetO*	Interaction with ribosomal protein and allosterically blocking the antibiotic binding site	Target modification	Chromosomal or plasmid	[217-219]
	Antibiotic efflux – *tetK*, *tetL*	Removal of antibiotic from the cell	Antibiotic efflux	Chromosome or plasmid	[202, 218]
Streptogramin A	Acetyltransferase – *vat*	Acetylation of antibiotic preventing interaction with target	Antibiotic modification	Plasmid	[220, 221]
Streptogramin B	Lactonase – *vgb*	Hydrolysis of the lactone ring	Antibiotic degradation	Plasmid	[220, 222]
Trimethoprim / Sulfonamide	Mutation in dihydropteroate synthase	Changes in protein sequence that alter drug binding	Target modification	Chromosomal	[223]
	Dihydrofolate reductase – *dfr*	Alternative dihydrofolate reductase with reduced binding to trimethoprim	Target replacement	Chromosomal or plasmid	[224]

Table 1.4 Continued

Antibiotic Class	Resistance Gene	Mechanism of Action	Type of Resistance	Transmission	Reference
Carbolic acid (mupirocin)	*mupA* and *mupB*	Alternative isoleucyl-tRNA synthetase	Target replacement	Plasmid	[225, 226]
	ileS mutation	Mutation alters ability of antibiotic to bind	Target modification	Chromosomal	[227]
Aminoglycoside	Aminoglycoside acetyltransferase	Acetylation of antibiotic preventing interaction with target	Antibiotic modification	Chromosomal or plasmid	[209]
	Aminoglycoside nucleotidyltransferae	Adenylation of antibiotic preventing interaction with target	Antibiotic modification	Chromosomal or plasmid	[209]
	Aminoglycoside phosphotransferase	Phosphorylation of antibiotic preventing interaction with target	Antibiotic modification	Chromosomal or plasmid	[209]
Phenicol	*fexA*	Removal of antibiotic from the cell	Antibiotic efflux	Chromosomal	[228, 229]
	Chloramphenicol acetyltransferases (CAT)	Acetylation of antibiotic preventing interaction with target	Antibiotic modification	Plasmid	[230] t
Lincosamide	lnuA, lnuB	Transfer of a nucleotide to the antibiotic preventing interaction with target (nucleotidyltransferase)	Antibiotic modification	Chromosomal or plasmid	[231, 232]
Macrolide	msrA, msrB	Removal of antibiotic from the cell	Antibiotic efflux	Plasmid	[233]
	mphC	Phosphorylation of antibiotic preventing interaction with target	Antibiotic modification	Plasmid	[234]

Table 1.4 Continued

Antibiotic Class	Resistance Gene	Mechanism of Action	Type of Resistance	Transmission	Reference
Multi-drug resistance genes					
Phenicol, lincosamide, oxazolidinone, pleuromutilin, streptogramin A	*cfr*	rRNA methylation to prevent antibiotic interaction	Target modification	Plasmid	[235, 236]
Macrolide, lincosamide, streptogramin B	*erm* genes	rRNA methylation to prevent antibiotic interaction	Target modification	Chromosomal or plasmid	[237, 238]
Lincosamide, pleuromutilin, streptogramin A	*lsaE*	Removal of antibiotic from the cell	Antibiotic efflux	Plasmid	[239]
Lincosamide, pleuromutilin, streptogramin A	*vga* genes	Removal of antibiotic from the cell	Antibiotic efflux	Chromosomal or plasmid	[240, 241]

The fourth and final mechanism of AMR is target replacement or modification, which is a widespread mechanism of resistance employed for many antibiotic classes. In *S. aureus*, the most well-known example is methicillin resistance encoded by the *mecA* gene (described in the Methicillin resistance section); however, this form of resistance is also employed in resistance to vancomycin, fluoroquinolones, and other antibiotics. Target modification is also the mechanism by which the multi-drug resistance gene *cfr* confers AMR. Vancomycin resistant *S. aureus* (VRSA) is mediated by the *vanA* gene, which is harbored on a plasmid highly similar to that found in vancomycin resistant *Enterococcus* species [242]. The *vanA* gene encodes production of D-alanine-D-lactate cross bridges, to which vancomycin is unable to bind [212, 213]. Alternatively, fluoroquinolone resistance is acquired through chromosomal mutation to the quinolone resistance determining region (QRDR) of DNA gyrase or DNA topoisomerase IV [197, 214]. Mutations in the QRDR reduce the ability of fluoroqinolones to bind to their target and alter the binding stability of the fluoroquinolone-target interaction [243, 244]. The *cfr* gene is another example of target modification conferring AMR in *S. aureus*. It encodes an rRNA methylase that modifies the adenine found at position 2503 in the 23S rRNA [235]. This causes multi-drug resistance by preventing ribosomal binding of antibiotics from the lincosamide, streptogramin A, phenicol, pleuromutalin, and oxazolidinone classes [236].

As discussed, selective pressures associated with antimicrobial exposure contribute to the development or acquisition of AMR genes. This has been seen in the health care industry where the use of penicillin and methicillin rapidly led to the presence and prevalence of *blaZ* and *mecA* in the hospital setting [245, 246]. These genes are

further disseminated generating “waves” of resistance that begin in the health care setting and spread into the community [247]. More recently, livestock production has been identified as a potential reservoir for AMR genes including *mecA* (see LA-MRSA characteristics section) and uncommon AMR genes, such as *cfr* and *vgaC* [248-251]. The diversity of AMR genes identified in *S. aureus* isolates from livestock species has triggered public health concerns surrounding antimicrobial use in the livestock industry and has encouraged continued monitoring for the development and dissemination of additional AMR genes [252, 253].

Methicillin Resistance

S. aureus isolates expressing resistance to methicillin are termed methicillin resistant *S. aureus* or MRSA. Resistance to methicillin was first reported in *S. aureus* in 1961 in an English hospital [246]. Clones of MRSA disseminated within the health care setting and over the next 30 years became a global burden for the health care industry that continues today [247]. In the time since its discovery, the impact of MRSA has broadened beyond the hospital setting and now causes community acquired infections as well as hospital acquired infections (see MRSA Epidemiology).

Methicillin resistance in *Staphylococcus* species is conferred through the acquisition of the Staphylococcal Cassette Chromosome *mec* (SCC*mec*) element, a MGE harboring the *mecA* gene [254]. SCC*mec* elements vary in size, which range from 21-67 kb, and composition through the presence of additional MGEs within the element including transposons and plasmids encoding other resistance genes [255]. The defining features of the SCC*mec* element are the *ccr* genes that function in recombination and the *mec* gene complex, which functions in methicillin resistance. These components are used

to classify SCC*mec* elements into 12 currently identified types (Table 1.5), with subtypes defined by the joining regions (J1-J3) oriented between chromosomal DNA, the *mec* gene complex, and the *ccr* genes (Figure 1.1) [256].

Table 1.5: Identified SCCmec elements and their associated ccr gene complex and mec gene compex. (Adapted from http://www.sccmec.org/)

SCC*mec* type	*ccr* gene complex	*mec* gene complex
I	Type 1	Class B
II	Type 2	Class A
III	Type 3	Class A
IV	Type 2	Class B
V	Type 5	Class C2
VI	Type 4	Class B
VII	Type 5	Class C1
VIII	Type 4	Class A
IX	Type 1	Class C2
X	Type 7	Class C1
XI	Type 8	Class E
XII	Type 9	Class C2

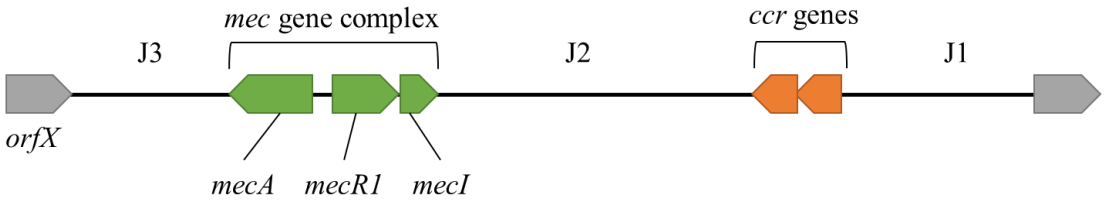


Figure 1.1: Structure of the SCCmec element. The SCC*mec* element is composed of the *mec* gene complex and the *ccr* genes. The *ccr* genes function to integrate the SCC*mec* element into the chromosome at *orfX* and the *mec* gene complex encodes the alternate penicillin binding protein (*mecA*) and the associated regulatory elements (*mecR1* and *mecI*). SCC*mec* elements are typed based on the *ccr* genes and the *mec* gene complex, with subtype of each being defined by the joining regions (J1-J3) indicated below. J1 is the region between the chromosome and the *ccr* genes. J2 is located between the *ccr* genes and the *mec* gene complex. J3 is the sequence found between the *mec* gene complex and the chromosome.

The *ccr* genes are present in the SCC*mec* element either as a combination of *ccrA*/*ccrB* alleles or *ccrC* allele alone (Table 1.6). They generate proteins that are involved in integration and excision of the SCC*mec* element in a site specific manner at the *orfX* gene [254]. The *mec* gene complex is composed of one or two insertion sequence(s), *mecA*, a complete or partial *mecR1*, and *mecI* in class A *mec* gene

complexes (Table 1.7). The *mecA* gene encodes an alternate penicillin binding protein (PBP2a), which has a lower binding affinity for β-lactam antibiotics and a reduced rate of inactivation through β-lactam mediated acetylation [215, 216]. PBP2a catalyzes the formation of cell wall cross bridges but is not inactivated by methicillin or other penicillin class antibiotics, preventing the bactericidal effects of these antibiotics [257, 258].

Table 1.6: Identified ccr gene complexes and their associated ccr alleles. (Adapted from http://www.sccmec.org/)

ccr gene complex	*ccr* genes
Type 1	A1B1
Type 2	A2B2
Type 3	A3B3
Type 4	A4B4
Type 5	C1
Type 6	A5B3
Type 7	A1B6
Type 8	A1B3
Type 9	C2

Table 1.7: Identified mec gene complexes and their composition. (Adapted from http://www.sccmec.org/)

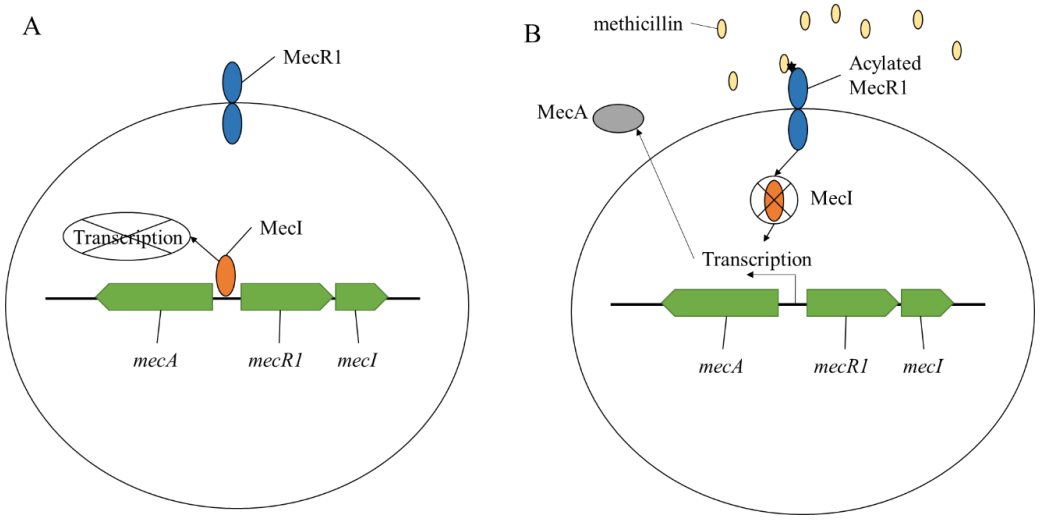
mec gene complex	Genes composing
Class A	IS*431-mecA-mecR1-mecI*
Class B	IS*431-mecA-ΔmecR1*-IS*1272*
Class C1	IS*431-mecA-ΔmecR1*-IS*431* [a]
Class C2	IS*431-mecA-ΔmecR1*-IS*431* [b]
Class D	IS*431-mecA-ΔmecR1*
Class E	*blaZ-mecA*$_{LGA251}$*- mecR1*$_{LGA251}$*- mecI*$_{LGA251}$ [c]

[a] – IS*431* in Class C1 are oriented in the same direction
[b] – IS*431* in Class C2 are oriented in opposing directions
[c] – *mecA*$_{LGA251}$ is also known as *mecC*

Production of *mecA* is regulated by *mecI* and *mecR1*, the transcriptional repressor and signal transduction protein encoded in the *mec* complex, respectively (Figure 1.2). The *mecI* gene is constitutively produced and binds to a 30 bp target found upstream of the *mecA* gene [259]. Through binding, *mecI* causes steric hindrance and prevents

polymerase access to the *mecA* gene, which results in marked reductions in transcription and translation [260]. The *mecR1* gene is a signal transduction protein that detects β-lactam antibiotics in the environment surrounding the cell. It is a homolog of and functions similarly to *blaR1*, the signal transduction protein associated with the β-lactamase gene *blaZ* [261]. The extracellular domain of the signal transducer is acetylated through contact with β-lactam antibiotics [261], which activates the cytoplasmic protease domain resulting in autocatalytic cleavage [204]. The proteolytic activity results in downstream cleavage of the transcriptional repressor, *mecI*, and releases the repression of the *mecA* gene [204]. Deletion of the *mecI* gene is seen in the Class B, Class C1, Class C2, and Class D *mec* gene complexes (Table 1.7). For these classes, *mecA* transcription is regulated by the repressor (*blaI*) and signal transducer (*blaR1*) from the β-lactamase operon or, if the *blaZ* locus is absent, constitutive expression of *mecA* is seen [260, 262].

Figure 1.2: Regulation of the mecA gene. The transcriptional repressor, MecI, binds upstream of the *mecA* gene in the absence of methicillin. Through this binding, MecI provides steric hindrance preventing RNA polymerase from accessing the *mecA* gene. When methicillin is present, MecR1 is activated through acetylation and cleaves MecI. In the absence of intact MecI, RNA polymerase can bind the promoter allowing transcription of *mecA* and the expression of phenotypic methicillin resistance.



More recently, a novel *mecA* homologue conferring methicillin resistance has been discovered in livestock isolates [263]. This gene has been named $mecA_{LGA251}$ or

mecC and was found to be 70% identical to the traditional *mecA* gene [263]. It is harbored by the class E *mec* gene complex, which is found within the type XI SCC*mec* element (Table 1.5 and 1.7).

The SCC*mec* element is hypothesized to have originated in coagulase negative *Staphylococcus* species, such as *Staphylococcus scuri* or *Staphylococcus haemolyticus* [264, 265]. Isolates of *S. scuri* were found to harbor a close homologue of the *mecA* gene, which is phenotypically silent in these isolates [264]. Evidence implicating *S. haemolyticus* as the source of SCC*mec* is associated with IS*1272*, an insertion sequence found in the Class B *mec* gene complex (Table 1.7), which is widely distributed in *S. haemolyticus* isolates [265]. Although the origin of the SCC*mec* element in *S. aureus* has not been definitively determined, the findings of these studies suggest that methicillin resistance originated in coagulase negative *Staphylococcus* species and was later transferred to *S. aureus* through acquisition of the SCC*mec* element.

MRSA Epidemiology

MRSA, similar to methicillin susceptible *S. aureus* (MSSA), is also found to colonize humans, although in most instances at a much lower rate than that seen for MSSA colonization. In 2008, the colonization rate of MRSA in the general population in the United States was 1.5% [42]; however, subsets of the population are at increased risk for colonization, such as healthcare workers, livestock caretakers and others (Table 1.8) [183]. There is evidence that MRSA isolates are able to take over the niche currently occupied by MSSA isolates, which has been noted as an increase in MRSA colonization with an associated decrease in MSSA colonization over time [266].

Table 1.8: Populations at risk for MRSA colonization. (Adapted from David and Daum, 2010 [183])

At risk individuals	Reference
Neonates and children	[267, 268]
Athletes (especially contact sports)	[269, 270]
Incarcerated individuals	[271]
Indigenous individuals	[272]
Military personnel	[273]
Veterinarians and livestock caretakers	[248, 274]
Individuals of low socieoeconomic status	[275]
HIV or AIDS affected individuals – intervenous drug users, men with male partners	[276-278]
Patients with cystic fibrosis	[279]
Household contacts of MRSA patients	[140, 280]
Emergency room patients	[281]
Health care professionals	[282]

MRSA isolates are classified based on epidemiologic characteristics into hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock associated (LA-MRSA). The predominant characteristic that defines each subset is the location from which the isolate is obtained, such that HA-MRSA isolates are obtained from contact with health care settings, CA-MRSA isolates are associated with sources outside of the health care setting, and LA-MRSA isolates are acquired from livestock species. The epidemiologic classifications are an important aide in the selection of antimicrobial therapy and play a role in understanding the virulence capacity of the isolate [283]. However, traditional definitions have blurred in recent years with HA-MRSA isolates circulating in the community and CA-MRSA isolates causing outbreaks in the health care setting [183]. This has led to a new scheme to define isolates based on other characteristics including: location of infection onset, recent health care exposure, risk factors associated with CA-MRSA colonization, genetic characteristics, AMR profile, and MRSA associated clinical syndrome [183, 284]. These characteristics are defined for each subset in Table 1.9.

Table 1.9: General characteristics of HA-, CA-, and LA-MRSA isolates

Characteristic	**HA-MRSA isolates**	**CA-MRSA isolates**	**LA-MRSA isolates**
Location of disease onset	Health care setting	Outside of health care setting	Outside of health care setting
Recent health care exposure	Yes	No	No
Risk factors associated with CA-MRSA	No	Yes – see Table 1.8	Yes – livestock contact
Health status of patient	Comorbidities present	Normal, healthy individual	Often possess comorbidities – such as an open wound
Virulence factors	Limited – PVL negative, ACME negative	More commonly harbor multiple virulence factors (PVL, ACME)	Typically lack human specific virulence factors (PVL, β-hemolysin converting bacteriophage, enterotoxins)
Antimicrobial resistance profile	Resistant to many non-β-lactam antibiotics	Susceptible to the majority of non-β-lactam antibiotics	Commonly resistant to tetracycline class antibiotics
SCC*mec* element	Type I-III	Type IV-V	Type III-V and many untypable
Common clinical syndrome	Severe, invasive infections	Skin and soft tissue infections	Skin and soft tissue infections

Another method that can aid in differentiating these subsets is MLST. As previously mentioned, MLST is an important tool used to define genetic lineages. Specific lineages have found success in different settings and some STs seem to possess characteristics that better enable them to adapt to their environment through the acquisition of MGE [12]. One example of this is the ST8 strain identified as USA300. The success of this CA-MRSA clone has been attributed to some of the MGEs it acquired, specifically the acquisition of ACME [182]. There are additional STs that have adapted to unique settings, such as the predominance of ST398 and ST9 in livestock species; however, there are also lineages that can persist in any environment and are found to thrive in both the hospital and community settings, such as the ST5 lineage [12, 285].

MRSA was first identified in the hospital setting in 1961 and developing strains of MRSA remained confined to this setting until the 1980s [246, 286, 287]. While initially discovered in Britain, dissemination occurred rapidly and the first outbreak of HA-MRSA in the United States was reported in 1968 [288]. HA-MRSA isolates were initially localized within the health care setting and were predominantly detected in patients with comorbidities [183, 289]. These patients tend to develop more severe disease associated with *S. aureus*, which is attributed to the immunocompromised state of many of these patients and the use of indwelling medical devices, such as intravenous catheters, that increase the risk of invasive infections [290]. The prevalence of MRSA in health care settings has increased since its introduction in both number of infections and as a percent of the total *S. aureus* infections, with over 60% of all *S. aureus* isolates reported in hospitals in 2004 [291, 292]. HA-MRSA isolates are characterized by

harboring the larger SCC*mec* elements (types I-III) and displaying phenotypic AMR to a wider range of antibiotics outside the β-lactam class [183, 293, 294]. Though HA-MRSA isolates tend to cause more severe clinical syndromes, they often lack virulence factors such as PVL and ACME [183].

The first reports of CA-MRSA were sporadic outbreaks in the 1980s and early 1990s [286, 287, 295-297]. CA-MRSA isolates have disseminated in the community setting since the mid-1990s and it is currently thought that around 90% of diagnosed MRSA infections are community onset [183, 298, 299]. In contrast to HA-MRSA, CA-MRSA infections occur in people with no recent exposure to the health care setting and tend to present as skin and soft tissue infections in healthy, younger individuals [300, 301]. CA-MRSA isolates are characterized by smaller SCC*mec* elements (type IV and V) and are generally susceptible to most of the non-β-lactam antibiotics [183]. Due to their ability to cause disease in otherwise healthy individuals, CA-MRSA isolates are considered more virulent and tend to harbor additional virulence factors found on MGE such as PVL and ACME [182, 183, 302]. These isolates have been detected in both urban and rural settings [268, 303, 304], but they tend to be maintained in locations with high density populations or where close contact between individuals occurs. Known CA-MRSA reservoirs include prisons, athletic centers, schools, and daycare centers [269, 305, 306]. It appears that CA-MRSA has added to the health care burden of *S. aureus* because the dissemination of CA-MRSA has been associated with a concomitant increase in doctor visits for abscesses, cellulitis, and other skin and soft tissue infections [307].

LA-MRSA is often considered a subset of CA-MRSA, since by definition CA-MRSA infections are those diagnosed in the community setting or within 48 hours of

hospital admission [308]. These isolates possess unique characteristics that enable them to be defined as a distinct subset of MRSA (Table 1.9). The discovery and characterization of these isolates are discussed more thoroughly below.

LA-MRSA Characteristics

The first reports of MRSA associated with livestock species were published in 1972 and detailed clinical mastitis in a Belgian dairy cow [309]. However, MRSA in livestock remained largely unreported until 2005, when the first report associated MRSA with swine in the Netherlands [248]. The connection with swine was discovered during a patient's pre-operative MRSA screening, which revealed the patient was colonized with MRSA [248]. Decolonization was attempted; however, routine decolonization procedures were ineffective [248]. The patient's history indicated no known risk factors for MRSA colonization and familial screening discovered the patient's parents were also colonized. Further testing indicated the swine on the farm where they lived served as the source of MRSA [248]. Furthermore, all of the identified isolates were unable to be typed using RFLP analysis. This was due to a novel restriction modification system that methylates DNA in the *SmaI* restriction site preventing digestion with *SmaI* [248, 310]. MLST later revealed the isolates were of the ST398 lineage, which became the prototypical LA-MRSA lineage [311].

Initial screening indicated MRSA was widespread in European swine [312-314]; however the prevalence varied depending on the study. Variable MRSA prevalence has been attributed to variable MRSA colonization rates as well as study design. One factor playing a major role in prevalence is location. For example, in Norway the prevalence of MRSA on swine farms was 0.6%, while studies in Germany found a MRSA prevalence

of 80% on swine farms [315, 316]. Differences in prevalence on farms have also been attributed to the age of pigs tested, the farm size and stocking density, and use of antimicrobials on farm [317-320]. While most studies look at prevalence as a snapshot, some longitudinal studies have been completed and they indicate that not all swine are permanently colonized with LA-MRSA [321, 322]. Because of the transient nature of colonization, the prevalence of LA-MRSA in individual animals on farms varies and herd level prevalence is a better indicator of the incidence of LA-MRSA in swine [316].

The discovery of LA-MRSA ST398 stimulated concerns within the public health community over the potential for swine to act as a reservoir for MRSA in humans. However, though LA-MRSA ST398 isolates are suspected to be derived from an ancestral human MSSA lineage [323], LA-MRSA ST398 isolates appear to present a reduced medical importance as compared to other lineages routinely found in the hospital or community but unassociated with livestock [320]. The majority of reported disease with LA-MRSA ST398 are skin and soft tissue infections and, rarely, severe invasive infections are noted, predominantly in patients with concurrent disease [324]. Reduced virulence is suspected to be due to the adaptation of the LA-MRSA ST398 lineage to colonizing animals, which caused the loss of virulence factors and led to a reduced capacity for causing disease in humans [323, 325-329]. The reduced pathogenicity and severity of disease associated with LA-MRSA ST398 isolates as compared to HA- and CA-MRSA isolates has been attributed to the absence of virulence factors that contribute to disease in humans and reduced adherence capacity and colonization capability. Additionally, AMR patterns differ in LA-MRSA isolates as compared to isolates from the hospital and community setting, which can alter treatment efficacy [330-332].

Comparisons of the virulence genes from LA-MRSA ST398 and MRSA isolates from the hospital or community settings have revealed differences in the complement of virulence genes harbored by the isolates [12, 323, 330]. The immune evasion cluster harbored by the β-hemolysin converting bacteriophage, while present in over 90% of human clinical isolates [29], is largely absent in LA-MRSA ST398 isolates [12, 323, 330]. Similarly, LA-MRSA ST398 isolates often lack enterotoxin genes commonly associated with foodborne illness and enterotoxin-like genes that function as superantigens [12, 323, 330, 333]. Many other exotoxins have also been absent or infrequent in screened LA-MRSA ST398 isolates including: leukocidin (*lukD* and *lukE*), PVL (*lukF-PV* and *lukS-PV*), and toxic shock syndrome toxin (*tst*) [12, 330, 334]. The absence of virulence genes is thought to contribute to the reduced pathogenicity exhibited by LA-MRSA ST398 and may represent an adaptation to colonizing animal hosts.

Investigation of humans in contact with swine that harbor LA-MRSA ST398 indicates carriage is common following interaction with swine [248, 318, 335-338]; however, colonization, when followed over time, appears to be dependent on animal contact and, without contact, it is rapidly lost [335, 336, 339]. Furthermore, epidemiologic analysis of LA-MRSA ST398 isolates indicates LA-MRSA isolates are less transmissible than HA-MRSA isolates within the hospital setting and fewer secondary contacts become positive with LA-MRSA isolates [340-342]. This also appears to be true in the community setting where colonization of household members and their contacts is less frequent than that seen in non-LA-MRSA isolates [338, 342, 343]. Reduced transmission and duration of colonization has been attributed to a demonstrated reduction in the capacity to adhere to human cells *in vitro* [344]. Genetic

analysis revealed mutations causing truncations of adhesion genes, such as the fibrinogen and fibronectin binding proteins (*clfA*, *clfB*, and *fnbpB*) as well as the absence of some adhesion genes in LA-MRSA ST398, such as *srdE*, which were thought to contribute to the differential adherence capability of these isolates *in vitro* [344].

Finally, LA-MRSA ST398 isolates are more variable in their resistance patterns than CA-MRSA isolates and they are typically resistant to more than the β-lactam antibiotic class [320]. These isolates are ubiquitously resistant to tetracycline class antibiotics and have variable resistance to many other classes including fluoroquinolone antibiotics (7-64%) and macrolide and lincosamide antibiotics (33.6-85%) [330-332, 345]. LA-MRSA ST398 isolates are under different selection pressures than HA-MRSA and CA-MRSA isolates, which promote the retention of different AMR genes. For example, LA-MRSA ST398 isolates often harbor SCC*mec* elements that are non-typeable or have smaller type IV and V elements [12, 320]. The type V element is common in LA-MRSA ST398 and may contribute to the fitness of the ST398 lineage in swine because it can harbor the *czrC* gene, a zinc and cadmium resistance gene, which provides resistance in the face of widespread zinc chloride use in the swine industry [323, 346-349]. LA-MRSA ST398 isolates also have the potential to act as a reservoir for diverse and uncommon AMR genes, including the multidrug resistance genes *cfr*, *vgaC*, and *vgaE* [249-251]. The prevalence of resistance genes beyond *mecA* and the identification of resistance genes uncommonly found in HA- and CA-MRSA isolates raises concerns about the potential for LA-MRSA isolates to disseminate AMR genes outside of the livestock setting.

While LA-MRSA ST398 isolates have been well studied since their discovery, more recent evaluation of LA-MRSA isolates worldwide indicates lineages associated with livestock are more complex than ST398 alone [335, 350-355]. While LA-MRSA ST398 is considered the predominant lineage in European swine, there are reports of other lineages as well including CC97 and CC30 [350, 351]. MRSA screening in Asian swine indicate LA-MRSA ST9 isolates predominate [354, 356-359]. This differs from the epidemiology of LA-MRSA in the United States, in which there is not a single prevalent lineage but a mix of ST398 and ST5 isolates with sporadic ST9 isolates detected [335, 360-362].

LA-MRSA in the United States was first detected in 2009 [318]. This report indicated LA-MRSA ST398 was widespread and found a prevalence of 50% in swine herds in Iowa and Illinois [318]; however, further investigation indicated the high prevalence was likely due to a sampling bias. It now appears that LA-MRSA is far less prevalent than this initial report, with later reports finding a prevalence of 2.8-30% [317, 335, 361, 363, 364]. These studies also indicated the lineages of LA-MRSA in the United States were more diverse than initially reported and isolates of the ST398, ST9, and ST5 lineages were detected [335, 361, 362, 365, 366].

Aims and Significance of Research

The prevalence of LA-MRSA ST398 revealed in initial investigations made it clear that swine had the potential to serve as the largest reservoir of MRSA outside of the hospital setting, making LA-MRSA a significant public health threat [333]. This threat is associated with direct risks of MRSA infection through human contact with animals harboring LA-MRSA and indirect risks through the dissemination of AMR genes and

virulence factors that LA-MRSA may harbor. While these risks have been well elucidated for LA-MRSA ST398 and investigated in LA-MRSA ST9, LA-MRSA ST5 has not been similarly evaluated. This is cause for concern because, unlike the MRSA ST398 and ST9 lineages, the ST5 lineage is a globally disseminated and widely successful cause of *S. aureus* infections in both the community and hospital settings [12]. The success of the ST5 lineage has been attributed to its ability to rapidly adapt to its environment through the acquisition of MGEs harboring virulence factors and AMR genes, many of which are widespread throughout the ST5 lineage [12].

The overarching goal of this dissertation was to address the public health concerns surrounding LA-MRSA ST5 isolates. More specifically, this dissertation aimed to evaluate the genetic background of LA-MRSA ST5 isolates as compared to clinical MRSA ST5 isolates from humans with no swine contact, determine the potential virulence of LA-MRSA ST5 isolates, and evaluate the antimicrobial resistance patterns of LA-MRSA ST5 isolates as compared to human clinical MRSA ST5 isolates. To address these aims, 82 swine-associated LA-MRSA ST5 isolates and 71 clinical MRSA ST5 isolates from humans with no swine contact were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA) (Appendix A-F). The resulting draft genome sequences were used for single nucleotide polymorphism (SNP) and phylogenetic analysis to detect the relatedness of LA-MRSA ST5 and human clinical MRSA ST5 isolates (Chapter 2). The genome sequences were also evaluated for MGEs encoding virulence factors and AMR genes, which were used to detect the potential for genetic exchange between these populations (Chapter 3-5). Finally, adherence capacity for these isolates was evaluated, including a genetic comparison of known adherence genes and a

phenotypic screen of a subset of isolates (Chapter 6). The results of genotypic and phenotypic analyses were used to determine the potential direct and indirect implications of LA-MRSA ST5 isolates on human health.

Organization of Dissertation

This dissertation is organized in journal format with 6 chapters. Chapter 1 contains a literature review of *S. aureus*. Each of chapters 2-5 contain a manuscript that is published, has been submitted for publication, or will be submitted for publication. Chapter 2 contains a core genome analysis of LA-MRSA ST5 and human clinical MRSA ST5 isolates in which SNPs were identified and used in a phylogenetic analysis to determine the relatedness of the subsets of isolates. Chapter 3 addresses the prevalence of the β-hemolysin converting bacteriophage, an integrating bacteriophage containing virulence genes involved in evasion of the host immune response, in both LA-MRSA and human clinical MRSA ST5 isolates. Chapter 4 and 5 evaluate the prevalence of AMR genes and compare the specific AMR genes found in both subsets of isolates. Chapter 6 examines the genetic potential and *in vitro* capacity for LA-MRSA ST5 and human clinical MRSA ST5 isolates to adhere to human keratinocytes. Finally, Chapter 7 contains a general conclusion that summarizes the studies contained within this dissertation and examines future directions for this research. References and Appendices are found following Chapter 7.

CHAPTER 2. SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS INDICATES GENETIC DISTINCTION AND REDUCED DIVERSITY OF AGRICULTURAL METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) ST5 ISOLATES AS COMPARED TO CLINICAL MRSA ST5 ISOLATES

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Abstract

Livestock associated MRSA (LA-MRSA) are lineages adapted to livestock species. LA-MRSA can be transmitted to humans and public health concerns exist because livestock may be the largest MRSA reservoir outside of hospital settings. Although the predominant European (ST398) and Asian (ST9) lineages of LA-MRSA are considered livestock adapted, North American swine also harbor ST5, a globally disseminated and highly pathogenic lineage. This study applied whole genome sequencing and single nucleotide polymorphism (SNP) typing to compare the population structure and genetic relatedness between swine associated and clinical MRSA ST5 isolates. The established high-resolution phylogenomic framework revealed that LA-MRSA and clinical MRSA ST5 are genetically distinct. LA-MRSA isolates were found to be clonal within farms, while greater genome plasticity was observed among sampled clinical MRSA ST5. Collectively, our data indicate LA-MRSA and clinical MRSA ST5 isolates are distinct and agricultural sources pose minimal significance as a source of clinical MRSA ST5 infections.

Introduction

Staphylococcus aureus is a commensal organism found in the nasopharynx and on the skin of humans and other mammals. It can also cause infections in these hosts and cause a range of diseases from mild skin and soft tissue infections to severe systemic infections. The treatment of *S. aureus* infections is hampered by the development of antimicrobial resistance in these isolates, such as the acquisition of the SCC*mec* element, which confers methicillin resistance. These isolates are designated as methicillin resistant

S. aureus (MRSA) and have become a significant burden for the health care system [367, 368].

MRSA isolates are classified based on epidemiologic characteristics into hospital acquired MRSA (HA-MRSA), community acquired MRSA (CA-MRSA), and livestock associated MRSA (LA-MRSA) [22]. These subsets are defined by the source of the isolate and the isolates within each subset tend to share genotypic and phenotypic characteristics, such as degree of antimicrobial resistance and virulence factors. Specific lineages or sequence types (STs) tend to predominate within each group, although there are regional variations. For example, in the United States, ST5 and ST8 are major HA-MRSA and CA-MRSA clones, respectively [369].

LA-MRSA became a significant public health concern in 2005, when the first report linked MRSA ST398 to swine production facilities [248]. Further investigations indicated swine may serve as the largest reservoir for MRSA outside of hospital settings and motivated considerable research into the potential health risks associated with LA-MRSA. Subsequent research showed the most prevalent lineage of LA-MRSA varied based on geographic location. In European swine populations, ST398 is the most common lineage [312], while the ST9 lineage predominates in swine in most Asian countries [357]. In the United States, the swine population was found to harbor more diverse sequence types with isolates of the ST398, ST9, and ST5 lineages [335, 361, 362]. The presence of MRSA ST5 isolates in US swine herds raised particular public health concerns because the ST5 lineage, unlike the ST398 and ST9 lineages, was not thought to be livestock adapted but is a highly successful and globally disseminated MRSA lineage among human clinical cases [12, 329, 370]. The widespread success of

the ST5 lineage has been partially attributed to its capacity to acquire mobile genetic elements (MGEs) that harbor virulence factors or antimicrobial resistance genes [12].

Phylogenetic studies employ whole genome sequencing (WGS) technology and data analysis to better understand the epidemiology, origin, and evolution of bacteria [323, 344, 355, 371]. In the case of LA-MRSA, these techniques have been used to assess the relatedness of isolates from the ST398 lineage. LA-MRSA ST398 isolates clustered separately from human ST398 isolates and are suspected to have evolved from an ancestral methicillin susceptible ST398 clade in humans [323]. Similar investigations into the CC97 lineage indicated that isolates causing clinical disease in humans comprise a sub-clade of the LA-MRSA isolates that may have developed an increased capacity for infecting and causing disease in humans [372]. WGS analysis can be used to assess isolate relatedness or determine genetic characteristics that define subsets of isolates, including information about the MGEs harboring virulence factors or antimicrobial resistance genes. Through SNP discovery within the core genome, the accuracy and resolution power is available to determine phylogenetic relationships and distinguish isolates within highly homogenous lineages, which provides insight in epidemiological investigations [355, 371, 373, 374].

Although previous reports have examined the relatedness of MRSA isolates from livestock species and humans within the ST398 and CC97 lineages [323, 372], to date there are no reports using WGS data to evaluate the relatedness of MRSA ST5 isolates obtained from swine and humans. In this study, we used SNP analysis of the core genome to evaluate the genetic relatedness of LA-MRSA ST5 isolates from a variety of swine associated sources and clinical MRSA ST5 isolates from humans with no swine contact

to investigate the potential for dissemination of LA-MRSA ST5 outside of the agricultural setting.

Methods

Isolate Acquisition

Swine associated LA-MRSA ST5 isolates were collected by Iowa State University (ISU) from swine nasal swabs (n = 38), the environment within swine facilities (n = 26), and healthy veterinary students after visiting a swine farm (n = 9) [335]. Isolates from healthy practicing swine veterinarians who have long term contact with pigs (n = 9) were provided by the University of Minnesota (UMN). MRSA ST5 isolates from humans with no swine contact were obtained from medical centers associated with the University of California Irvine (UCI) (n = 64) [375] and the University of California San Francisco (UCSF) (n = 8). Isolates were characterized for MLST and *Spa* typed prior to acquisition [335, 375]. Isolate information can be found in Appendix A.

Whole Genome Sequencing

Draft genomes were generated as previously described [376-381]. Briefly, total genomic DNA was extracted from isolates grown in Trypticase Soy Broth (BD Biosciences, Sparks, MD) using a High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN). The Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA) was used to generate paired-end DNA libraries with 250-bp read length that were sequenced using the MiSeq v2 500 Cycle reagent kit on the Illumina MiSeq platform (Illumina, San Diego, CA). Sequence reads were assembled with MIRA v. 4.0.2 (http://mira-

assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html) and annotated using the NCBI Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Draft genomes and sequence reads have been submitted to GenBank and the Sequence Read Archive (SRA). Accession numbers are provided in Appendix A.

Core Genome SNP Discovery

For reference based SNP discovery we used a custom developed pipeline implemented on Galaxy [382], that was successfully applied for the high resolution genomic epidemiology profiling of various microbial human pathogens [371, 373, 374]. Strategies and phylogenetic principles have been described in detail in Rusconi et al., 2016 [374]. The MRSA core genome in this study is defined in the samples as the set of genic and intragenic regions that are not repeated and do not contain mobile genetic elements (which evolve at different rates and are not indicative of evolutionary relationships), such as phages, IS elements, genomic islands or plasmids. These excluded regions were determined for the reference genome Mu50 as follows: NUCmer was used to detect repeat regions by running the reference against itself [383], integrated bacteriophages were identified using PHAST [384], ISFinder was used for detection of insertion sequences [385], and antibiotic resistance cassettes were detected with ResFinder [386]. The SNP discovery and verification pipelines are implemented on Galaxy, and contain the following modules: (i) SNP discovery and typing, (ii) SNP curation, (iii) SNP annotation, (iv) SNP distribution, and (v) SNP phylogeny.

SNP discovery and typing: Illumina reads of the 72 MRSA ST5 isolates from humans with no swine contact and 82 LA-MRSA ST5 were uploaded into Galaxy along

with two ST5 representative closed genomes: a HA-MRSA isolate from Japan (Mu3) and a representative poultry-adapted MRSA ST5 isolate (ED98) [325, 387]. For read-based SNP discovery, reads were aligned with Bowtie2 to the reference genome Mu50 [388]. The resulting alignments were processed with Freebayes using the following threshold settings: mapping quality 30, base quality 20, coverage 30, and allelic frequency 0.9 [389]. The two closed representative genomes, Mu3 and ED98, were analyzed using the contig-based workflow. Briefly, a panel of SNPs for each genome was obtained by aligning the genome against the reference strain, Mu50, using NUCmer. SNPs were called with delta-filter and show-snps distributed with the MUMmer package [383].

SNP curation: Several SNP curation strategies were used to correct for false positive calls [371, 373]. First, reads were mapped against the reference genome Mu50 and false positives identified by Freebayes with the settings described. If reads were unavailable (Mu3 and ED98), the post-assembly workflow generated a reference-based NUCmer alignment and extracted SNPs as described above with filtering of false SNPs. SNPs located within excluded regions (repeat regions, bacteriophages, resistance cassettes, and IS elements) were removed. SNPs were further curated by extracting the 40 nucleotides surrounding each predicted SNP in the reference genome and completing a nucleotide BLAST against the query genomes [390]. Finally, resulting alignments were parsed to remove SNP locations derived from ambiguous hits (≥2), low alignment quality or misalignments, non-uniformly distributed regions, and InDels, as previously described [371, 373, 391]. Also, multinucleotide insertions and deletions of polymorphic bases were not considered SNPs and were excluded.

SNP annotation: The curated catalogued SNPs from each query genome were merged into a single SNP panel that reported the allele, genic/intergenic status, SNP position, and annotation [344, 372, 374]. This SNP discovery and validation pipeline allows for rapid typing of strains of unknown provenance by interrogating the captured allelic states from established SNP panels [373].

SNP distribution: From the distribution of SNPs along the Mu50 chromosome, potential mutational hotspots and genes under positive selection could be identified using custom scripts implemented on Galaxy [382, 391, 392].

SNP phylogeny: The identified curated SNP panel was used for phylogenetic reconstruction by maximum parsimony with PAUP v4.0a146 with 100 bootstrap replicates [393]. The SNP tree was visualized in Geneious (vR9) and the majority consensus tree was built in Mesquite [394, 395]. Tree decorations were added using Evolview [396]. Calculation of the consistency index for each SNP allowed for identification of parsimony informative SNPs and flag homoplastic SNPs, as described in our previous works [371, 374].

Mobile genetic element analysis

Mobile genetic elements harboring antimicrobial resistance genes and virulence factors were detected as described previously and verified using Geneious 9.0.5 [397-399]. Antimicrobial resistance genes detected with ResFinder were verified through draft genome analysis [399]. The SCC*mec* type was determined using PCR and confirmed *in silico* [397]. Immune evasion genes associated with the β-hemolysin converting bacteriophage were identified in these isolates using PCR and confirmed *in silico* [398].

Results

Isolate provenance and sequence information

Swine associated LA-MRSA ST5 isolates were obtained from nasal swabs of healthy pigs or humans, none of which exhibited signs of MRSA infection, or from swabs of the environment within swine facilities. Clinical MRSA ST5 isolates were obtained from patients with MRSA related disease at two urban, university affiliated hospitals in California where the likelihood that patients had contact with swine was considered negligible. Draft genomes confirmed the MLST data indicating all isolates were ST5.

Single nucleotide polymorphism typing

For reference based SNP discovery, genomes were aligned to the closed genome of reference strain Mu50, a vancomycin resistant HA-MRSA isolate from Japan [387]. The core genome was determined by excluding identified mobile genetic elements and repeats. Core genome SNP discovery identified 759 SNPs comprised of: 150 intergenic, 186 synonymous, and 423 non-synonymous SNPs (Table 2.1). Further evaluation of non-synonymous SNPs indicated that eight were shared by swine associated LA-MRSA ST5 isolates and not present in isolates from humans with no swine contact, as listed in Table 2.2. There were also two SNPs found only in MRSA ST5 isolates from humans with no swine contact (Table 2.2) that were specific to clinical isolates. The genes harboring the non-synonymous SNPs distinct to each subset of isolates have not been implicated in virulence of *S. aureus* and are unlikely to contribute to the pathogenicity of these isolates. SNPs were distributed throughout the Mu50 reference genome and no mutational hotspots were observed (Figure 2.1).

Table 2.1. SNP discovery results.

	Total	Number of Genes	NSYN	SYN	INT	Genic
SNPs	759	362	423	186	150	609
Non-Informative	514	279	287	134	93	421
Parsimony Informative	245	166	136	52	57	188
Stop Gain	25	22	25	0	0	25
Stop Loss	2	2	2	0	0	2
Hypothetical Proteins	150	87	106	44	0	150
Transition	509	280	264	150	95	414
Transversion	250	166	159	36	55	195
Multiallelic	0	0	0	0	0	0

NSYN – nonsynonymous
SYN – synonymous
INT – intergenic

Table 2.2. Non-synonymous SNPs unique to LA-MRSA ST5 isolates or MRSA ST5 isolates from humans with no swine contact.

Number of Swine Associated Isolates	Number of Isolates from Humans with No Swine Contact	SNP Position[a]	Gene Product[b]
82/82	0/72	160799	2'-3'-cyclic-nucleotide 2'-phosphodiesterase
82/82	0/72	292343	sorbitol dehydrogenase homologue
82/82	0/72	806489	putative transporter
82/82	0/72	848820	putative P-loop-containing kinase
82/82	0/72	1012841	similar to ATP-dependent nuclease subunit A
82/82	0/72	1928498	O-succinylbenzoic acid-CoA ligase
82/82	0/72	2695325	ferrous iron transport protein B homolog
82/82	0/72	2720180	regulatory protein
0/82	69/72	192929	hypothetical protein
0/82	69/72	2277937	conserved hypothetical protein

[a] SNP position indicates the location of the SNP in the reference genome of Mu50
[b] Gene product represents the protein produced by the gene containing the SNP of interest

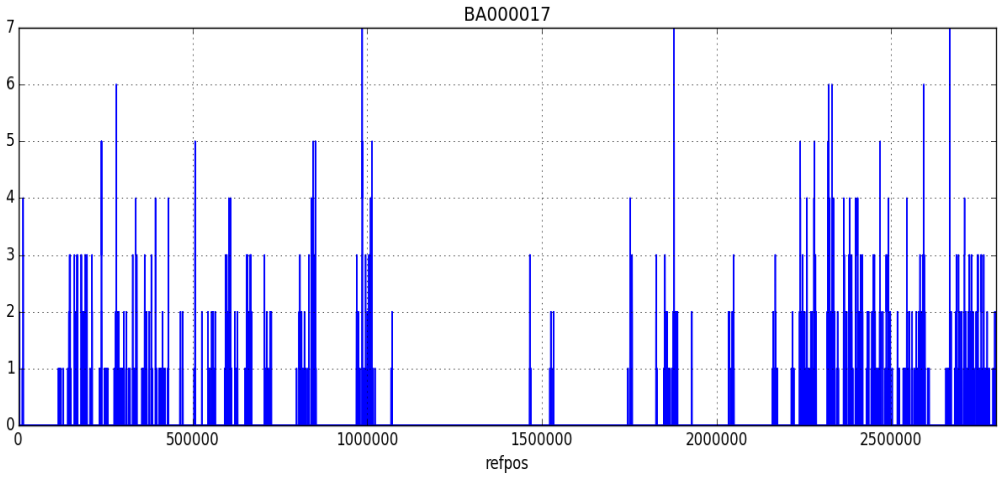

Figure 2.1: Genomic distribution of SNPs. The position of the 759 identified SNPs were plotted on the Mu50 chromosome using a sliding window of 1000 bp. SNPs were distributed throughout regions included in the analysis and did not indicate any locations for mutational hotspots. Regions lacking SNP predictions are associated with locations of mobile genetic elements and repeat regions that were excluded from the SNP discovery and encode elements such as the SCC*mec* element and the β-hemolysin converting bacteriophage.

Phylogenetic analysis

A phylogenetic hypothesis was constructed from the identified core genome SNPs and rooted using the MRSA ST5 isolate Mu50. This tree depicts the evolutionary relationships between the 154 MRSA ST5 isolates and the reference isolates Mu3 and ED98 (Figure 2.2). The tree topology shows that swine associated LA-MRSA ST5 isolates cluster together and are separated from MRSA ST5 isolates from humans with no swine contact. These groups will be referred to as Clades I and II, representing MRSA ST5 isolates from humans with no swine contact and LA-MRSA ST5, respectively. A single MRSA ST5 isolate (UCSF14436) from a human with no swine contact was contained within Clade II. This isolate was the most distantly related of the Clade II isolates, harboring 24 unique/strain-specific SNPs. USCF14436 harbors a type IV SCC*mec* element and two antimicrobial resistance genes (*mecA* and *blaZ*), which is a strong indication this isolate is a CA-MRSA strain (Figure 2.3).

MRSA ST5 isolates from humans with no swine contact (Clade I) are divided into subclades Ia and Ib. The SNP-level plasticity between MRSA ST5 isolates from humans with no swine contact is greater than that from LA-MRSA ST5 isolates. Despite the northern and southern geographic regions represented by the two groups of California isolates, the tree topology shows that UCSF isolates were found interspersed throughout Clade I and did not cluster by geographic location.

The swine associated isolates within Clade II formed four subclades, IIa-d. Each subclade represents a subset of isolates from a specific farm or farms; such that, Clade IIa represents isolates from Farm 10, Clade IIb represents isolates from Farm 24, Clade IIc represents isolates from Farm 46, and Clade IId represents isolates from Farm 38-42. Isolates originating from individual farms were genetically homogenous, possessing fewer than 5 SNPs (distinguishing them from other isolates from the same farm). These results suggest that LA-MRSA ST5 populations residing on farms are clonal and intermingling/transfer of isolates among farms or reintroduction of MRSA ST5 onto farms had likely not occurred. This was true for all swine associated isolates except those from farms 38-42 (Clade IId). The high degree of genetic relatedness of the isolates within Clade IId suggests these farms are likely from a single production system or share a common genetic source, with exposure of pigs to LA-MRSA ST5 early in the production system (farrowing unit) and disseminated throughout the later stages as pigs are moved (finishing barns). High resolution profiling provides important phylogenetic signals for strain attribution [371, 374]. In this case, isolates from humans with short-term contact with swine farms could be traced back to a specific farm or production system. For example, isolates ISU886-ISU889 were traced to Farm 24, through SNP analysis.

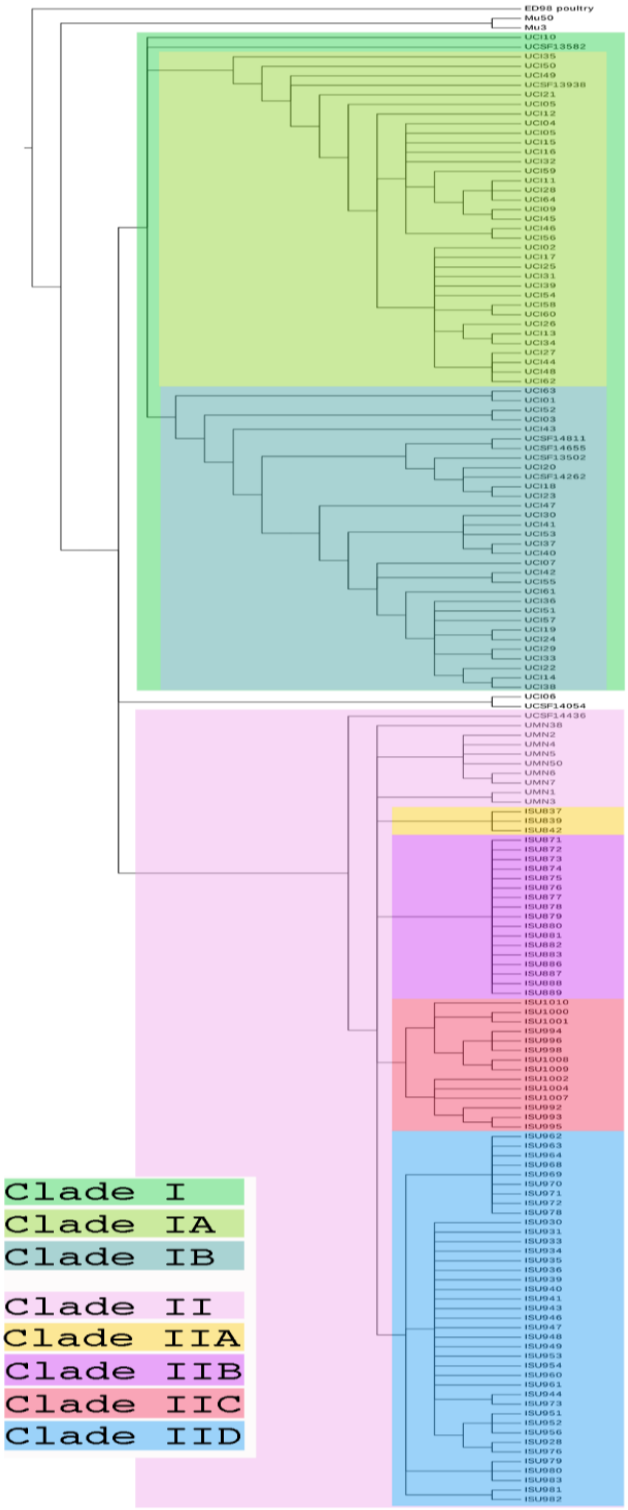

Figure 2.2: Maximum parsimony SNP tree of ST5 MRSA isolates. Comparison of 156 genomes yielded a total of 759 SNPs, of which 245 were parsimony informative. The tree shown is a majority-consensus tree of 4440 equally parsimonious trees with a consistency index of 0.9428. Trees were recovered using a heuristic search in Paup 4.0b10 [393]. This tree is broken into clades, with Clade I representing clinical MRSA ST5 isolates from humans with no swine contact representing and Clade II representing LA-MRSA ST5 isolates. Clade IIa-d are subsets of LA-MRSA ST5 isolates and each subclade represents an individual farm or production system.

These isolates possessed the unique pattern of SNPs present on that farm or within that production system (Clade IIb, IIc, and IId), indicating exposure to the farm harboring that specific clone. The remaining isolates in Clade II include the isolates from humans with long-term swine contact and UCSF14436. Isolates from humans with long term swine contact did not cluster with isolates from the tested farms (Figure 2.2). This is consistent with the source of these isolates as it was unlikely the swine veterinarians sampled had contact with the specific farms sourcing the other swine associated isolates in this study. Though these isolates were distinct from the isolates obtained from swine, swine facilities, and humans with short term swine contact, they clustered together within Clade II and were representative of the livestock associated ST5 genotype.

Mobile genetic element analysis

The draft genomes of both LA-MRSA ST5 isolates and clinical MRSA ST5 isolates from humans with no swine contact were evaluated for mobile genetic elements. LA-MRSA ST5 isolates could also be distinguished from clinical MRSA ST5 isolates by the antimicrobial resistance genes and virulence factors they harbored (Figure 2.3). For LA-MRSA ST5 isolates, the SCC*mec* elements were of type III, IV, or untypable. Alternatively, clinical MRSA ST5 isolates harbored predominantly type II SCC*mec* elements and two type IV elements. Evaluation of the antimicrobial resistance genes revealed the primary macrolide resistance gene was *ermA* in clinical MRSA ST5 isolates, while LA-MRSA ST5 isolates harbored *ermC*. Additionally, tetracycline resistance genes were found exclusively in LA-MRSA ST5 isolates. Virulence factors harbored by the subsets were also different, with the majority (65/72, 90.3%) of clinical MRSA ST5 isolates harboring innate immune evasion genes within the β-hemolysin converting

bacteriophage and none of the LA-MRSA ST5 isolates harboring these genes. This analysis revealed that MGE were not commonly shared between the two subsets of isolates.

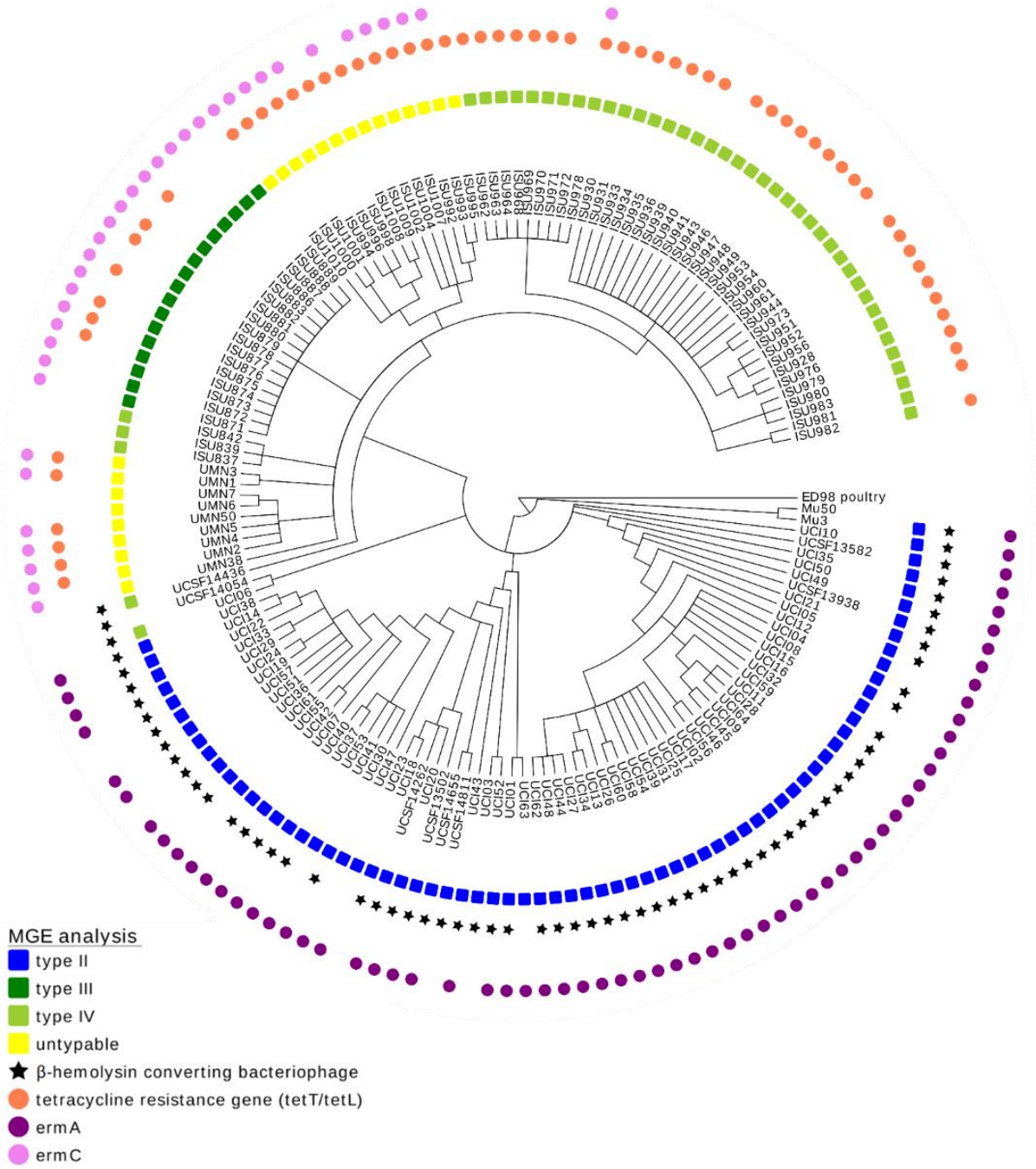

Figure 2.3: Maximum parsimony SNP tree of ST5 MRSA isolates with MGE analysis. The SNP tree developed for Figure 2.2 was decorated using Evolview [396, 400]. The tree shows the MGE complement of these isolates, specifically describing the SCC*mec* element, the β-hemolysin converting bacteriophage (harboring virulence factors involved in innate immune evasion), and antimicrobial resistance genes involved in tetracycline resistance (*tetT/tetL*) and macrolide resistance (*ermA* and *ermC*).

Discussion

MRSA in livestock species was first identified in the 1970s [309]; however, discovery of a high prevalence of MRSA ST398 in swine in 2005, and subsequently in other livestock, brought debate over its public health implications to the forefront [248]. Previous studies investigating LA-MRSA ST398 demonstrated these isolates have adapted to livestock hosts. This was represented by the absence of human specific virulence factors, such as the β-hemolysin converting bacteriophage [323], and reduced adherence and transmission among humans [339, 344]. LA-MRSA ST9 isolates, similar to ST398, are thought to be livestock adapted, and clinical cases appear to be rare and attributed to animal contact [401]. While the ST5 lineage in humans is globally distributed and successful in both the hospital and community settings [12], there are currently no reports of MRSA ST5 related disease occurring due to contact with livestock species; however, the genetic potential for swine isolates to adhere, invade, and cause disease in humans has not been investigated.

Here, we examined the phylogenetic relatedness applying high resolution core genome SNP profiling strategies on MRSA ST5 isolates from swine associated sources and humans with no swine contact to determine if distinct subpopulations of ST5 isolates exist within different host populations, which may provide evidence of host adaption. Similar analyses have been conducted for the ST398 lineage, where researchers identified that the studied LA-MRSA ST398 isolates were within the same clade and that clade appeared to have evolved from an ancestral human methicillin susceptible *S. aureus* (MSSA) ST398 clade [323].

In this study, isolates from humans with no swine contact clustered distinctly from swine associated LA-MRSA ST5 isolates. All swine associated LA-MRSA ST5 isolates were contained within Clade II, while all but one MRSA ST5 from humans with no swine contact belonged to Clade I. The MGE complement of this clinical MRSA ST5 isolate (UCSF14436) suggests that it is likely a CA-MRSA isolate. CA-MRSA isolates tend to be genetically distinct from HA-MRSA isolates [22], which may explain why UCSF14436 is found within the LA-MRSA clade. The separation of LA-MRSA ST5 isolates from clinical MRSA ST5 isolates based on their core genome relatedness supports our previous work, which indicated that swine associated LA-MRSA ST5 isolates harbor different MGEs containing virulence and resistance genes than MRSA ST5 from humans with no swine contact [397-399]. The data reported here further demonstrate the phylogenetic distinction of isolates into different groups and may reflect the adaptation of LA-MRSA ST5 isolates to colonization of swine. Furthermore, similar to LA-MRSA ST398, evaluation of known virulence genes provides evidence that swine associated LA-MRSA ST5 may be less capable of causing disease in humans (Figure 3) [398].

Our results indicate frequent introduction of MRSA ST5 onto swine farms is unlikely and dominant clones of MRSA ST5 circulated within each farm at the time of sampling. A high degree of genetic relatedness was identified among swine-associated isolates. As expected, the population of isolates found on individual farms were dominated by clonal populations. This indicates either a single introduction of LA-MRSA ST5 onto a farm or dominance of a single variant that precludes colonization with alternative isolates introduced by human caretakers. However, multiple *S. aureus*

lineages and spa types often occur simultaneously on swine farms and in individual pigs [402], so the diversity of *S. aureus* in these animals may be underrepresented when looking specifically at MRSA ST5 isolates. This clonality was not present in isolates from humans with no swine contact, where genome plasticity was greater and individual isolates showed more diversity than that observed among LA-MRSA ST5 isolates.

While the evidence here suggests the populations of LA-MRSA ST5 and MRSA ST5 from humans with no swine contact are distinct, the isolates used in this analysis were sourced from geographically limited regions to ensure the clinical isolates were from patients with no swine contact. Because of the unlikelihood of these populations mingling, the SNPs identified can be attributed to the geographic separation or the host species of the isolates. In the future, the inclusion of clinical MRSA ST5 isolates from the hospital and community setting in areas of swine production could further elucidate the distribution and impact of LA-MRSA ST5 isolates outside of the livestock setting and the potential of swine associated isolates to cause disease in humans. As evidenced in this study, WGS followed by high resolution SNP profiling is a powerful molecular genomic epidemiology approach to gain insights into the population structure and transmission dynamics of LA-, CA-, and HA-MRSA and can provide important phylogenetic signals for strain attribution.

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CHAPTER 3. COMPARATIVE PREVALENCE OF IMMUNE EVASION COMPLEX GENES ASSOCIATED WITH BETA-HEMOLYSIN CONVERTING BACTERIOPHAGES IN MRSA ST5 ISOLATES FROM SWINE, SWINE FACILITIES, HUMANS WITH SWINE CONTACT, AND HUMANS WITH NO SWINE CONTACT

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Abstract

Livestock associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) draws concern from the public health community because in some countries these organisms may represent the largest reservoir of MRSA outside hospital settings. Recent studies indicate LA-MRSA strains from swine are more genetically diverse than the first

reported sequence type ST398. In the US, a diverse population of LA-MRSA is found including organisms of the ST398, ST9, and ST5 lineages. Occurrence of ST5 MRSA in swine is of particular concern since ST5 is among the most prevalent lineages causing clinical infections in humans. The prominence of ST5 in clinical disease is believed to result from acquisition of bacteriophages containing virulence or host-adapted genes including the immune-evasion cluster (IEC) genes carried by β-hemolysin converting bacteriophages, whose absence in LA-MRSA ST398 is thought to contribute to reduced rates of human infection and transmission associated with this lineage. The goal of this study was to investigate the prevalence of IEC genes associated with β-hemolysin converting bacteriophages in MRSA ST5 isolates obtained from agricultural sources, including swine, swine facilities, and humans with short- or long-term swine exposure. To gain a broader perspective, the prevalence of these genes in LA-MRSA ST5 strains was compared to the prevalence in clinical MRSA ST5 strains from humans with no known exposure to swine. IEC genes were not present in any of the tested MRSA ST5 strains from agricultural sources and the β-hemolysin gene was intact in these strains, indicating the bacteriophage's absence. In contrast, the prevalence of the β-hemolysin converting bacteriophage in MRSA ST5 strains from humans with no exposure to swine was 90.4%. The absence of β-hemolysin converting bacteriophage in LA-MRSA ST5 isolates is consistent with previous reports evaluating ST398 strains and provides genetic evidence indicating LA-MRSA ST5 isolates may harbor a reduced capacity to cause severe disease in immunocompetent humans.

Introduction

Staphylococcus aureus is a gram positive coccus that forms part of the normal nasal microflora in humans and other animal species. In developed countries, approximately one-quarter to one-third of healthy people harbor *S. aureus* in the nose, but prevalence appears to be lower in developing countries [403]. Although considered to be a commensal in the nasopharynx, *S. aureus* is an opportunistic pathogen causing a wide range of disease in humans. Skin and soft tissue infections are most commonly reported [58], but *S. aureus* also causes severe, invasive diseases including necrotizing pneumonia, bacteremia, osteomyelitis, and toxin mediated diseases such as toxic shock syndrome and staphylococcal enterotoxicosis. In 2005, the incidence rate for invasive MRSA infections per 100,000 individuals in the US was estimated to be 31.8 infections and 6.3 fatalities [367].

Resistance to methicillin was first reported in *S. aureus* in 1961 [246]. It is mediated by a mobile genetic element containing the *mecA* gene that confers resistance to methicillin and other β-lactam antibiotics. These isolates are designated methicillin resistant *S. aureus* (MRSA) and are difficult to treat, particularly if they have acquired multiple antibiotic resistance elements. Prevalence reports indicate MRSA may colonize as much as 1.5% of the healthy US population [42], and caused over 400,000 infections and millions of dollars in healthcare costs and lost productivity in 2009 [404].

Based on epidemiological characteristics, MRSA isolates are classified into three types: hospital-acquired (HA-MRSA), community-acquired (CA-MRSA), and livestock-associated (LA-MRSA). HA-MRSA isolates are obtained through contact in a healthcare setting. CA-MRSA isolates are not associated with a healthcare environment and are

more commonly found in younger and healthy persons [58]. They are typically obtained from close contact environments, such as dormitories or athletic centers [405, 406]. CA-MRSA isolates tend to possess fewer antimicrobial resistance elements, but exhibit increased virulence compared to HA-MRSA. The association between MRSA and swine was first reported in 2005 [248] and these isolates were referred to as LA-MRSA.

MRSA isolates are typically characterized by their genetic lineage through multi-locus sequence typing (MLST). MLST involves sequencing seven housekeeping genes (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, *yqiL*) to obtain an allelic profile that defines the isolate's sequence type (ST) [407]. STs present in the human population vary regionally and this trend is also true for animal populations, including swine. Initial reports of LA-MRSA from swine and other livestock species described isolates which all belonged to a novel MLST type (ST398). Subsequent research revealed a more complex epidemiology of *S. aureus* in pigs. The predominant swine-associated LA-MRSA lineage in Europe is ST398 [312], while in Asia ST9 isolates are most prevalent [357]. However, LA-MRSA isolates in the United States are a diverse population containing ST398, ST9, and ST5 isolates [335]. The presence of ST5 isolates in swine has raised additional public health concern because, in contrast to ST398 and ST9, ST5 is a highly successful and globally disseminated MRSA lineage in humans with both HA- and CA-MRSA clones reaching pandemic levels [12].

The clinical significance of ST5 strains is thought to be due to their ability to acquire mobile genetic elements (MGE) containing virulence factors or antibiotic resistance genes. Of particular importance is the β-hemolysin converting bacteriophage that is commonly acquired by MRSA ST5 isolates [12]. The β-hemolysin converting

bacteriophage is a lysogenic phage that integrates into and disrupts the β-hemolysin gene of *S. aureus*. This bacteriophage contains an immune evasion cluster (IEC) encoding a combination of 1–4 known virulence factors that enhance the capacity of *S. aureus* strains to colonize, disseminate, and persist within a human host. These genes include: staphylococcal complement inhibitor (*scn*), chemotaxis inhibitory protein (*chp*), staphylokinase (*sak*), staphylococcal enterotoxin A (*sea*), and staphylococcal enterotoxin P (*sep*) [29]. These genes have been shown by in vitro assays to disrupt the normal function of the human immune system by inhibiting chemotaxis of phagocytes to the site of infection or inflammation (*chp*), preventing receptor-mediated phagocytosis (*sak*), inactivating antimicrobial peptides (*sak*), and inhibiting the complement cascade (*scn*) [165, 168, 169]. These proteins allow *S. aureus* to survive and replicate within host tissues causing local disease. Staphylokinase also acts through plasminogen activation to break down the extracellular matrix enabling bacterial dissemination from the initial site of infection [170]. Each of these proteins has been shown to be highly specific for human immune cells and serum proteins and are therefore considered human-specific virulence factors [165, 168, 408]. The toxin genes (*sea* and *sep*) are less specific to humans; however, they are important virulence determinants in disease. The enterotoxin proteins are superantigens, which when introduced systemically, non-specifically activate large populations of T cells in the host and cause dysregulation of the host's adaptive immune response [409]. This limits the host's ability to form an adaptive response specific to *S. aureus* antigens, preventing bacterial elimination [410]. While not all IEC genes are present within an individual prophage, the presence of a combination of several IEC genes confers increased virulence to an individual isolate. Previous studies indicate that

prophage integration was present in 90.5% of human clinical isolates [29]; however, because not all genes are present on a given bacteriophage, the prevalence of individual genes differs.

In spite of the pathogenicity attributed to HA- and CA-MRSA ST5 isolates and detection of LA-MRSA ST5 in the nasal cavity of persons with swine contact, there are currently no known reports of clinical infection with ST5 isolates being attributed to animal contact. Epidemiological data has indicated that LA-MRSA ST398 isolates have reduced person-to-person transmission rates and are less virulent than their HA- and CA-MRSA counterparts [339, 411]. Studies from pig dense regions in Europe suggest low risks of clinical infection with ST398 MRSA and there are few reports of severe clinical infections with LA-MRSA in people with animal contact despite high levels of exposure [412, 413]. Previous studies focusing on ST398 isolates have demonstrated the absence of MGE associated virulence factors, including the IEC genes described here which is believed to contribute to the decreased pathogenicity and zoonotic potential of LA-MRSA isolates [323, 414, 415]. Reports showing a comparatively low prevalence of β-hemolysin converting bacteriophages in swine-associated ST398 isolates along with the lack of reports implicating swine exposure in MRSA ST5 related disease have led to the hypothesis that swine-associated LA-MRSA ST5 isolates would similarly have a low prevalence of prophage integration compared to their counterparts causing human clinical infections.

Methods

Strain acquisition

Swine associated isolates were obtained from Iowa State University [335] and the University of Minnesota. Sources for these isolates were swine (38 isolates), the environment within swine facilities (26 isolates), humans with short-term contact with swine (9 isolates), and swine veterinarians representing humans with long-term contact with swine (9 isolates). Clinical isolates from humans with no swine contact, representing both HA- and CA- *S. aureus* isolates, were obtained from the University of California Irvine (64 MRSA isolates) [375] and the University of California San Francisco (7 MRSA and 1 MSSA isolates). All isolates were MLST and Staphylococcal protein A (*spa*) typed prior to acquisition. *Spa* types can be found in Supplemental Table 1 (available at https://doi.org/10.1371/journal.pone.0142832.s001) and Appendix A. ATCC strains Mu3 (ATCC #700698), Mu50 (ATCC #700699), Newman (ATCC #25904) were obtained for use as controls for the IEC genes, and a ST398 isolate from Iowa State University was used as a control for a strain encoding an intact β-hemolysin gene.

DNA isolation

Strains were grown overnight on Trypticase Soy Agar (BD Biosciences, Sparks, MD) at 37°C to obtain isolated colonies. Individual colonies were selected to start an overnight culture of Trypticase Soy Broth (BD Biosciences, Sparks, MD). After 12–18 hours of growth, 750 μl of the liquid culture was pelleted and the supernatant was removed. The pelleted cells were stored at -80°C until DNA extraction. To isolate DNA, each pellet was resuspended in 200 μl 1× Phosphate Buffered Saline with 0.2 M EDTA. To lyse the cells, the following were added to each suspension: 12 μl of Lysozyme

solution (Sigma, St. Louis, MO), 1 µl RNase (Roche, Mannheim, Germany), 7.5 µl Lysostaphin solution (Sigma, St. Louis, MO), and 7.5 µl Mutanolysin solution (Sigma, St. Louis, MO). The cells were then incubated for 1 hour at 37°C. Forty microliters of Proteinase K (Roche, Mannheim, Germany) was added and the suspension was incubated overnight at 55°C. The following day, a Roche High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) was used to isolate DNA according to the manufacturer's protocol. The Elution Buffer containing DNA samples was centrifuged for 5 minutes at 8000xg to remove visible debris. The supernatant was transferred into a clean 1.5 mL tube and stored at 4°C until further analysis.

PCR reactions

The primers, reaction conditions, and expected product size are listed in Table 3.1 [29, 182, 326, 416]. Primers were designed based on a multiple sequence alignment of Mu3, Mu50, and Newman. PCR screening was conducted in 50 uL reaction volume using either AmpliTaq (Applied Biosystems, Carlsbad, CA) or AccuPrime Taq (Invitrogen, Carlsbad, CA) depending on the primer set. An MJ Research PCT-200 DNA Engine thermocycler (GMI, Ramsey, MN) was used for amplification using the following settings: 30 cycles of 30 second denaturation at 94°C, 30 seconds annealing at the temperature listed, 1 minute extension at 72°C (AmpliTaq) or 68°C (AccuPrime). PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light. Nucleotide sequence determination of PCR products was completed by Sanger sequencing methods.

Table 3.1: Primer sets with reaction components, expected product size and primer source.

Primer	Sequence	Annealing Temp	DNA Polymerase Used	Expected Product Size	Primer Source
Int-F	GCTTTGAAATCAGCCTGTAGAGTC	54º	AmpliTaq	499 bp	This study
Hlb-R3	GTTGATGAGTAGCTACCTTCAGT				Jarraud et al [416]
Scn-F4	TGAGGCACAAGCTAGCACAAGCT	63º	AccuPrime	224 bp	This study
Scn-R4	TGAAGTTGATATTTTGCTTCTGACATTTTC				This study
Sak-F2	TGAGGTAAGTGCATCAAGTTCA	53º	AmpliTaq	403 bp	Sung et al [326]
Sak-R2	CCTTTGTAATTAAGTTGAATCCAGG				Sung et al [326]
Chp-F	TTTACTTTTGAACCGTTTCCTAC	51.5º	AccuPrime	404 bp	van Wamel et al [29]
Chp-R2	TGCATATTCATTAGTTTTTCCAGG				Sung et al [326]
Sea-F5	GGTTATCAATGTGCGGGTGG	54º	AmpliTaq	322 bp	This study
Sea-R4	CAAATAAATCGTAATTAACCGAAGGTTC				Jarraud et al [416]
Sep-F2	GACCTTGGTTCAAAAGACACC	54º	AmpliTaq	275 bp	Diep et al [182]
Sep-R2	TGTCTTGACTGAAGGTCTAGC				Diep et al [182]
Hlb-TNF1	TATGTTATCGACCGTGTTGTATCC	58º	AmpliTaq	766 bp	This study
Hlb-TNR1	ATCCCATGGCTTAGGTTTTTCAGT				This study

Southern blotting

Genomic DNA (500ng) was digested overnight with BamH1 in a 25 uL reaction volume and run on a 1% agarose gel. Each gel was depurinated in 0.2M HCl for 10 minutes and rinsed with distilled water. It was then placed in denaturing solution for 1 hour followed by neutralizing solution for 1 hour. Each gel was set up for transfer via capillary action to a nylon membrane overnight. The DNA was crosslinked in UV light to the membrane. Prehybridization was done at 42°C for 2 hours and hybridized at 42°C overnight using a DIG labeled probe (Roche, Mannheim, Germany). The membrane was washed with 2× and 0.5× wash solution and blocked for 1 hour with maelic acid solution containing 5% powdered milk. It was then probed with anti-DIG antibody (Roche, Mannheim, Germany) at 1:10,000 in 5% powdered milk in maelic acid solution. The membrane was washed in washing buffer to remove excess antibody and CSPD (Roche, Mannheim, Germany) was added for visualization. Imaging was done using a myECLImager (Life Technologies, Grand Island, NY) or x-ray film development.

Phage typing

Phage types were designated based on the complement of IEC genes present in the isolate as determined by PCR results. The typing scheme employed is the same as previously reported by van Wamel and colleagues [29]. Briefly, type A included isolates carrying the genes *sea*, *sak*, *chp*, and *scn*. Isolates containing *sak*, *chp*, and *scn* were designated type B. Type C comprised isolates containing *chp* and *scn*. Those isolates carrying *sea*, *sak*, and *scn* were labeled type D. Isolates harboring *sak* and *scn* were designated type E. Type F were isolates containing *sep*, *sak*, *chp*, and *scn*. Those isolates

containing *sep*, *sak*, and *scn* were designated type G. Type H isolates were those containing only the *scn* gene as reported by Price and colleagues [323].

Statistical analysis

The prevalence results were analyzed with a two-tailed Fisher's exact test using the program GraphPad Prism (GraphPad Software, La Jolla, CA). A P value of 0.05 was used as the cutoff for statistical significance.

Results

IEC gene prevalence

PCR screens of the ST5 isolates were used to detect the presence of each of the five IEC genes known to be carried by β-hemolysin converting bacteriophages. Results of all screening tests are listed in Table 3.2. The staphylococcal complement inhibitor (*scn*) gene was not found in any of the swine-associated ST5 isolates (0/82). It was detected in 90.3% (65/72) of the isolates from humans with no swine contact ($p < 0.0001$).

The gene for the chemotaxis inhibitory protein (*chp*) was absent in the swine-associated isolates (0/82), but was detected in 88.9% (64/72) of isolates from humans with no swine contact ($p < 0.0001$). The staphylokinase (*sak*) gene was also lacking in all of the swine-associated isolates (0/82), but found to be present in 90.3% (65/72) of isolates from humans with no swine contact ($p < 0.0001$). The gene encoding staphylococcal enterotoxin-like P (*sep*) was absent from all swine-associated isolates (0/82), but detected in 37.5% (27/72) of isolates from humans with no swine contact ($p < 0.0001$). The staphylococcal enterotoxin A (*sea*) gene was not found in any of the swine-associated isolates (0/82), and the prevalence of this gene was also low (1.4%, 1/72) in isolates from humans with no swine contact ($p = 0.4675$).

Table 3.2: Immune-evasion complex and β-hemolysin converting bacteriophage screening results for all isolates.

		Gene Tested						
Isolate Source		***scn***	***chp***	***sak***	***sea***	***sep***	***int***	**Intact *hlb*[a]**
Agricultural	Human: short-term contact	0 (0/9)[b]	0 (0/9)	0 (0/9)	0 (0/9)	0 (0/9)	0 (0/9)	100 (9/9)
	Human: long-term contact	0 (0/9)	0 (0/9)	0 (0/9)	0 (0/9)	0 (0/9)	0 (0/9)	100 (9/9)
	Pig	0 (0/38)	0 (0/38)	0 (0/38)	0 (0/38)	0 (0/38)	0 (0/38)	100 (38/38)
	Environment	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	0 (0/26)	100 (26/26)
Clinical		90.3 (65/72)	88.9 (64/72)	90.3 (65/72)	1.4 (1/72)	37.5 (27/72)	90.3 (65/72)	9.7 (7/72)

[a] Data reported represents the results from Southern blotting.
[b] Data reported as percent of isolates positive for each gene tested. Number of positive isolates is noted in parenthesis.

Table 3.3: Phage types in human clinical isolates.

Phage Type	Genes	van Wamel et al [29]	Price et al [323]	This Study
A	sea—sak—chp—scn	[a]12.2 (11)	0 (0)	1.4 (1)
B	sak—chp—scn	26.7 (24)	31.6 (6)	50 (36)
C	chp—scn	13.3 (12)	52.6 (10)	0 (0)
D	sea—sak— —scn	15.6 (14)	0 (0)	0 (0)
E	sak— —scn	14.4 (13)	0 (0)	1.4 (1)
F	sep—sak—chp—scn	4.4 (4)	0 (0)	37.5 (27)
G	sep—sak— —scn	3.3 (3)	0 (0)	0 (0)
H	scn	0 (0)	10.5 (2)	0 (0)
None	None	10 (9)	5.3 (1)	9.7 (7)

[a]Data are reported as the percent of isolates of each phage type out of the number of isolates evaluated. The number of isolates found of each phage type is listed in parenthesis.

β-hemolysin converting bacteriophage prevalence via integrase gene screening

Although PCR results for the IEC genes indicated the presence or absence of the β-hemolysin converting bacteriophage in the tested isolates, PCR analysis for the integrase gene insertion site was used to verify the presence of an integrated phage. The integrase gene enables lysogenic bacteriophages to integrate into the bacterial genome. Due to the high degree of nucleotide sequence conservation of bacteriophage integrase genes, the primers used for this PCR analysis were developed to create a product that spanned the 3' end of the integrase gene and the 5' end of the β-hemolysin gene (Figure 3.1). This ensured specificity to the integrase gene of the β-hemolysin converting bacteriophage. None of the swine isolates were found to contain the integrase/β-hemolysin gene junction (0/82), while the gene was present in 90.3% (65/72) of the isolates from humans with no swine contact ($p < 0.0001$).

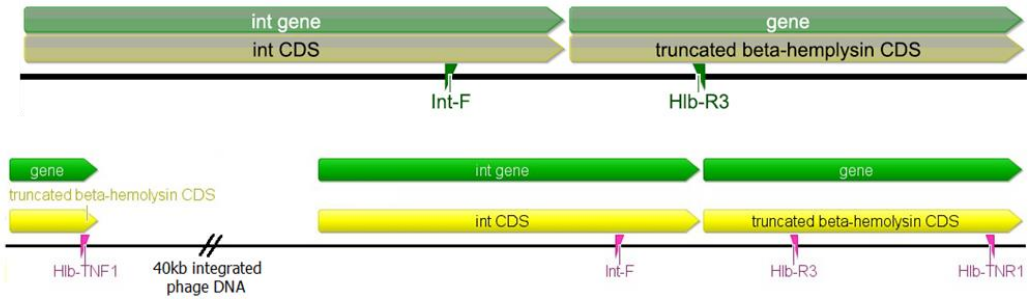

Figure 3.1. Location of primers used for PCR used to test for the presence of the integrase gene and intact β-hemolysin gene. The integrase gene associated with the β-hemolysin converting bacteriophage (int CDS) and the 5' end of the disrupted β-hemolysin gene (truncated beta-hemolysin CDS) are shown along with the primer specific for the the integrase gene (Int-F) and the primer specific for the β-hemolysin gene (Hlb-R3). The primers used for detection of an intact β-hemolysin gene (Hlb-TNF1 and Hlb-TNR1) spanned the disrupted portion of the gene and no product should be generated when the phage has integrated.

β-hemolysin gene analysis

To further confirm the absence of the β-hemolysin converting bacteriophage in the isolates tested, PCR screening was undertaken using primers Hlb-TNF1 and Hlb-TNR1 (Figure 3.1). An ST398 strain was used as a control for the absence of the bacteriophage and produced a 750 base pair product, while the controls for the presence of the bacteriophage, ST8 Newman, ST5 Mu3, ST5 Mu50, produced a 300 base pair product. After further analysis, the nucleotide sequence of 300 base pair product produced by the control isolates containing the bacteriophage was determined to be a portion of bacteriophage DNA at the 5' end of the integrated sequence. All swine-associated isolates produced a band 750 base pairs in length (82/82). These results demonstrate that the β-hemolysin gene is intact and no bacteriophage is present in these strains. Of isolates from humans with no swine contact, 9.7% (7/72) produced a band 750 base pairs in length. However, only 31.9% (23/72) of the isolates produced a single band 300 base pairs in length. Many of the isolates (56.9%, 41/72) produced two bands, one at 750 base pairs and the other at 300 base pairs in length (Figure 3.2). After comparing these results with the PCR results for the IEC genes and the integrase gene, it was determined that the isolates producing a 300 base pair band, even with the presence of a 750 base pair band, were carrying a disrupted β-hemolysin gene. One explanation for the multiple PCR products is that they may have been produced due to the induction of beta-converting phage during bacterial culture, subsequently leading to the loss of phage in a sub-population of bacterial cells.

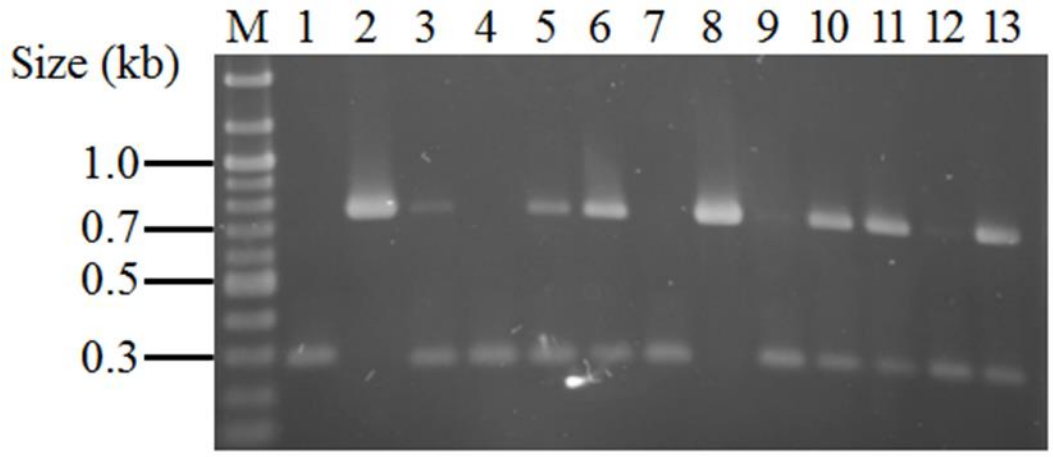

Figure 3.2. Agarose gel electrophoresis demonstrating inconclusive banding pattern resulting from PCR used to test an intact β-hemolysin gene in isolates obtained from humans with no swine contact. Those isolates producing only a 750bp band were found to contain an intact β-hemolysin gene, while isolates producing only a 300bp band or both bands were found to contain a disrupted β-hemolysin gene. A disrupted β-hemolysin gene is represented by the negative control ST8 Newman (lane 1), which produced a band 300bp in size. An intact β-hemolysin gene is represented by the positive control ST398 (lane 2), which produced a band 750bp in size. Of the isolates from humans with no swine contact, 9.7% (7/72) produced a 750bp band (lane 8) and 31.9% (23/72) produced a 300bp band (lane 4, 7). However, 56.9% (41/72) of the isolates produced both a 750bp and a 300bp band (lane 3, 5, 6, 9–13).

Due to the inconclusive banding pattern, Southern blotting was used to confirm whether the β-hemolysin gene was intact or disrupted using a probe derived from the 750bp PCR product of ST398 and the Hlb-TNF1 and Hlb-TNR1 primers. The restriction enzyme BamH1 was selected to digest the genomic DNA because no restriction sites were present within the β-hemolysin gene or any of the bacteriophage genes (confirmed using Newman, Mu3, and Mu50 genomes in GenBank accession numbers NC_009641, NC_009782, NC_002758 respectively). The genome fragments produced by BamH1 digestion of isolates with an intact and disrupted β-hemolysin genes were approximately 20kb and 65kb respectively and were readily distinguished during analysis of the Southern blots (Figure 3.3). Intact genes produced a distinct band around 20kb, while disrupted genes showed background extending beyond 48kb with no distinct band. The lack of a distinct band seen with the disrupted genes was attributed to reduced annealing

strength of the probe to the disrupted β-hemolysin gene and the reduced transfer rate seen with larger band sizes. Southern blotting was able to confirm β-hemolysin gene disruption in 90.3% (65/72) of the isolates from humans with no swine contact. The β-hemolysin gene was intact in 9.7% (7/72) of isolates from humans with no swine contact and in 100% (82/82) of the swine-associated isolates ($p < 0.0001$).

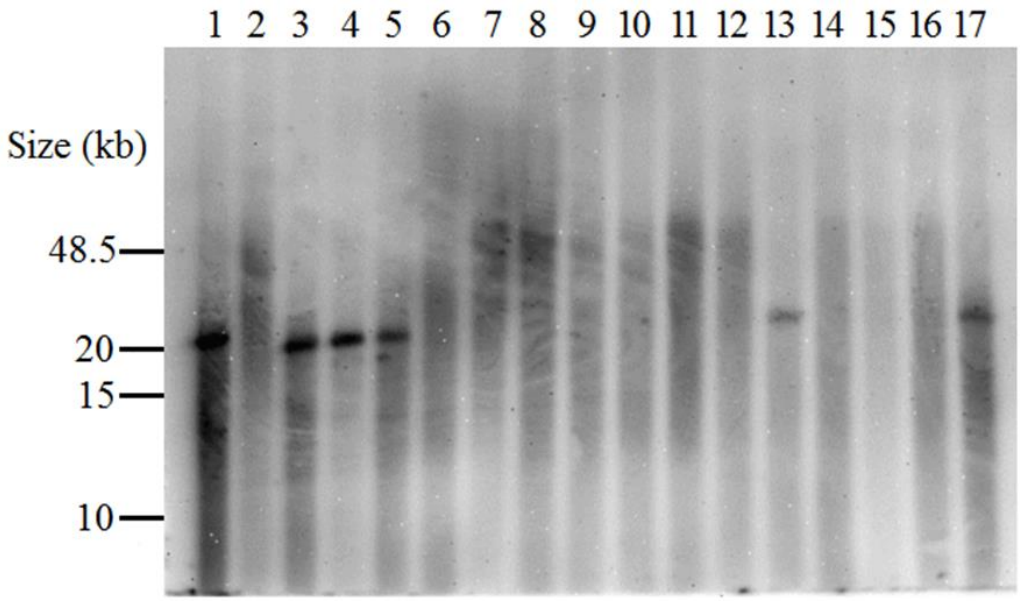

Figure 3.3. Southern blot demonstrating the presence of an intact or disrupted β-hemolysin gene. Isolates containing an intact β-hemolysin gene produced a distinct band (20kb) as seen with the control isolate ST398 that lacked the bacteriophage (lane 1). Isolates containing a disrupted β-hemolysin gene did not produce a distinct band at 20kb and background extended in the lanes to a size greater than 40kb, as seen with the control ST8 Newman (lane 2). Swine-associated MRSA ST5 isolates are represented by lanes 3–5 and MRSA ST5 from humans with no swine contact are represented by lanes 6–17. Lanes 13 and 17 contained isolates from humans with no swine contact bearing an intact β-hemolysin gene..

Phage typing

The different combinations of the immune evasion complex genes carried by the β-hemolysin converting bacteriophage have been previously defined into seven types (A-G) by van Wamel and colleagues [29]. A novel phage type was discovered by Price and colleagues and will be referred to here as type H [323]. The phage types found in both studies can be found in Table 3.3 along with the prevalence rate found in the isolates

from humans with no swine contact analyzed in this study. The ST5 isolates evaluated in this study were found to contain primarily type B (50%, 36/72) and type F (37.5%, 27/72) prophages.

Discussion

It has long been known that *S. aureus* commonly colonizes many mammalian and avian species and particular lineages are more adapted to different host species [417]. The recent recognition that livestock may represent a substantial reservoir of MRSA and people having regular contact with animals were commonly colonized with LA-MRSA isolates represented a shift in MRSA epidemiology and raised urgent questions about the public health significance of these organisms. Fundamental questions remain about the ability of *S. aureus* lineages adapted to animals to both colonize and cause disease in humans. The capacity of ST398 LA-MRSA to cause clinical disease in humans is established, but reports of severe infections in people with occupational exposure to livestock remain uncommon, despite continued exposure to these organisms. There is increasing evidence that ST398 MRSA isolates of animal origin are less likely to be transmitted between people and are less likely to be associated with severe infections than are human adapted variants. Genomic studies have indicated that distinct livestock and human variants are identifiable even within a given sequence type and *spa* type, such as ST398/t571 and ST1/t127 [344, 418]. More specifically, the absence of MGE associated virulence factors, including IEC genes has been linked to host adaptation and loss of virulence in ST398 LA-MRSA [323, 414, 415].

The MRSA lineages ST398 and ST9, which predominate in swine populations in Europe and Asia respectively, do not appear to have a significant impact on human health

in the US. Unlike in Europe and Asia where ST5 MRSA have only rarely been reported, several studies indicated that ST5 S. aureus (both MRSA and MSSA) are relatively common in the North American swine industry [335, 353, 362, 402, 419]. Because the ST5 lineage is a major contributor to both hospital and community associated MRSA and MSSA infections in this country and worldwide [12, 420], it is important to address the question about the potential contribution, if any, of the swine reservoir to the burden of clinical disease associated with ST5 *S. aureus*.

At this time, no human disease due to swine-associated LA-MRSA ST5 isolates has been reported. This may be due to differences in the composition of the accessory genome seen in LA-MRSA versus HA- and CA-MRSA isolates, similar to that previously noted in ST398 isolates [323, 415]. HA- and CA-MRSA ST5 isolates are known to carry several MGE that enhance their virulence and antibiotic resistance, which have contributed to their dissemination and pathogenicity [12].

This is the first report examining β-hemolysin converting bacteriophage prevalence within human clinical MRSA ST5 isolates specifically. The incidence of prophage integration in ST5 isolates obtained from humans with no swine contact was consistent with that found in previous reports of human clinical S. aureus isolates, both MRSA and methicillin-susceptible [29, 323, 415]. Additionally, this is the first study investigating the prevalence of the β-hemolysin converting bacteriophage in LA-MRSA ST5 isolates. The results obtained in this study are consistent with previous publications, and comparative statistical analysis showed no significant difference ($p = 0.3987$) in the prevalence of β-hemolysin converting bacteriophages between LA-MRSA ST5 isolates

evaluated in this study (0/82) and LA-MRSA ST398 isolates evaluated previously (1/63) by Price and colleagues [323].

Due to the restricted host specificity of the most prevalent genes (*sak*, *scn*, *chp*), it has been suggested that this prophage may be absent in MRSA isolates after adapting to a livestock niche [323]. These genes would not confer an advantage during colonization or disease development in livestock species and are therefore unnecessary to retain within the genome of LA-MRSA isolates. The loss of these important virulence factors is likely one of the reasons LA-MRSA isolates are rarely known to cause invasive disease in immunocompetent humans. The absence of IEC genes carried by β-hemolysin converting bacteriophages in LA-MRSA ST398 and ST5 strains parallels the findings for poultry adapted ST5 strains in that the human-specific IEC genes were lost and subsequently replaced by genes encoding avian-specific factors after the human-to-poultry transition [325]. However, unlike poultry adapted ST5 strains, swine-associated LA-MRSA ST398 and ST5 strains harbor an intact β-hemolysin gene, indicating that the bacteriophage is absent from these strains rather than being replaced by genes encoding swine-specific factors.

There were several interesting differences noted between the prevalence of phage types found in the human clinical isolates evaluated in this study compared to those previously reported by van Wamel and colleagues (Table 3.3). Their initial investigation and description of the β-hemolysin converting bacteriophage types was completed using 85 clinical *S. aureus* isolates from several hospitals in the Netherlands and 5 *S. aureus* reference strains [29]. These isolates were not characterized using MLST typing and are therefore thought to have a more diverse genetic background than the isolates evaluated

here. Specifically, the enterotoxin A (*sea*) and enterotoxin-like P (*sep*) gene prevalence varied considerably between these investigations. In this study, significantly more ($p < 0.0001$) isolates were found to harbor the *sep* gene and significantly less ($p < 0.0001$) isolates were found to harbor the *sea* gene compared to the prevalences previously reported. The results reported here correlate with a decrease in the prevalence of phage types A and D and an increase in phage type F. This was surprising in that *sea* is considered to be the most common enterotoxin involved in staphylococcal food borne illness [409]. The increased prevalence of *sep* seen in this study may indicate a larger role in the development of clinical disease than previously described. Additionally, significantly more isolates were found to harbor the *chp* gene ($p < 0.0001$) and the *sak* gene ($p = 0.0352$) in this study. These results correlate with an increase in prevalence of the type B and F prophages that contain the three innate immune evasion genes. Variability in phage types seen within this population of clinical MRSA ST5 isolates indicates multiple acquisitions of the β-hemolysin converting bacteriophage even within a regional population. The differences in phage prevalence reported in this study compared to the van Wamel study may be due to the sample populations evaluated. The isolates evaluated previously were clinical isolates from several hospitals in the Netherlands and no MLST types were indicated. In contrast, the isolates evaluated in this study comprised of 73 clinical ST5 isolates from University of California associated medical facilities. The differences in phage prevalence reported could be attributed to the disease profile of the isolates, the narrowed genetic background of the isolates evaluated, or a regional difference (California versus the Netherlands) not previously noted.

ST5 *S. aureus* appear to be widespread in the North American swine population and have likely been endemic in this reservoir for some time, yet livestock contact has not been identified as a risk factor for clinical staphylococcal disease [361]. This investigation identified clear genomic differences between ST5 MRSA isolates linked to swine and isolates from human clinical infections. These differences parallel previous observations with ST398 isolates. We hypothesize that the genetic changes observed may reflect general processes related to host adaptation of *S. aureus* to pigs. More extensive genomic investigations of ST5 S. aureus in pigs are warranted, as is investigation of other lineages *S. aureus* associated with pigs such as ST9.

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CHAPTER 4. ZINC RESISTANCE WITHIN SWINE-ASSOCIATED METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* ISOLATES IN THE UNITED STATES IS ASSOCIATED WITH MULTILOCUS SEQUENCE TYPE LINEAGE

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Abstract

Zinc resistance in livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) sequence type 398 (ST398) is primarily mediated by the *czrC* gene colocated with the *mecA* gene, encoding methicillin resistance, within the type V staphylococcal cassette chromosome *mec* (SCC*mec*) element. Because *czrC* and *mecA* are located within the same mobile genetic element, it has been suggested that the use of

zinc in feed as an antidiarrheal agent has the potential to contribute to the emergence and spread of methicillin-resistant *S. aureus* (MRSA) in swine, through increased selection pressure to maintain the SCC*mec* element in isolates obtained from pigs. In this study, we report the prevalence of the *czrC* gene and phenotypic zinc resistance in U.S. swine-associated LA-MRSA ST5 isolates, MRSA ST5 isolates from humans with no swine contact, and U.S. swine-associated LA-MRSA ST398 isolates. We demonstrated that the prevalence of zinc resistance in U.S. swine-associated LA-MRSA ST5 isolates was significantly lower than the prevalence of zinc resistance in MRSA ST5 isolates from humans with no swine contact and swine-associated LA-MRSA ST398 isolates, as well as prevalences from previous reports describing zinc resistance in other LA-MRSA ST398 isolates. Collectively, our data suggest that selection pressure associated with zinc supplementation in feed is unlikely to have played a significant role in the emergence of LA-MRSA ST5 in the U.S. swine population. Additionally, our data indicate that zinc resistance is associated with the multilocus sequence type lineage, suggesting a potential link between the genetic lineage and the carriage of resistance determinants.

Importance

Our data suggest that coselection thought to be associated with the use of zinc in feed as an antimicrobial agent is not playing a role in the emergence of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) ST5 in the U.S. swine population. Additionally, our data indicate that zinc resistance is more associated with the multilocus sequence type lineage, suggesting a potential link between the genetic lineage and the carriage of resistance markers. This information is important for public health professionals, veterinarians, producers, and consumers

Introduction

Staphylococcus aureus commonly colonizes the skin and mucosal surfaces of mammalian and avian species and is present in the anterior nares of 20 to 30% of healthy humans [42]. *S. aureus* is also a major opportunistic human pathogen with diverse clinical manifestations, ranging from mild skin and soft tissue infections to severe systemic infections and fatal sepsis. Prior to the availability of antibiotics, fatality rates for human cases of *S. aureus* bacteremia were estimated at 80% [186]. Increased access to antibiotics has reduced the case fatality rate of *S. aureus* bacteremia to around 20 to 30% [186], but the capacity of *S. aureus* to acquire resistance to antibiotics has made multidrug-resistant strains a major public health concern [368].

Methicillin-resistant *S. aureus* (MRSA) was first reported in 1961 [246] and rapidly became endemic in hospitals (i.e., hospital-associated MRSA [HA-MRSA]) in many countries. During the 1990s, an increasing number of MRSA infections occurred in persons with no known risk factors for HA-MRSA infection [421]. These infections developed in healthy members of the general community and were termed community-associated MRSA (CA-MRSA). *S. aureus* is considered a clonal organism, and genotypes associated with hospital infections typically differed from those associated with community infections, as well as varying geographically [422].

Although MRSA was first reported in food animals (dairy cattle) in 1972 [309], animal reservoirs were not considered to play a significant role in MRSA epidemiology until 2004, when an atypical MRSA variant was detected in three people in the Netherlands and was attributed to their residence on a swine farm [248]. These initial isolates could not be typed by pulsed-field gel electrophoresis using SmaI restriction

digestion, due to a variation in methylation by the type I restriction modification system. Multilocus sequence typing (MLST) revealed that these isolates belonged to a novel sequence type (ST), ST398 [248]. This genotype was found to be widespread in the Dutch pig industry and to be present in other animal species, including cattle, poultry, and horses [12, 312, 423, 424]. Subsequent research revealed more complex epidemiology, and the predominant genotypes of MRSA found in swine vary geographically. In most Asian countries, ST9 variants are most common [357, 359, 425]; in the United States and Canada, both ST398 and ST5 MRSA variants appear to be relatively common, with ST9 MRSA being detected sporadically [335, 353, 360, 362].

Resistance to tetracycline antibiotics has been almost universal in *S. aureus* isolates from pigs. Additionally, a prominent feature of livestock-associated MRSA (LA-MRSA) ST398 isolates from Europe and North America is the high prevalence (61 to 74%) of zinc resistance seen in swine-associated isolates [323, 346, 347, 349], relative to isolates from veal calves (42%) or humans (48%) [346, 347]. Zinc resistance in these MRSA isolates has been attributed to colocalization of the *czrC* gene (conferring zinc and cadmium resistance) on the type V staphylococcal cassette chromosome *mec* (SCC*mec*) element, which contains the *mecA* gene (conferring methicillin resistance). A strong correlation between phenotypic zinc resistance and the presence of *czrC* was reported previously, with 99% of MRSA ST398 isolates harboring *czrC* showing phenotypic zinc resistance and 96% of isolates exhibiting zinc resistance harboring the *czrC* gene [346]. Dietary zinc supplementation at >2,400 ppm (compared with the minimum nutritional requirement of 100 to 165 ppm) for 5 to 10 days is commonly used in weaned pigs to control enteric disease [426]. Since *czrC* and *mecA* are colocated on the SCC*mec*

element, it has been suggested that the use of high concentrations of zinc in feed might have contributed to the emergence and spread of MRSA in swine, by increasing the selection pressure to maintain the SCC*mec* element in swine-associated ST398 isolates [348, 349, 427].

While many reports detailing the prevalence of zinc resistance in LA-MRSA ST398 and ST9 isolates have been published, little to no information exists regarding the prevalence of zinc resistance in LA-MRSA ST5 isolates [346]. Here we report the prevalence of zinc resistance in U.S. swine-associated LA-MRSA ST5 isolates and compare it with the prevalence in MRSA ST5 isolates obtained from humans with no swine contact and that in U.S. swine-associated LA-MRSA ST398 isolates, as well as that in previous studies reporting zinc resistance in LA-MRS ST398 isolates.

Materials and Methods

Isolate acquisition

Swine-associated LA-MRSA ST5 cultures were isolated from swine (n = 38), the environment within swine facilities (n = 26), and persons with short-term (n = 9) and long-term (n = 9) swine contact. These isolates were provided by Iowa State University and the University of Minnesota [335]. Clinical isolates from humans with no swine contact were obtained from the University of California, Irvine (n = 64) [375], and the University of California, San Francisco (n = 7 MRSA and 1 methicillin susceptible *S. aureus*), hospitals servicing urban populations in Orange County (southern California) and the San Francisco area (northern California), respectively. Swine-associated LA-MRSA ST398 cultures obtained from Iowa State University were isolated from swine (n = 8) or the environment within swine facilities (n = 6) [335]. Isolates were subjected to

MLST and *spa* typing prior to acquisition [335, 375]. Isolate sources and *spa* types are provided in Supplemental Table 1 (available at https://doi.org/10.1128/AEM.00756-17) and in Appendix A.

Zinc susceptibility testing

Zinc chloride MICs were determined by agar dilution, as described by Aarestrup and Hasman [428]. Briefly, plates of Mueller-Hinton agar with an adjusted pH of 5.5 were supplemented with zinc chloride in 2-fold dilutions, with concentrations ranging from 0.25 to 16 mM. The isolate *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 was used as a positive control, and *S. aureus* ATCC 29213 and ATCC 43300 were used as negative controls. A MIC value of >2 mM was used as the cutoff value to designate resistance, in accordance with previously published reports and the result for the positive control in this study [346, 347].

***czrC* PCR testing**

The presence of the *czrC* gene was determined by PCR using previously reported primers and protocols [347]. Briefly, PCR was carried out in an MJ Research PCT-200 DNA Engine thermocycler (GMI, Ramsey, MN) using 50 ng of purified genomic DNA from the appropriate strains, the forward primer 5'-TAGCCACGATCATAGTCATG-3', and the reverse primer 5'-ATCCTTGTTTTCCTTAGTGACTT-3'. Reaction mixtures included 0.4 μM primers, 1 U of AmpliTaq polymerase (Applied Biosystems, Foster City, CA), 2.5 μl of 10× buffer II (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 2.5 mM $MgCl_2$, and 200 μM deoxynucleoside triphosphates (dNTPs), in a final volume of 50 μl. Cycling conditions were 95°C for 2 min, 30 cycles of 95°C for 15 s, 52°C for 30 s, and

72°C for 1.5 min, and a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light.

SCC*mec* typing

SCC*mec* typing was completed using previously designed primer sets (Table 4.1) [429-434]. Briefly, PCR was carried out in a MJ Research PCT-200 DNA Engine thermocycler (GMI) using 50 ng of purified genomic DNA from the appropriate strains; reaction mixtures included 0.4 μM primers, 1 U of AmpliTaq polymerase (Applied Biosystems), 2.5 μl of 10× buffer II (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 2.5 mM $MgCl_2$, and 200 μM dNTPs, in a final volume of 50 μl. PCR for the *ccrA* and *ccrB* genes was a multiplex reaction with cycling conditions of 95°C for 2 min, 10 cycles of 95°C for 15 s, 65°C for 30 s, and 72°C for 1.5 min, 25 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension step of 72°C for 7 min. PCR of the *ccrC* gene used cycling conditions of 95°C for 2 min, 30 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 2 min, and a final extension step of 72°C for 7 min. PCR of the *mec* element genes was completed with cycling conditions of 95°C for 2 min, 30 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 2 min, and a final extension step of 72°C for 7 min. PCR products were run on a 1% agarose gel, stained with ethidium bromide, and visualized using UV light.

Table 4.1: Primer sets used to SCCmec type isolates.

	Nucleotide Sequence	Expected Product (Forward Primer Used)	Source
ccrB-F	ATTGCCTTGATAATAGCCITCT		Ito et al. 2001 [429]
ccrA1-R	AACCTATATCATCAATCAGTACGT	694 (ccrB-F)	Ito et al. 2001 [429]
ccrA2-R	TAAAGGCATCAATGCACAAACACT	937 (ccrB-F)	Ito et al. 2001 [429]
ccrA3-R	AGCTCAAAAGCAAGCAATAGAAT	1791 (ccrB-F)	Ito et al. 2001 [429]
ccrA4-R	GTATCAATGCACCAGAACTT	1287 (ccrB-F)	Kondo et al. 2007 [431]
ccrC-F	CGTCTATTACAAGATGTTAAGGATAAT		Kondo et al. 2007 [431]
ccrC-R	CCTTTATAGACTGGATTATTCAAAATAT	518 (ccrC-F)	Kondo et al. 2007 [431]
mecI-F	CAAGTGAATTGAAACCGCCT		Okuma et al. 2002 [432]
mecI-R	CAAAAGGACTGGACTGGAGTCCAAA	187 (mecI-F)	Okuma et al. 2002 [432]
mecR1-R	GTCTCCACGTTAATTCCATT	1920 (mecI-F)	Kobayashi et al. 1996 [433]
Class B-F	TATACCAAACCCGACAAC		Katayama et al. 2001 [430]
IS1272-R	AACGCCACTCATAACATATGGAA	1996 (Class B-F)	Okuma et al. 2002 [432]
Class C-F	AACGTTGTAACCACCCCAAGA		Hiramatsu et al. 1992 [434]
IS431-R	TGAGGTTATTCAGATATTTCGATGT	2072 (Class C-F)	Katayama et al. 2001 [430]

Whole-genome sequencing and analysis

Draft genome sequence data for 14 *czrC*-carrying isolates (UCI3, UCI9, UCI11, UCI19, UCI21, UCI24, UCI27, UCI43, UCI45, UCI46, UCI48, UCI52, UCI56, and UCI64) were generated using the Illumina MiSeq platform (Illumina, San Diego, CA) [376]. Indexed libraries were generated and run on the MiSeq platform using the 500-cycle MiSeq v2 reagent kit. The data were assembled using MIRA 4.0.2 (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html) [435]. Closed genomes were obtained for UCI28 and UCI62 as described previously [436]. Briefly, genomic DNA was sequenced with a PacBio RSII instrument using a 10-kb insert library and one SMRT cell for each isolate. The data were assembled using PacBio SMRT Analysis 2.3.0 and CANU 1.3 software. The genomes were then polished and error corrected using Illumina MiSeq data and Broad Institute Pilon 1.18 software. Whole-genome sequence data were analyzed using Geneious 9.0.5 (Biomatters Ltd., Auckland, New Zealand). The SCC*mec* region was extracted from the closed genome sequences of Mu3 (GenBank accession number AP009324.1), UCI28 (GenBank accession number CP018768), UCI62 (GenBank accession number CP018766), and S0385 (GenBank accession number AM990992.1); these were compared visually in Geneious, using multiple sequence alignments to determine similarity. For the 14 draft genomes, the contig harboring the *czrC* gene was extracted and used for comparison. These regions were analyzed for similarity to UCI28 and UCI62 using multiple sequence alignments.

Statistical analysis

Comparisons between isolates from humans with no swine contact and swine-associated isolates were completed using Fisher's exact test using GraphPad Prism (GraphPad Software, La Jolla, CA).

Accession numbers

The whole-genome sequences for isolates UCI28 and UCI62 were deposited in DDBJ/ENA/GenBank with the following accession numbers: UCI28, CP018768 and CP018769; UCI62, CP018766 and CP018767 [436]. The draft genome sequences obtained for 14 *S. aureus* ST5 isolates were deposited in DDBJ/ENA/GenBank with the following accession numbers: UCI3, LKYU00000000; UCI9, LKZA00000000; UCI11, LKZC00000000; UCI19, LKZK00000000; UCI21, LKZM00000000; UCI24, LKZP00000000; UCI27, LKZS00000000; UCI43, LLAI00000000; UCI45, LLAK00000000; UCI46, LLAL00000000; UCI48, LLAN00000000; UCI52, LLAR00000000; UCI56, LLAV00000000; UCI64, LLBD00000000 [376].

Results

Prevalence of the *czrC* gene

The *czrC*-specific PCR demonstrated that none of the tested swine-associated MRSA ST5 isolates (0/82 isolates) harbored the *czrC* gene (Table 4.2). In contrast, all LA-MRSA ST398 isolates (14/14 isolates) tested harbored the *czrC* gene. The prevalence of *czrC* in LA-MRSA ST5 isolates associated with swine was significantly lower than that in swine-associated LA-MRSA ST398 isolates ($P < 0.0001$) (Table 4.2) and also was lower than that reported for other LA-MRSA ST398 isolates ($P < 0.0001$) [346]. Over one-fifth of MRSA ST5 isolates obtained from humans with no swine contact (16/72

isolates [22%]) contained the *czrC* gene (Table 4.2). The prevalence of the *czrC* gene among MRSA ST5 isolates obtained from humans with no swine contact was significantly higher than that among swine-associated ST5 isolates ($P < 0.0001$) (Table 4.2). Information on individual isolates is provided in Supplemental Table 1 (available at https://doi.org/10.1128/AEM.00756-17).

Table 4.2: The prevalence of phenotypic zinc chloride resistance and *czrC* presence in isolates from human with no swine contact and swine-associated isolates.

	Number of Isolates/Total Number of Isolates (%)		
Characteristic	**MRSA ST5 from Humans with no Swine Contact**	**LA-MRSA ST5**	**LA-MRSA ST398**
czrC PCR Prevalence	16/72 (22.2%)[a]	0/82 (0%)	14/14 (100%)[a]
Phenotypic Zinc Chloride Resistance	18/72 (25%)[a]	0/82 (0%)	14/14 (100%)[a]

[a] – Statistical significance ($p < 0.0001$) as compared to LA-MRSA ST5

Zinc chloride susceptibility testing

Susceptibility testing revealed that no swine-associated MRSA ST5 isolates (0/82 isolates) were resistant to zinc chloride, while phenotypic resistance was seen for all LA-MRSA ST398 isolates (14/14 isolates). The prevalence of phenotypic resistance to zinc among MRSA ST5 isolates obtained from humans with no swine contact was 25% (18/72 isolates), greater than among swine-associated MRSA ST5 isolates ($P < 0.0001$) (Tables 4.2 and 4.3). Two MRSA ST5 isolates obtained from humans with no swine contact exhibited phenotypic resistance despite not harboring the *czrC* gene. Phenotypic zinc chloride resistance in the absence of *czrC* was reported previously for MRSA ST398 and non-ST398 isolates by Cavaco et al. [346], which indicates that an alternative mechanism for zinc resistance is also present in MRSA ST5 isolates.

Table 4.3: Results from phenotypic zinc chloride resistance screen.

Isolate Type	Number of isolates with susceptibility of*:						
	0.25 mM	**0.5 mM**	**1 mM**	**2 mM**	**4 mM**	**8 mM**	**16 mM**
MRSA ST5 from Humans with no Swine Contact (n = 73)	7	0	18	29	14	4	0
LA-MRSA ST5 (n = 82)	0	20	53	9	0	0	0
LA-MRSA ST398 (n = 14)	0	0	0	0	14	0	0

* >2 mM designates resistance

SCC*mec* typing

The swine-associated LA-MRSA ST5 isolates carried SCC*mec* type III (17/82 isolates [21%]) or type IV (42/82 isolates [51%]) or could not be typed using the primer sets published previously (23/82 isolates [28%]) (Table 4.4). Of the 23 untypeable isolates, 20 (24.4% of LA-MRSA ST5 isolates) carried a class D *mec* gene complex, which has not been assigned to a *mec* type, and 3 carried a class A *mec* gene complex without the traditional *ccrA-ccrB* gene combination. All LA-MRSA ST398 isolates tested harbored *SCCmec* type V (14/14 isolates). The MRSA ST5 isolates from humans with no known swine contact mostly carried SCC*mec* type II (69/72 isolates [96%]); the others were type IV (2/72 isolates [2.8%]) or lacked a SCC*mec* element (1/72 isolates [1.4%]).

Table 4.4: SCCmec type and czrC gene prevalence in swine associated LA-MRSA ST5 and ST398 and MRSA ST5 isolates from humans with no swine contact.

Isolate type	**SCC*mec* type**	***czrC* prevalence**
LA-MRSA ST5	III	0/17
	IV	0/42
	Untypable[a]	0/23
MRSA ST5 from Humans with no Swine Contact	II	16/69
	IV	0/2
	None	0/1
LA-MRSA ST398	V	14/14

[a] – Isolates that are unable to be classified into an SCC*mec* type due to the presence of a *ccr* gene or *mec* complex unable to be determined using available primer sets or the presence of a *ccr* and *mec* complex combination not currently assigned an SCC*mec* type.

***czrC* localization**

To determine the location of the *czrC* gene within the genomes of the 16 MRSA ST5 isolates obtained from humans with no swine contact that harbored the *czrC* gene, draft genome sequences were obtained, along with the complete genome sequences for two of the strains (UCI28 and UCI62) [376, 436]. The gene content and organization of the *SCCmec* region and the surrounding mobile genetic elements for strains UCI28 and UCI62, along with strains Mu3 and S0385 for reference, are shown in Figure 4.1. The *SCCmec* region for strains Mu3 and UCI28 contained pUB110 within the J3 region of the SCC*mec* element (Figure 4.1-A and B). Strain UCI28 and 12 other isolates (UCI3, UCI9, UCI19, UCI21, UCI28, UCI43, UCI45, UCI46, UCI48, UCI52, UCI56, and UCI64) harbored *czrC* downstream of *speG* and upstream of pUB110 and the SCC*mec* element (Figure. 4.1-B) [376, 436]. The nucleotide sequences containing *speG* and *czrC* located between the 23S methyltransferase and the second transposase were observed to be 100% identical between strains UCI28 and UCI62. The SCC*mec* elements of Mu3, UCI28, and UCI62 were observed to be 95.8% identical, with nucleotide differences being found in the J2 region. Isolate UCI62, as well as UCI11and UCI27, harbored *czrC* downstream of *speG* and upstream of the arginine catabolic mobile element (ACME) genes and the SCC*mec* element (Figure 4.1-C) [376, 436]. The *czrC* gene for all 16 clinical isolates, even those lacking ACME, was located downstream of *speG*, a spermidine acetyltransferase that functions in the detoxification of spermidine and is often found within the ACME composite island. The location of the *czrC* gene within the SCC*mec*

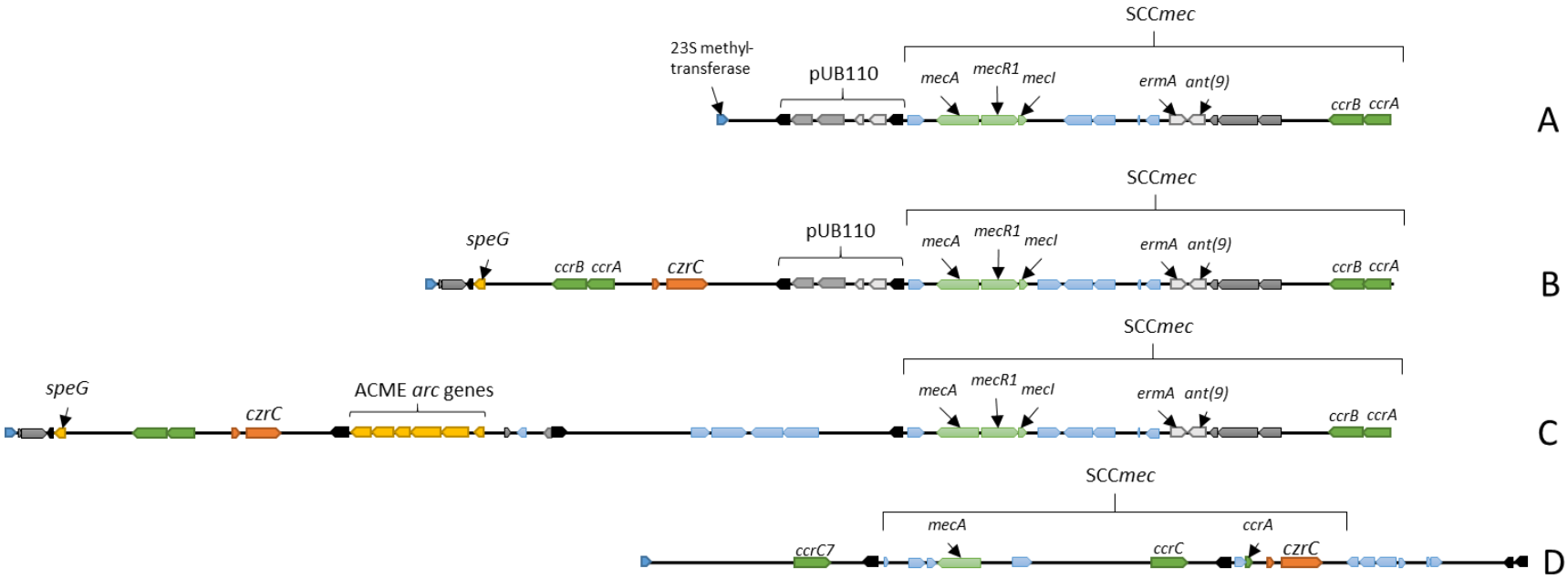

Figure 4.1. Localization of the czrC gene within the SCCmec region. SCC*mec* region and surrounding mobile genetic elements for Mu3 (A), UCI28 (B), UCI62 (C), and S0385 (D). All regions start at the 23S methyl-transferase (indicated by the blue pentagons). Insertion sequences are depicted by solid black pentagons ( ). The location of the *SCCmec* elements, *czrC*, *speG*, and pUB110 are indicated along with other previously annotated genes of interest. The SCC*mec* element of S0385 (D) is given as a reference for the location of *czrC* within the type V SCC*mec* element in LA-MRSA ST398.

region of these isolates is different than the location of *czrC* in the LA-MRSA ST398 reference strain S0385, in which *czrC* is located downstream of the *mecA* gene within the type V SCC*mec* (Figure 4.1-D) [333].

Discussion

The recent emergence of MRSA in livestock throughout the world has become a focal point in discussions regarding the role of antibiotic use in food animal production and the development of antibiotic-resistant clinical infections in humans. However, the mechanisms and factors responsible for this emergence, as well as the factors contributing to the geographical variations in genotypes of swine-associated MRSA found globally, are poorly understood. Although some causal role of antibiotic use in the emergence of LA-MRSA is hypothesized and may seem obvious, epidemiological evidence of such relationships has not been readily demonstrated [437]. It is clear that other factors, including disinfectants and metals, may play selective roles in the emergence of particular MRSA clones in humans and animals [349, 438, 439].

In this study, zinc resistance mediated by the *czrC* gene was examined as a potential contributor to the prevalence of LA-MRSA ST5 on swine farms in the United States. A documented association exists between the presence of *czrC* and the *mecA* gene in LA-MRSA ST398 isolates obtained from swine farms, with swine-associated isolates having a higher prevalence of *czrC* than LA-MRSA ST398 isolates obtained from veal calves or humans [346, 347]. The strong correlation (99%) reported between isolates harboring the *czrC* gene and phenotypic zinc resistance in LA-MRSA ST398 indicates that this gene orchestrates the predominant mechanism mediating zinc resistance in this lineage [346]. The specific importance of the *czrC* gene and the physical link between

mecA and *czrC* within the SCC*mec* element provide a mechanism by which dietary supplementation of zinc in swine rations could contribute to the persistence of methicillin resistance through coselection [347-349, 427]. Evidence of the practical relevance of this mechanism comes from Denmark, where widespread use of zinc in weaned pig diets as an alternative to antibiotic therapy for controlling enteric disease followed the banning of antibiotics for growth promotion in 2000, approximately a decade before LA-MRSA ST398 became highly prevalent in the Danish swine industry [348].

Sequencing studies have demonstrated that the *czrC* gene is located within the type V and type VIII SCC*mec* elements of ST398 MRSA [347, 440]. The majority of European LA-MRSA ST398 isolates investigated carried the type V SCC*mec* element containing the *czrC* gene downstream of the *mecA* gene [333], but none of the LA-MRSA ST5 isolates we examined carried the SCC*mec* type V element. All of the MRSA ST5 isolates from humans with no swine contact that carried the *czrC* gene contained the SCC*mec* type II element (Table 4.4), which has not been previously associated with the *czrC* gene. In those isolates, the *czrC* gene was located upstream of the SCC*mec* element and possibly transferred with the *speG* gene, which confers resistance to spermidine and a potential selective advantage for isolates colonizing and infecting humans [176]. Importantly, the fact that none of the swine-associated LA-MRSA ST5 isolates harbored the SCC*mec* types seen in ST5 MRSA isolates from clinical infections provides further evidence that the animal and human reservoirs of ST5 MRSA appear to be phylogenetically distinct [398].

Previous reports examining *czrC* in LA-MRSA isolates indicated a higher prevalence of this gene in MRSA clonal complex 398 (CC398) isolates (72.5%),

compared to all non-CC398 isolates evaluated (25.5%) [346]. An absence of *czrC* in European and Asian LA-MRSA isolates of the CC5 and CC9 lineages has been reported, which is consistent with our results evaluating LA-MRSA ST5 isolates from the United States. Collectively, our results and previously published data indicate that the *czrC* gene has a lineage association and is prevalent in the ST398 lineage but is absent or rare among livestock-associated ST5 and ST9 lineages [346]. An alternate explanation for the elevated prevalence of the *czrC* gene in the ST398 lineage is the selection pressure incurred with the use of elevated levels of zinc in feed. However, the prevalence of the *czrC* gene in non-ST398 LA-MRSA isolates from European swine was reported to be 30% in the tested isolates, while the phenotypic zinc resistance was reported to be 60% in the same isolates [346], arguing against selection pressure incurred with the use of elevated levels of zinc in feed being the sole factor controlling MRSA prevalence in swine, because the majority of these isolates lacked an SCC*mec* element carrying *czrC*. Although no national data concerning the use of zinc in swine rations exist, the practice is thought to be widespread in the United States (M. Tokach, personal communication). This appears not to have played the same role in propagating methicillin resistance in livestock isolates of *S. aureus* in the United States, as the majority of herds tested in recent studies were MRSA negative [335, 361].

Our results reported here, combined with previously reported results [346], open new avenues of research to be explored. First, the *czrC* gene has been identified in two methicillin-susceptible *S. aureus* ST398 isolates [323]. The presence of this gene without the SCC*mec* element should be evaluated to determine whether *czrC* is a remnant from a previously methicillin-resistant isolate or whether the *czrC* gene has been integrated

through a different mechanism. Both LA-MRSA isolates [346] and swine-associated methicillin-susceptible *S. aureus* isolates (J. Sun, R. S. Singer, S. J. Hau, T. L. Nicholson, and P. R. Davies, unpublished data) that show phenotypic zinc resistance without carrying the *czrC* gene have been identified. Such isolates should be screened for other mechanisms of zinc resistance, to determine the impact of other genes in conferring a resistant phenotype. Evaluation of the impact of *czrC* in non-ST398 LA-MRSA, specifically the ability of LA-MRSA ST5 isolates to acquire and to harbor *czrC*, and the impact of zinc in feed on the capability of LA-MRSA ST398 isolates to outcompete other lineages in swine also bears further investigation. Ultimately, zinc resistance in LA-MRSA is more complex than the presence or absence of *czrC* or the use of zinc in feed as an antimicrobial agent to combat disease in livestock. Further investigation is needed to determine the mechanisms leading to zinc resistance and to illuminate the impact of selective pressure on the emergence of particular MRSA clones in humans and animals.

Overall, the data reported here indicate that coselection associated with zinc supplementation in feed has not contributed to the persistence or prevalence of LA-MRSA ST5 in the U.S. swine population. This conclusion is contrary to theories surrounding the dissemination of LA-MRSA ST398 in Europe and, considering the presence of *czrC* in LA-MRSA ST398 isolates in the United States, indicates a potential link between the genetic lineage and the carriage of specific resistance markers, such as that seen for *qacA* resistance in CC22 in the hospital setting [439]. Furthermore, the data reported here indicate that multiple mechanisms contribute to fitness and the ability of LA-MRSA ST5 and other lineages to compete and to persist in the nasal microbiota of pigs.

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CHAPTER 5. DIFFERENCES IN ANTIMICROBIAL RESISTANCE PATTERNS AMONG METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* SEQUENCE TYPE 5 ISOLATES FROM HEALTH CARE AND AGRICULTURAL SOURCES

Adapted from an article prepared for *Journal of Antimicrobial Chemotherapy*

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Abstract

Background

Antimicrobial resistance is a prominent public health concern and methicillin resistant *Staphylococcus aureus* (MRSA) is a notable example. The discovery of

livestock associated MRSA (LA-MRSA) has heightened public health concerns due to the potential of LA-MRSA isolates to serve as a reservoir for antimicrobial resistance determinants.

Objectives

To compare swine associated LA-MRSA ST5 and human clinical MRSA ST5 isolates for phenotypic antimicrobial resistance and genetic determinants of resistance and evaluate for evidence of genetic exchange between swine associated and clinical MRSA ST5 isolates.

Methods

Minimum inhibitory concentrations for antibiotics were determined using microbroth dilution techniques. Genotypic determinants of antimicrobial resistance were detected through draft genome analysis.

Results

Swine associated LA-MRSA ST5 isolates exhibited resistance to fewer antibiotics than clinical MRSA ST5 isolates from humans with no swine contact. Distinct genomic antimicrobial resistance elements were harbored by each subgroup, with little overlap in shared antimicrobial resistance genes between swine associated LA-MRSA ST5 and clinical MRSA ST5 isolates.

Conclusions

Phenotypic antimicrobial susceptibilities and genotypic determinants of antimicrobial resistance among swine associated LA-MRSA ST5 and clinical MRSA ST5 isolates are separate and distinct suggesting ST5 MRSA isolates from agricultural sources

were not a source of antimicrobial resistance elements for these clinical human ST5 MRSA isolates.

Introduction

Treatment of *Staphylococcus aureus* infections is complicated by the bacterium's ability to acquire mobile genetic elements (MGEs) encoding antimicrobial resistance (AMR). Most notable of these is the SCC*mec* element harboring *mecA* (or less commonly *mecB* or *mecC*) which encodes resistance to the beta-lactamase resistant beta-lactam antibiotic methicillin [246]. Methicillin resistant *S. aureus* (MRSA) are typically classified epidemiologically based on their putative source into hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock associated (LA-MRSA); however, these designations have become blurred with some MRSA clones being identified in multiple settings.

MRSA infections were limited to hospital settings until the 1990s, when CA-MRSA isolates were detected in community members with no risk factors for HA-MRSA [370]. Although CA-MRSA isolates are considered more virulent than HA-MRSA isolates, HA-MRSA isolates typically harbor a greater number of AMR determinants [367, 370]. LA-MRSA was first reported in swine in 2004 and raised concerns that swine and other livestock may serve as reservoirs for MRSA isolates that can transmit to humans [248]. While LA-MRSA are less able to colonize and cause disease in humans than HA- and CA-MRSA isolates [274, 338, 344], they often harbor multiple antimicrobial resistance genes and can be a source for diverse AMR determinants, such as the multidrug resistance gene *cfr* and the lincosamide, pleuromutilin, and streptogramin A resistance genes *vgaC* and *vgaE* [249-251].

Multi-locus sequence typing (MLST) has been employed to classify *S. aureus* isolates by genetic lineage. The distribution of sequence types (STs) in both human and animal populations, including swine, vary regionally. In Europe, ST398 is the predominant swine associated LA-MRSA lineage and in Asia, ST9 isolates dominate [312, 357]. In contrast, swine herds in North America harbor a mixed population of LA-MRSA isolates containing ST398, ST9, and ST5 [335, 419]. While ST9 and ST398 MRSA isolates are considered livestock adapted and are uncommon causes of human infections [370, 441], ST5 isolates compose a globally disseminated and highly successful lineage with both CA- and HA-MRSA clones reaching pandemic levels [12]. The success of the ST5 lineage in and out of a hospital setting is attributed to the capacity of these isolates to acquire MGEs containing genes encoding virulence factors and AMR determinants [12].

MGEs are components of the accessory genome and function in adaption of bacteria through genome plasticity [30]. Under selective conditions, these elements confer advantageous phenotypes that improve fitness or enable organism survival. The selective advantage of MGEs facilitates genome evolution through adaption to environmental and host derived selection pressures. This is true for the numerous AMR genes carried on MGE including transposons, insertion sequences, and plasmids.

AMR is a significant public health concern due to the economic and societal cost associated with increased morbidity, mortality, and treatment costs [442]. Both ST398 and ST9 LA-MRSA isolates can harbor diverse resistance elements [249, 443], raising concerns over the potential for LA-MRSA isolates to disseminate AMR beyond the agricultural setting. In this report, we compared the AMR phenotypes and genetic

determinants conferring AMR in MRSA ST5 isolates obtained from swine-associated sources and from humans with no known swine contact.

Materials and methods

Isolate Acquisition

Swine associated LA-MRSA ST5 isolates were obtained from swine (n = 38), environmental samples from swine facilities (n = 26), humans after short-term swine contact (n = 9), and swine veterinarians with long-term contact (n = 9) [335, 444]. Clinical MRSA isolates with no known livestock connection were obtained from hospitals at University of California San Francisco (n = 7) and University of California Irvine (n = 64) [375]. All isolates were determined to be *mecA* positive and were MLST, SCC*mec*, and *spa* typed prior to acquisition (Appendix A).

DNA Sequencing

As previously described, genomic DNA was extracted from overnight cultures using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN) [376-381]. The Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA) was used to generate indexed libraries sequenced on an Illumina MiSeq instrument using the MiSeq v2 500 Cycle reagent kit (Illumina, San Diego, CA). Sequence reads were assembled using MIRA v. 4.0.2 (http://mira-assembler.sourceforge.net/) [435]. Genome sequences are available from GenBank under accession numbers listed in Appendix A.

Genomic AMR Analysis

ResFinder 2.1 from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/) and the Comprehensive Antibiotic Resistance

Database (CARD) (https://card.mcmaster.ca/home) [445] were employed for AMR determinant identification. Draft genomes were submitted to ResFinder 2.1 using a threshold ID of 70% and a minimum length of 60% and CARD using the criteria "default – perfect and strict hits only".

AMR genetic elements were analyzed using Geneious 9.0.5 (Biomatters Ltd., Auckland, New Zealand). Multiple sequence alignments were used to compare sequence identity of genes and plasmids. AMR determinants found on transposons were evaluated for location of integration. Geneious 9.0.5 was used for image generation.

Phenotypic AMR Analysis

Phenotypic antibiotic resistance was determined using the microbroth dilution method by standard operating procedures. Each isolate was tested using the Trek BOPO6F plate (Thermo Fisher Scientific Inc., Oakwood Village, OH) and the Trek GPALL1F plate (Thermo Fisher Scientific Inc., Oakwood Village, OH) and minimum inhibitory concentrations (MICs) were determined. MICs were evaluated in accordance with Clinical Laboratory Standards Institute (CLSI) recommendations to give resistance interpretations for 29 antibiotics in 14 antibiotic (Table 5.1). AMR index, defined as the proportion of antibiotics tested to which an isolate exhibited phenotypic resistance, was determined for each isolate using the results of the microbroth dilution analysis

Statistical Analysis

Statistical analysis was completed using GraphPad Prism 7.01 (GraphPad Software, Inc., La Jolla, CA). Phenotypic antimicrobial resistances were compared using contingency analyses. Comparisons of AMR index and resistance gene numbers were

completed using Mann-Whitney tests. Results were considered significant using a P-value cutoff of $P < 0.05$.

Results

Phenotypic AMR distribution

Phenotypic resistance prevalence was determined and compared between swine-associated and human clinical MRSA ST5 isolates (Table 5.1). Swine associated isolates had AMR indices ranging from 0.14-0.66, while clinical isolates had AMR indices ranging from 0.21-0.59 (Figure 5.1). Clinical MRSA ST5 isolates had significantly higher AMR index (median=0.52) than swine associated LA-MRSA ST5 isolates (median=0.38) ($P < 0.0001$), which equated to isolates exhibiting phenotypic resistance to an average of 14.6 and 11.7 (median 15 and 11) antibiotics respectively (Figure 5.1). These data indicate AMR was generally less extensive among swine associated LA-MRSA ST5 isolates than clinical MRSA ST5 isolates.

Isolates were screened for resistance to vancomycin and linezolid, antibiotics of choice for MRSA treatment in a hospital setting. Neither swine associated nor clinical MRSA ST5 isolates displayed phenotypic resistance to vancomycin or linezolid (Table 5.1). Genetic determinants conferring vancomycin resistance and the multidrug resistance gene *cfr* were absent from all isolates.

Fluoroquinolone Resistance

Fluoroquinolone resistance was significantly more prevalent among clincail MRSA ST5 isolates than swine associated LA-MRSA ST5 isolates ($P < 0.0001$) (Table 5.1). Of the fluoroquinolone resistant LA-MRSA ST5 isolates, 13/25 (52.0%) were obtained from a single farm and 4/25 (16%) were from humans visiting that farm. The

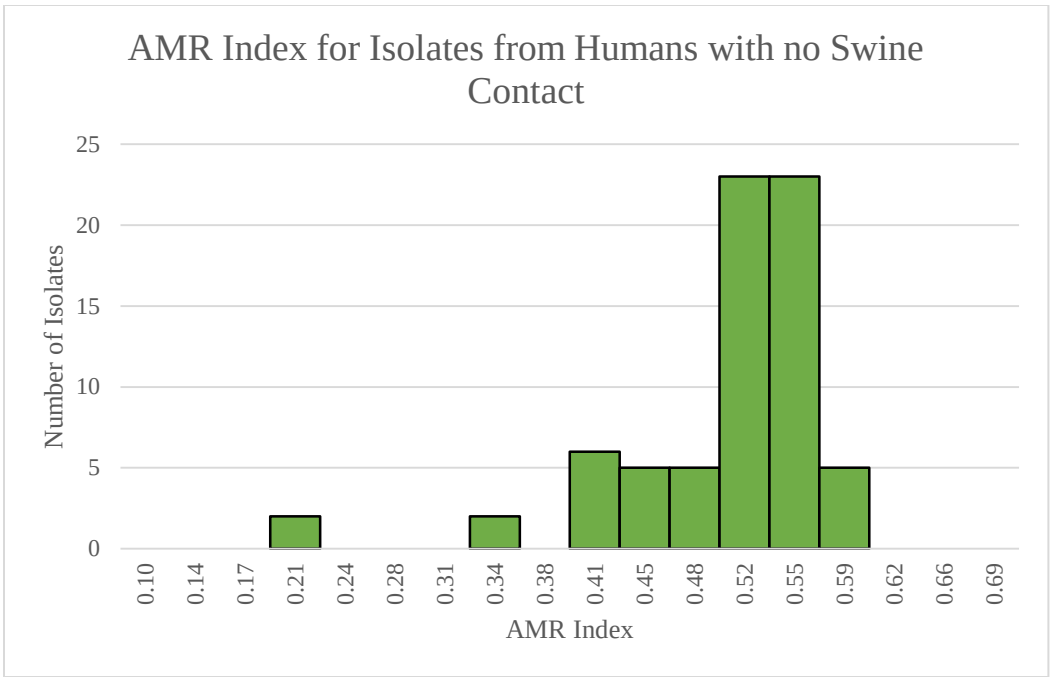
remaining fluoroquinolone resistant isolates (8/25, 32.0%) were distributed on two other farms (n = 4), from humans contacting swine on those farms (n = 2), or from humans with long term swine contact (n = 2).

Table 5.1: Antibiotic resistance prevalence for screened antibiotics in LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact

Antibiotic Class	Antibiotic	Swine Associated LA-MRSA ST5 [a]	MRSA ST5 from Humans with No Swine Contact	Statistics [b]
Penicillin	Penicillin	82/82 (100%)	71/71 (100%)	NS, P = 1.0
	Ampicillin	82/82 (100%)	71/71 (100%)	NS, P = 1.0
	Oxacillin	78/82 (95.1%)	71/71 (100%)	NS, P = 0.1240
Cephalosporin	Cefoxitin	74/82 (90.2%)	71/71 (100%)	P = 0.0075
	Ceftiofur	73/82 (89.0%)	67/71 (94.4%)	NS, P = 0.2628
	Ceftriaxone	49/82 (59.8%)	60/71 (84.5%)	P = 0.0011
Aminoglycoside	Gentamicin	22/82 (26.8%)	12/71 (16.9%)	NS, P = 0.1735
	Neomycin	69/82 (84.1%)	67/71 (94.4%)	NS, P = 0.0689
	Streptomycin	1/82 (1.2%)	1/71 (1.4%)	NS, P = 1.0
Tetracycline	Chlortetracycline	65/82 (79.3%)	0/71 (0%)	P < 0.0001
	Oxytetracycline	65/82 (79.3%)	0/71 (0%)	P < 0.0001
	Tetracycline	65/82 (79.3%)	0/71 (0%)	P < 0.0001
Phenicol	Chloramphenicol	9/82 (11.0%)	1/71 (1.4%)	P = 0.0206
	Florfenicol	30/82 (36.6%)	54/71 (76.1%)	P < 0.0001
Macrolide	Erythromycin	36/82 (43.9%)	69/71 (97.2%)	P < 0.0001
	Tilmicosin	36/82 (43.9%)	55/71 (75.3%)	P < 0.0001
Sulfonamides	Sulfadimethoxine	0/82 (0%)	35/71 (49.3%)	P < 0.0001
	Trimethoprim + Sulfamethoxazole	0/82 (0%)	0/71 (0%)	NS, P = 1.0
	Trimethoprim	0/82 (0%)	0/71 (0%)	NS, P = 1.0
Fluoroquinolone	Ciprofloxacin	19/82 (23.2%)	69/71 (97.2%)	P < 0.0001
	Enrofloxacin	25/82 (30.5%)	69/71 (97.2%)	P < 0.0001
	Levofloxacin	15/82 (18.3%)	69/71 (97.2%)	P < 0.0001
	Moxifloxacin	18/82 (22.0%)	69/71 (97.2%)	P < 0.0001
Nitrofuran	Nitrofurantoin	0/82 (0%)	0/71 (0%)	NS, P = 1.0
Lincosamide	Clindamycin	39/82 (47.6%)	55/71 (77.5%)	P = 0.0002
Lipopeptide	Daptomycin	0/82 (0%)	0/71 (0%)	NS, P = 1.0
Pleuromutilin	Tiamulin	7/82 (8.5%)	1/71 (1.4%)	NS, P = 0.0654
Glycopeptide	Vancomycin	0/82 (0%)	0/71 (0%)	NS, P = 1.0
Oxazolidinone	Linezolid	0/82 (0%)	0/71 (0%)	NS, P = 1.0

[a] – Number resistant out of total isolates tested (percent resistant)
[b] – Statistical significance designated at P < 0.05; NS = not significant

A)



B)

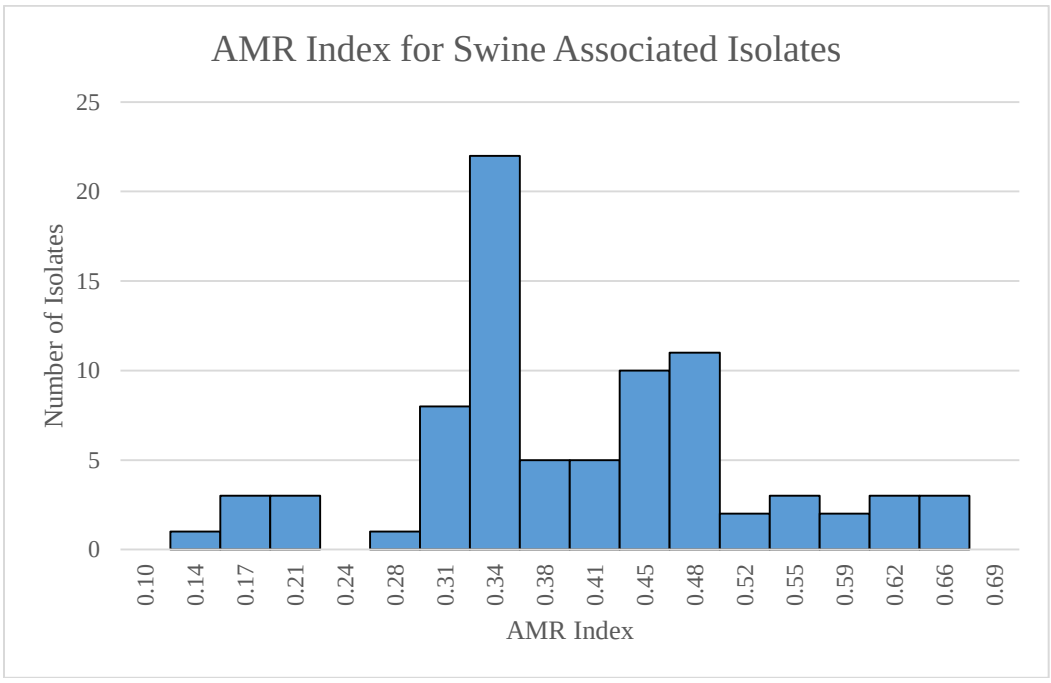


Figure 5.1: AMR index of isolates from MRSA ST5 from humans with no swine contact and swine associated LA-MRSA ST5. AMR index is defined as the proportion of the tested antibiotics to which an isolates is phenotypically resistant. (A) The AMR indexes determined for isolates from humans with no swine contact show a range of 0.21-0.59 with a median AMR index of 0.52. (B) The AMR indexes determined for swine associated LA-MRSA ST5 isolates. Swine associated isolates had AMR indexes with a wider range (0.14-0.66) and higher maximum AMR index; however, the median AMR index was 0.38, which was significantly less than that of humans with no swine contact ($P < 0.0001$).

In clinical MRSA ST5 isolates, fluoroquinolone resistance was associated with mutations in *gyrA*, *parC*, and/or *parE*, while resistance in swine associated isolates was primarily associated with mutations in both *gyrA* and *parC* (Table 5.2). Eight clinical isolates did not harbor any fluoroquinolone resistance determinants. This may be a result of gaps in the draft genome, novel mutations, or novel genes conferring fluoroquinolone resistance. Eight swine associated isolates also lacked a mutation or AMR gene conferring fluoroquinolone resistance. One of these isolates was from the farm harboring isolates with *parC* and *gyrA* mutations, indicating the mutations may be absent due to gaps in the draft genome sequence. Of the remaining seven isolates, six exhibited limited phenotypic resistance, being resistant only to enrofloxacin but susceptible to ciprofloxacin, levofloxacin, and moxifloxacin. These isolates also exhibited a lower enrofloxacin MIC (2.0 μg/mL) when compared to isolates with *gyrA*, *parC*, or *parE* mutations (MIC of >2.0 μg/mL). Other isolates from the same farms exhibited a range of MICs from <0.12-1.0 μg/mL and 21/33 (63.6%) of the non-resistant isolates from these farms were deemed of "intermediate" susceptibility.

Table 5.2: Quinolone resistance mechanisms

Mutant Genes	Swine Associated LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
gyrA	0/82 (0%)	10/71 (14.1%)
parC	1/82 (1.2%)	11/71 (15.5%)
parE	0/82 (0%)	3/71 (4.2%)
gyrA + parC	16/82 (19.5%)	33/71 (46.5%)
gyrA + parC + parE	0/82 (0%)	4/71 (5.6%)
Unknown	8/82 (9.8%)	8/71 (11.3%)

Tetracycline Resistance

Phenotypic tetracycline resistance was seen exclusively in swine associated LA-MRSA ST5 isolates (Table 5.1). Tetracycline resistance was observed on all but one farm

(7/8, 87.5%). Genetic analysis indicated swine associated LA-MRSA ST5 isolates harbored zero to two tetracycline resistance genes. The tetracycline resistance genes identified in LA-MRSA ST5 isolates were *tetT* (61/82, 74.4%) and *tetL* (62/82, 75.6%) (Table 5.3), and the majority (61/65, 93.8%) of tetracycline resistant isolates harbored both *tetT*, a ribosomal modification gene and *tetL*, a gene encoding antibiotic efflux. Further examination of the location of tetracycline resistance genes indicated they were encoded on a plasmid also harboring the aminoglycoside resistance gene *aadD* (Figure 5.2). Consistent with the lack of phenotypic resistance, no tetracycline resistance genes were identified in the genomes of clinical MRSA ST5 isolates (0/71, 0%) (Table 5.3).

Table 5.3: Tetracycline resistance genes

Tetracycline Resistance Genes	Swine Associated LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
tetL	62/82 (75.6%)	0/71 (0%)
tetT	61/82 (74.4%)	0/71 (0%)
Unknown	3/82 (3.7%)	0/71 (0%)

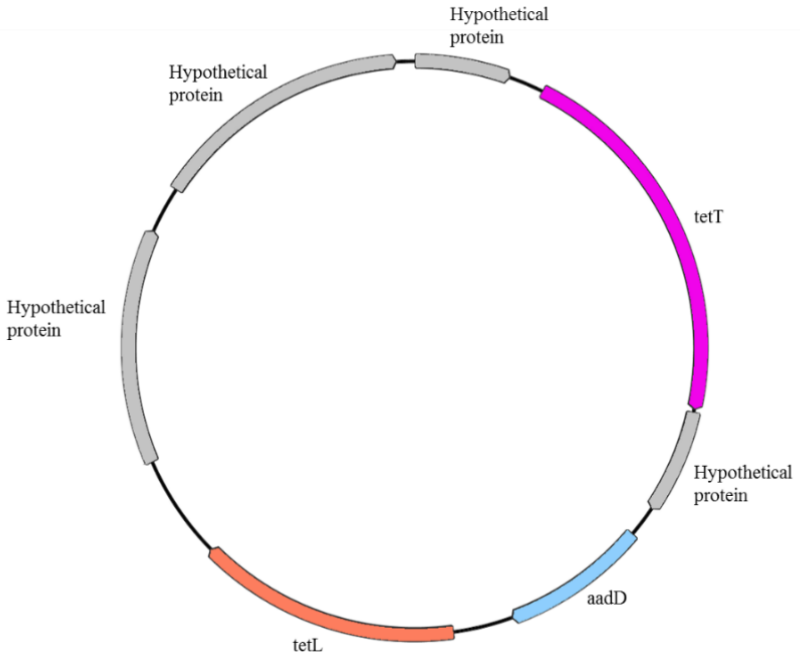


Figure 5.2: LA-MRSA ST5 tetracycline resistance plasmid. The tetracycline resistance genes *tetT* (magenta) and *tetL* (coral) were harbored on a 9,269bp multidrug resistance plasmid that also harbored the aminoglycoside resistance gene *aadD* (light blue). This plasmid was found in 62/82 (75.6%) of all swine associated LA-MRSA ST5 isolates and had a 95.4% (62/65) correlation with tetracycline resistance.

Macrolide, Lincosamide, and Streptogramin (MLS) Resistance

Phenotypic resistance to macrolide ($P < 0.0001$) and lincosamide ($P = 0.0002$) antibiotics was significantly higher in clinical MRSA ST5 isolates than swine associated LA-MRSA ST5 isolates (Table 5.1). Genomic screening for determinants conferring resistance to MLS antibiotics revealed differences between isolate subsets. The majority of clinical MRSA ST5 isolates harbored the *ermA* gene (65/71, 91.5%) (Table 5.4). These isolates exhibited two distinct phenotypes: 14/65 (21.5%) were resistant to erythromycin and susceptible to tilmicosin and clindamycin and 51/65 (78.5%) were resistant to erythromycin, tilmicosin, and clindamycin. The *ermA* gene was identified within the type II SCC*mec* element in 65/69 (94.2%) of the erythromycin resistant isolates. The remaining four isolates also harbored a type II SCC*mec* element, indicating the *ermA* gene may be present but missing from the draft sequence. The remaining isolates (2/71, 2.8%) were susceptible to tested MLS antibiotics and harbored a type IV SCC*mec* element, which did not harbor *ermA*.

Clinical isolates also harbored several MLS resistance genes in addition to *ermA* (Table 5.4). Two isolates harbored *lnuA*, a gene that functions to inactivate lincosamide antibiotics. There were also 20 isolates (28.2%) that harbored *mphC*, a gene involved in macrolide antibiotic inactivation, and *msrA*, a streptogramin and macrolide efflux pump (Table 5.4). These genes (*lnuA*, *mphC*, and *msrA*) were found in isolates harboring *ermA* or isolates suspected to have *ermA* based on their SCC*mec* type. Due to the resistance profile of *ermA* harboring isolates, the contribution of *lnuA*, *mphC*, and *msrA* to macrolide resistance could not be determined.

Table 5.4: Genes conferring resistance to macrolide, lincosamide, or streptogramin (MLS) antibiotics

Resistance Genes	Swine Associated LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
ermA	0/82 (0%)	65/71 (91.5%)
ermC	36/82 (43.9%)	0/71 (0%)
vgaA	78/82 (95.1 %)	0/71 (0%)
vgaE	2/82 (2.4%)	0/71 (0%)
mphC	0/82 (0%)	20/71 (28.2%)
msrC	0/82 (0%)	20/71 (28.2%)
lnuB	1/82 (1.2%)	0/71 (0%)
lnuA	0/82 (0%)	2/71 (2.8%)
Unknown	2/82 (2.4%)	4/71 (5.6%)

Swine associated isolates with phenotypic resistance to MLS antibiotics harbored primarily *ermC* (36/82, 43.9%) (Table 5.4). These isolates displayed only one phenotype: resistance to erythromycin, tilmicosin, and clindamycin. Sequence analysis determined *ermC* was plasmid mediated. Two different plasmids were identified with the majority of *ermC* positive isolates (35/39, 89.7%) containing a 2.4 kbp plasmid encoding only *ermC* and a maintenance and replication protein (Figure 5.3). One swine associated isolate harbored *lnuB*, which functions to inactivate lincosamide antibiotics, and the isolate exhibited phenotypic lincosamide resistance.

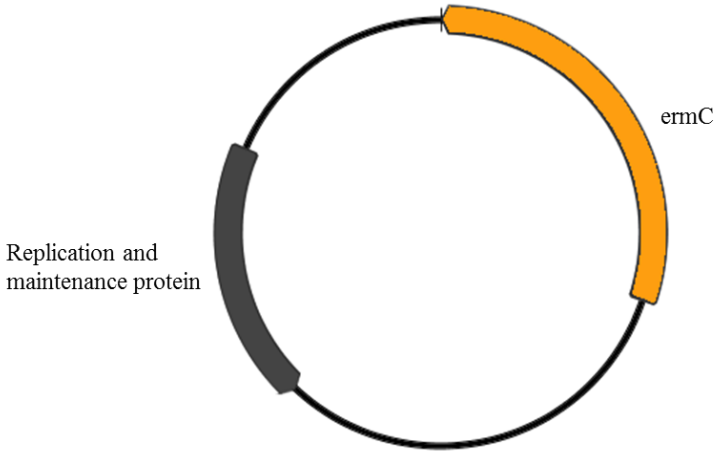


Figure 5.3: LA-MRSA ST5 plasmid containing ermC. MLS resistance in LA-MRSA ST5 was predominantly mediated by *ermC* (orange) in 92.3% (36/39) of isolates. This gene was found most commonly on a 2,432bp plasmid (35/36, 97.2%). Isolates carrying this plasmid were resistant to erythromycin, tilmicosin, and clindamycin.

The majority of swine associated LA-MRSA strains harbored AMR genes conferring resistance to streptogramin A. The most common streptogramin A resistance gene was *vgaA* (78/82, 95.1%), which functions as an efflux pump with activity toward streptogramin A and lincosamide antibiotics. Two isolates harbored *vgaE* along with *vgaA*. No correlation between phenotypic lincosamide resistance and the presence of *vgaA* or *vgaE* was detected. Streptogramin resistance genes were not found in any clinical MRSA ST5 isolates.

Aminoglycoside Resistance

Phenotypic aminoglycoside resistance (gentamicin, neomycin, or streptomycin) was not significantly different between swine associated and clinical MRSA ST5 isolates (Table 5.1). Neomycin resistance was widely distributed in LA-MRSA ST5 isolates and was present in isolates from all farms sampled (8/8, 100%). Phenotypic gentamicin and streptomycin resistance were limited in LA-MRSA ST5 isolates, with all gentamicin resistant isolates obtained from a single farm. Only one swine associated isolate exhibited streptomycin resistance.

Genetic determinants conferring aminoglycoside resistance were more prevalent in clinical MRSA ST5 isolates (range 0-5, average 2.3, median 2) than isolates from swine associated sources (range 0-3, average 1.1, median 1) ($p < 0.0001$) (Table 5.5). The aminoglycoside resistance genes unique to clinical MRSA isolates were *spc* (ant(9)-Ia), aph(3')-III, and ant(6)-Ia. Isolates from swine associated sources uniquely harbored *aadE* and *str* (aph(6)-Ia) (Table 5.5). Three resistance genes were shared between the two groups of isolates: *aadD*, aph(2")-Ih, and aac(6')-aph(2") (Table 5.5).

Table 5.5: Aminoglycoside resistance genes

Resistance Genes	Swine Associated LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
aadD (aka ant(4')-Ia)	71/82 (86.6%)	62/71 (87.3%)
aadE	1/82 (1.2%)	0/71 (0%)
aph(2")-Ih	2/82 (2.4%)	3/71 (4.2%)
aac(6')-aph(2")	14/82 (17.1%)	9/71 (12.7%)
spc (aka ant(9)-Ia)	0/82 (0%)	61/71 (85.9%)
str (aka aph(6)-Ia)	1/82	0/71 (0%)
aph(3')-III	0/82 (0%)	15/71 (21.1%)
ant(6)-Ia	0/82 (0%)	15/71 (21.1%)
Unknown	3/82 (3.7%)	0/71 (0%)

Neomycin resistance was distributed in both swine associated LA-MRSA ST5 isolates (69/82, 84.1%) and clinical MRSA ST5 isolates (67/71, 94.4%) and correlated with the presence of *aadD* in both subsets of isolates. A strong association (100% in clinical isolates and 72.7% in swine associated isolates) between phenotypic resistance to gentamicin and the aminoglycoside resistance gene aac(6')-aph(2") or aph(2")-Ih was also observed. Further genetic investigation indicated the gene identified as aph(2")-Ih is likely a truncated version of aac(6')-aph(2") found at the end of the contig. A single swine associated isolate harbored the resistance gene *str* and was the only swine associated isolate to display phenotypic streptomycin resistance. The single clinical ST5 isolate exhibiting phenotypic resistance to streptomycin did not harbor an identified streptomycin resistance determinant.

The precise location of the shared aminoglycoside resistance genes was determined to detect potential transfer of AMR between the isolate subsets. The location of *aadD* varied among LA-MRSA ST5 isolates. The majority of isolates (62/71, 87.3%) harbored *aadD* on a multidrug resistance plasmid that also harbored the tetracycline resistance genes *tetL* and *tetT* (Figure 5.2). In the remaining nine LA-MRSA isolates, *aadD* was harbored on a different plasmid. Some of these plasmids contained other

antimicrobial resistance genes, such as the beta-lactamase, *blaZ*. In the majority (59/62, 95.2%) of clinical MRSA ST5 isolates, *aadD* was identified within plasmid sequence that also contained a bleomycin resistance gene (Figure 5.4). Of the remaining isolates, two harbored *aadD* on a contig encoding a bacitracin ABC transporter permease; however, whether this contig was plasmid or chromosomal sequence could not be determined. Evaluation of the location of aac(6')-aph(2") indicated all isolates harbored a similar insertion sequence containing the gene (Figure 5.5). BLAST results indicated the insertion sequence was present in several plasmids, none of which were common between the subsets of isolates.

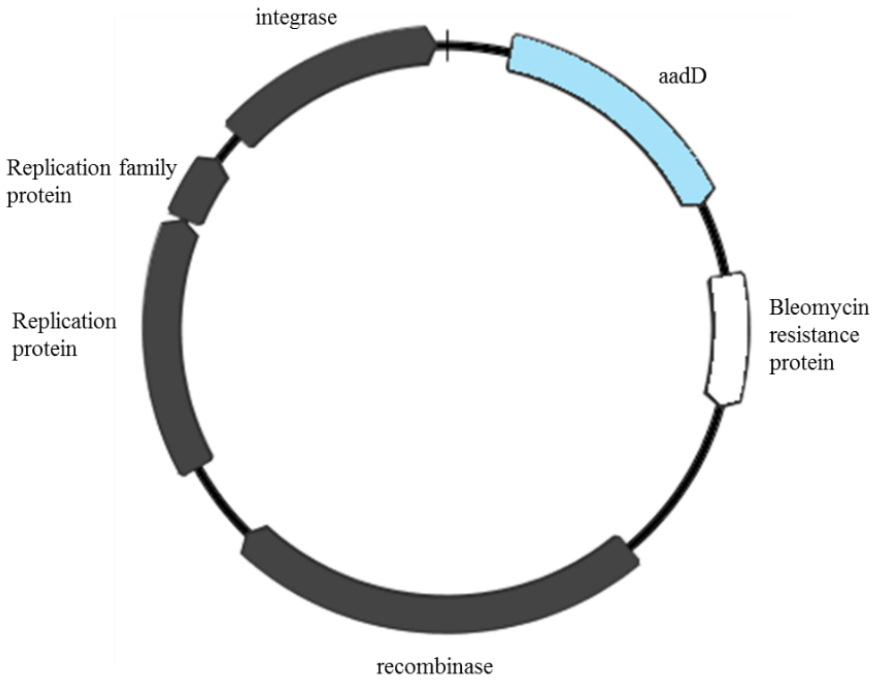


Figure 5.4: Small plasmid carrying aadD found in MRSA ST5 isolates from humans with no swine contact. The *aadD* gene (light blue) was harbored on a 5,370bp plasmid in the majority of MRSA ST5 isolates from humans with no swine contact (60/62, 96.8%). The plasmid also harbored a bleomycin resistance protein (white), which confers resistance to belomycin a glycopeptide antibiotic used for chemotherapeutic treatment of cancer.

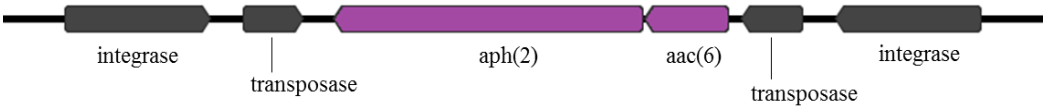


Figure 5.5: Transposon harboring aac(6')-aph(2"). The gentamicin resistance gene aac(6')-aph(2") was harbored on the same transposon in both LA-MRSA ST5 isolates (14/82, 17.1%) and MRSA ST5 isolates from humans with no swine contact (9/71, 12.7%). The aminoglycoside resistance gene aph(2")-Ih was found in LA-MRSA ST5 (2/82, 2.4%) and MRSA ST5 from humans with no swine contact (3/71, 4.2%). This gene was determined to be a truncated aac(6')-aph(2") due to its location at the end of a contig. This transposon was highly correlated with the presence of gentamicin resistance and was found in 72.7% (16/22) of gentamicin resistant LA-MRSA ST5 isolates and 100% (12/12) of isolates from humans with no swine contact that were phenotypically resistant to gentamicin.

Phenicol Resistance

Phenotypic resistance to phenicol class antibiotics differed between swine associated and clinical MRSA ST5 isolates (Table 5.1). Significantly more swine associated isolates exhibited phenotypic resistance to chloramphenicol ($P = 0.02$); while resistance to florfenicol was more prevalent in clinical ST5 isolates ($P < 0.0001$).

All swine associated isolates exhibiting phenotypic chloramphenicol resistance (9/9, 100%) harbored the phenicol resistance gene *fexA* (Table 5.6). These isolates were also phenotypically resistant to florfenicol. The presence of *fexA* and chloramphenicol resistance was clustered in swine associated isolates and all *fexA* containing isolates were obtained from a single farm. No genetic determinants conferring chloramphenicol resistance were identified in the clinical isolate that exhibited phenotypic chloramphenicol resistance. All florfenicol resistant clinical ST5 isolates and the majority of florfenicol resistant swine associated isolates (21/30, 70%) did not harbor any recognized florfenicol resistance determinants.

Table 5.6: Phenicol resistance genes

Resistance Genes	Swine Associated LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
fexA	9/82 (11.0%)	0/71 (0%)
Unknown	21/82 (25.6%)	54/71 (76.1%)

Discussion

While previous studies have reported AMR prevalence among LA-MRSA ST398 and ST9 isolates [330, 345, 446-449], little to no information exists regarding AMR prevalence among LA-MRSA ST5 isolates. Here, we found clinical ST5 isolates exhibited a higher AMR index than ST5 isolates obtained from swine associated sources. In contrast to this general trend, 13 (15.9%) LA-MRSA ST5 isolates displayed resistance to 15-19 of the antibiotics tested (AMR index of 0.52-0.66) (Figure 5.1). Nine of these isolates were from a single farm and one was obtained from a human with short-term swine contact on that farm. Multi-drug resistance patterns similar to that observed in this farm have also been reported in swine associated ST398 LA-MRSA isolates [330, 332, 446, 450].

AMR distribution among swine associated MRSA ST5 isolates predominantly reflected patterns consistent with antimicrobial use in the swine industry. Tetracycline resistance was found in 79.3% of the LA-MRSA ST5 isolates and on 7 of the 8 farms sampled. Tetracycline resistance has been previously reported in LA-MRSA ST398 isolates, where phenotypic resistance has approached 100% of isolates evaluated [330, 331, 345, 450, 451]. This has been attributed to the long term use of chlortetracycline and oxytetracycline antibiotics in the swine industry. Similarly, the much lower prevalence of fluoroquinolone resistance in LA-MRSA ST5 is unsurprising given the relatively recent approval of fluoroquinolones for swine in the United States and the ban on extralabel fluoroquinolone use food animals under the Animal Medicinal Drug Use Clarification Act (AMDUCA) [452]. While the prevalence of chloramphenicol resistance in LA-MRSA ST5 isolates was similar to that reported for LA-MRSA ST398 [331, 345], we

noted higher chloramphenicol resistance in swine associated LA-MRSA ST5 isolates compared to clinical ST5 isolates, despite the ban on chloramphenicol use in food animals in the USA under AMDUCA. The presence of chloramphenicol resistance among both LA-MRSA ST398 and ST5 may reflect coselection related to the use of florfenicol in the swine industry.

Evaluation of the draft genome sequences indicated the AMR genes identified in the MRSA ST5 isolates were harbored on MGEs in all cases except for fluoroquinolone resistance. The AMR genes were found on plasmids (such as *tetL, tetT, aadD, ermC, msrA,* and *mphC*), transposable elements (such as aac(6')-aph(2"), *lnuB, aadE,* and *fexA*), and within the SCC*mec* element (*ermA* and ant(9)-Ia). The presence of AMR genes detected in this study on MGEs underlies the potential for transfer of AMR genes among bacteria. *S. aureus* ST5 isolates are highly susceptible to transfer of MGEs [12], which may facilitate transmission of uncommon AMR genes to and from LA-MRSA ST5 isolates.

Notable differences in genetic determinants underlying AMR were identified in LA-MRSA ST5 isolates compared to clinical MRSA ST5 isolates. MLS resistance was mediated by the *ermA* gene in clinical isolates and the *ermC* gene in swine associated isolates. While a genetic determinant conferring resistance to phenicol class antibiotics was unidentified in the majority of florfenicol resistant isolates, *fexA* was harbored by a portion of LA-MRSA ST5 isolates expressing phenicol resistance (9/30, 30%) yet absent from all phenicol resistant clinical MRSA ST5 isolates. There were two shared AMR genes between swine associated and clinical MRSA ST5 isolates: *aadD* and aac(6')-aph(2"). For these genes, although sequence analysis showed high sequence identity for

both genes (>90% across all isolates), the genes were harbored on different plasmids in the two groups of isolates, indicating it is unlikely the presence of these genes in clinical MRSA ST5 isolates was associated with transfer from LA-MRSA ST5 isolates. Similarly, separate and distinct sets of AMR genes harbored by human isolates compared to livestock associated isolates were previously reported for *Salmonella* Typhmurium DT104 and in comparisons between LA-MRSA and HA-MRSA isolates in the same location [330, 453].

LA-MRSA ST5 isolates harbored a different complement of AMR genes than previously identified in LA-MRSA ST398 isolates. For example, tetracycline modification genes and tetracycline efflux genes were widespread in the LA-MRSA ST5 isolates tested here and in LA-MRSA ST398 [323, 330, 331, 345]. While both sequence types can harbor *tetL*, tetracycline resistance genes (*tetM* and *tetK*) previously identified in LA-MRSA ST398 were not found in our LA-MRSA ST5 isolates. Similarly, MLS resistance genes in LA-MRSA ST398 have been more diverse than those in LA-MRSA ST5 [330, 331, 345]. The prevalence of *ermC* ranged from 16-40% in previous reports of LA-MRSA ST398 isolates [330, 331, 345], in which *ermA* (0-34% of isolates) and *ermB* (10-38% of isolates) also occurred. However, only *ermC* was detected in the LA-MRSA ST5 isolates tested here. The prevalence of *fexA* in LA-MRSA ST5 was similar to that reported among LA-MRSA ST398 isolates, where 2-13% of isolates harbored *fexA* [331, 345]; however, these studies also identified the multidrug resistance gene *cfr* in 1-3% of ST398 isolates screened [330, 331, 345], but *cfr* was not found in the LA-MRSA ST5 isolates tested here.

The identified differences in the repertoire of AMR genes harbored by ST5 isolates compared to LA-MRSA ST398 isolates could reflect differences in the specific study populations or may represent lineage specific adaptions. Lineage specific adaptions have been previously identified for disinfectants in HA-MRSA isolates and zinc resistance in LA-MRSA isolates [397]. In the present study, LA-MRSA ST5 isolates harbored *tetL* and *tetT* on an extrachromosomal plasmid. In contrast, the *tetM* gene is integrated into the chromosome of LA-MRSA ST398 isolates and reports indicate it is widespread in isolates from livestock [323, 330, 331, 345, 454]. This may be an adaption specific to the ST398 lineage, while ST5 isolates harbor plasmid encoded tetracycline resistance. Differences in study populations may also play a role, as most studies investigating LA-MRSA ST398 have evaluated European isolates, which are geographically distinct and may be under different selection pressures than isolates in the U.S. We observed substantial between-farm and within-farm variation in both phenotypic resistance and antimicrobial resistance genes. Variation between farms may be associated with differences in on-farm selection pressures, in the case of mobile genetic elements encoding resistance, or the genetic background of isolates prior to introduction, such as isolates with *parC* and *gyrA* mutations. Within farm variation of resistance phenotypes and resistance elements was also seen in LA-MRSA ST5 isolates. For example, ISU839 expressed phenotypic resistance to tetracycline antibiotics, clindamycin, and streptomycin; while ISU837 and ISU842 were isolated from the same farm and susceptible to these antibiotics. ISU839 harbored the tetracycline resistance plasmid (Figure 5.2) and the insertion sequence ISSsu5, discovered in *Streptococcus suis*, containing the lincosamide resistance gene *lnuB* and the aminoglycoside resistance gene

aadE,[455] which was absent in other isolates from the farm. Overall, this indicates isolates gain and/or lose MGEs encoding AMR genes and supports concerns regarding the capacity of these isolates to potentially disseminate AMR beyond the agricultural setting.

To begin addressing the public health concerns over the potential for LA-MRSA isolates to disseminate AMR beyond the agricultural setting, we specifically selected clinical isolates from a geographically distinct population in an urban environment to ensure the clinical isolates were from humans with no swine contact. We found swine associated LA-MRSA ST5 isolates exhibited resistance to fewer antibiotics than clinical MRSA ST5 isolates. More importantly, we identified separate and distinct genetic determinants of AMR harbored by clinical ST5 isolates compared to swine associated LA-MRSA ST5 isolates. Collectively, our data suggest that the swine reservoir of ST5 MRSA in the USA is unlikely to serve as a source for AMR determinants in human clinical MRSA ST5 isolates. To fully evaluate the contribution of LA-MRSA ST5 isolates to the risk of human MRSA infections and antimicrobial resistance in human MRSA isolates, follow up studies including human clinical isolates obtained from regions of swine production are warranted.

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CHAPTER 6. ADHERENCE CAPACITY OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* SEQUENCE TYPE 5 ISOLATES FROM HEALTH CARE AND AGRICULTURAL SOURCES

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Abstract

Staphylococcus aureus is part of the nasal microbiome of many humans and has become a significant public health burden due to infections with antibiotic resistant strains, including methicillin resistant *S. aureus* (MRSA). Several lineages of *S. aureus* including MRSA are found in livestock species and can be acquired by humans through

contact with animals. These livestock associated MRSA (LA-MRSA) isolates raise public health concerns because of the potential for livestock to act as reservoirs for MRSA outside of the hospital setting. In the United States, swine harbor a mixed population of LA-MRSA isolates with the sequence type (ST) 398, ST9, and ST5 lineages being detected. LA-MRSA ST5 isolates are particularly concerning to the public health community because, unlike ST398 and ST9 lineages, the ST5 lineage is a significant cause of human disease in both the hospital and community setting globally. The ability of swine associated LA-MRSA ST5 isolates to adhere to human keratinocytes *in vitro* was investigated and adherence genes harbored by these isolates were evaluated and compared to clinical MRSA ST5 isolates from humans with no swine contact. The two subsets of isolates adhered equivalently to human keratinocytes *in vitro* and contained an indistinguishable complement of adherence genes that possessed a high degree of sequence identity. Collectively our data indicate that, unlike LA-MRSA ST398 isolates, LA-MRSA ST5 isolates did not exhibit a reduced genotypic or phenotypic capacity to adhere to human keratinocytes.

Importance

Our data indicate swine associated livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) ST5 isolates are equally capable of adhering to human skin and have the same genetic potential to adhere as clinical MRSA ST5 isolates from humans. This suggests humans in contact with livestock have the potential to become colonized with LA-MRSA ST5 isolates; however, genes that contribute to persistence of *S. aureus* on human skin were absent in LA-MRSA ST5 isolates. The data

presented here is important evidence in evaluating potential risks LA-MRSA ST5 isolates pose to humans with livestock contact.

Introduction

Humans and several other mammals harbor *Staphylococcus aureus* as a component of their nasal and skin microbiome. Although this organism is a commensal in 25-33% of humans in developed countries [403], it can cause opportunistic infections that range in severity from mild skin infections to severe systemic infections [8, 58]. Treatment of these infections is challenging due to the rapid acquisition of antimicrobial resistance genes, including the SCC*mec* element that encodes the *mecA* gene conferring methicillin resistance [247, 254]. These isolates, deemed methicillin resistant *S. aureus* (MRSA), have become a significant public health burden in the United States causing thousands of infections annually, which result in significant healthcare costs and losses in productivity [404, 456].

MRSA isolates are classified by the source from which they are acquired to form the following categories: hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock associated (LA-MRSA) [22]. These subsets of isolates possess unique characteristics that allow them to thrive in each environment. For example, HA-MRSA isolates tend to possess a large number of antimicrobial resistance genes that enable them to survive in a hospital setting where the use of antimicrobial agents is more common [182]. Alternatively, CA-MRSA isolates more commonly possess the arginine catabolic mobile element (ACME), which improves their survival on the skin of healthy humans through the degradation of polyamines and pH modulation at the skin surface [457]. Finally, LA-MRSA isolates are thought to have adapted to colonizing livestock

species through the loss of human specific virulence factors and, in some cases, gain of virulence factors specific to their livestock host species [251, 323, 325].

With the discovery of LA-MRSA sequence type (ST) 398 in swine [248], significant concerns arose due to the potential that livestock species can be reservoirs for MRSA, and LA-MRSA may contribute to the risk for human infections in the community. This precipitated research investigating the prevalence of LA-MRSA and the associated infection risk. Studies found, while ST398 was the predominant lineage in Europe [312], outside of Europe other lineages were more prevalent. In Asian swine, LA-MRSA ST9 was the most common lineage. North America harbored a more diverse population of LA-MRSA, with isolates of the ST398, ST9, and ST5 lineages being found [335, 357]. Evidence indicates the ST398 and ST9 lineages are animal adapted and less able to colonize and cause disease in humans [251, 323, 344]. This has not been shown for the ST5 lineage, which is a globally disseminated and highly successful *S. aureus* clone in humans [12].

Nasal colonization with *S. aureus* contributes to *S. aureus* infections in the host, especially within the hospital setting [54, 458]. This becomes important for humans with livestock contact, as these individuals are significantly more likely to carry MRSA than their counterparts with no livestock contact [248, 353]. Persistent colonization is a complex interaction between host tissues and the microbiota of the nasal cavity or skin. While the impact of few genes has been experimentally verified [76-78], many genes are thought to contribute to adherence and colonization of *S. aureus* through their interaction with host proteins, such as fibrinogen and fibronectin [97, 101]. Furthermore, there are many genes suspected to function in adherence based on identified motifs consistent with

other adherence genes [91], although their specific ligand has yet to be identified. Genetic investigation examining known and suspected adherence genes indicates variability in the presence or absence of these genes in different lineages, such as the *sdr* genes that are not uniformly present in all lineages [69]. Additional genes, such as the arginine catabolic mobile element (ACME) and *speG*, are found on mobile genetic elements (MGEs) and are thought to promote bacterial survival on the skin and contribute to long term colonization with *S. aureus* [176, 182, 457].

Reports indicate LA-MRSA ST398 isolates have a reduced ability to adhere to human keratinocytes, seem to colonize humans more transiently than other lineages, and seem to be less transmissible between humans than their HA-MRSA counterparts [334, 338, 339, 344]. These LA-MRSA ST398 isolates possess genetic differences, such as truncation of the adherence genes *clfA*, *clfB*, and *fnbB* and the absence of *sdrE* [344], which are thought to contribute to the reduced capacity of LA-MRSA ST398 to colonize and cause disease in humans. The adherence properties and genetic factors contributing to adherence have not been investigated for LA-MRSA ST5 isolates. In this study, we present a comparison of the *in vitro* adherence capability of LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact and compare identified and suspected adherence genes in these populations of *S. aureus*.

Materials and methods

Isolate acquisition and selection

S. aureus isolates were acquired from Iowa State University (n = 73), University of Minnesota (n = 9), University of California Irvine (n = 64) and University of California San Francisco (n = 8) [335, 375]. These isolates included 82 LA-MRSA ST5 isolates from swine associated sources including pigs (n = 38), the environment within swine buildings (n = 26), veterinary students with short term swine contact were sampled after a one time visit to a swine farm (n = 9) and swine veterinarians with long term, occupational swine exposure (n = 9). Seventy-one clinical MRSA ST5 isolates and one clinical methicillin susceptible *S. aureus* ST5 isolate were included from humans residing in urban areas with no known swine contact (n = 72). All isolates were confirmed to be ST5 by MLST and *spa* typed prior to acquisition (Appendix A). *Staphylococcus aureus* Mu3 (ATCC #700698, ATCC, Manassas, VA) was used for comparison during analysis of the adherence assays and as a reference genome for adherence factor gene analysis.

Isolates were purposively selected from swine associated sources to represent the potential diversity among the isolates during adherence assays. Ten swine associated LA-MRSA isolates were selected from the 82 total isolates. They were selected such that each farm was represented in the adherence assay by an environmental (n = 3) or swine isolate (n = 4) or represented by an isolate from a human after visiting the farm (n = 1). Two isolates were from humans with long term swine contact were also selected for phenotypic screening. Nine isolates from humans with no known swine contact were randomly selected for inclusion in the adherence assay. Name and source information for isolates included in adherence assays can be found in Table 6.1.

Table 6.1: Isolates selected for adherence assays

Isolate Name	Isolate Source
ISU837	Environment (Farm 10)
ISU876	Pig (Farm 24)
ISU936	Pig (Farm 39)
ISU949	Pig (Farm 42)
ISU960	Environment (Farm 38)
ISU978	Pig (Farm 39)
ISU980	Environment (Farm 41)
ISU1007	Human – short term contact
UMN4	Human – long term contact
UMN38	Human – long term contact
UCI08	Human clinical isolate
UCI15	Human clinical isolate
UCI22	Human clinical isolate
UCI27	Human clinical isolate
UCI30	Human clinical isolate
UCI45	Human clinical isolate
UCI52	Human clinical isolate
UCI64	Human clinical isolate
UCSF13938	Human clinical isolate

Genome sequencing

Draft genome sequences were generated using the protocol previously described [376-381]. In short, isolates were grown in Trypticase Soy Broth (TSB) (BD Biosciences, Sparks, MD) and genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN). The Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA) was used to generate DNA libraries that were sequenced using the MiSeq v2 500 Cycle reagent kit (Illumina, San Diego, CA) on an Illumina MiSeq instrument. Sequence reads were assembled using MIRA v. 4.0.2 (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html) and resulting sequences can be found in GenBank with the accession numbers listed in Supplemental Table 1. The whole genome sequence for Mu3 was obtained from NCBI (GenBank Sequence #AP009324.1) to use for adherence gene comparisons.

Gene comparisons

The adherence genes analyzed can be found in Table 6.2 and Table 6.3. For each gene, the percent nucleotide sequence identity as compared to that gene in the reference genome Mu3 was determined using multiple sequence alignments in Geneious 9.0.5 (Biomatters Ltd., Auckland, New Zealand). Percent identities relative to Mu3 were then used to generate heatmaps using R [459]. For *sasG*, the gene was designated as present or absent due to the gene structure including "B repeats", 384 nucleotide repeats, which prevented adequate alignment of the entire gene. The genes composing ACME, *cna*, and *sasX* were also a present or absent designation as these genes are not found in Mu3.

Accessory gene regulator (*agr*) typing

The *agr* type of the isolates was determined by screening the draft genome sequences *in silico* with Geneious 9.0.5 for type specific regions using the following primer sets: *agrI* (5′-GTC ACA AGT ACT ATA AGC TGC GAT-3′), *agrII* (5′-TAT TAC TAA TTG AAA AGT GGC CAT AGC-3′), *agrIII* (5′-GTA ATG TAA TAG CTT GTA TAA TAA TAC CCA G-3′), and *agrIV* (5′-CGA TAA TGC CGT AAT ACC CG-3′) (as described previously [460]).

Human epidermal keratinocyte adherence assay

Human epidermal keratinocytes (HEKs) (ATCC #PCS-200-010, ATCC, Manassas, VA) were obtained to screen isolates for *in vitro* adherence to human keratinocytes. These cells were grown in Dermal Cell Basal Medium (DCBM) (ATCC, Manassas, VA) supplemented with the Keratinocyte Growth Kit (ATCC, Manassas, VA). For the assay, HEKs of passage 5 or lower were plated into 24-well plates (Becton,

Dickinson and Company, Franklin Lakes, NJ) at 5000 cells/cm^2 and allowed to grow to confluence.

An overnight MRSA culture grown in TSB was used to inoculate 5mL of TSB and was incubated at 37ºC until logarithmic growth. The bacterial culture was diluted to an optical density at 600nm (OD_{600}) ranging from 0.50-0.59 and 60µL was inoculated into 10mL of supplemented DCBM to reach an average inoculum concentration $3.96x10^6$ colony forming units (CFU)/mL. Wells of HEKs were inoculated with 750 µl of diluted culture from each isolate (n = 3) resulting in an average multiplicity of infection (MOI) of 45, with an uninoculated DCBM control on each plate (n = 3). The plate was centrifuged at 400 *x g* for five minutes followed by a one hour incubation at 37ºC with 5% CO_2. After incubating, each well was washed gently eight times with phosphate buffered saline (PBS). Each well was then treated with 200µL of 0.1% trypsin to dislodge cells and adherent bacteria. The trypsin was collected and combined with 800µL of PBS.

Serial dilutions of the inoculum and the recovered contents from each well were generated and plated onto Trypticase Soy Agar (BD Biosciences, Sparks, MD) plates and incubated for 24 hours at 37ºC. Three non-treated control wells from each plate were collected in the same manner as the test wells and a Scepter 2.0 cell counter (Millipore, Billerica, MA) was used to calculate the average keratinocytes/well in the plate. Each isolate was screened with three biological replicates generated from the average of three technical replicates. For each biological replicate, the CFU per HEK was calculated for comparison.

Swine skin biopsy adherence assay

The external epidermis of the pinna and the base of the ear was cleaned of visible surface debris, scrubbed with gauze soaked in 7.5% povidone-iodine surgical scrub (Purdue Pharma L.P., Stamford, CT), and rinsed with gauze soaked in 95% ethanol three times. A section of scrubbed skin was excised and washed in Gibco EpiLife cell culture media (Thermo Fisher Scientific Inc., Oakwood Village, OH) supplemented with Gibco Human Keratinocyte Growth Supplement (Thermo Fisher Scientific Inc., Oakwood Village, OH) containing 100 IU/mL Penicillin G, 100µg/mL Streptomycin, 50µg/mL Gentamycin sulfate and 1.0µg/mL Amphotericin B. The skin segment was moved to supplemented EpiLife media containing 100 IU/mL Penicillin G and incubated for 2 hours at 37ºC. Skin was then transferred to supplemented EpiLife media without antibiotic and incubated for 30 minutes at 37ºC. The hypodermis was removed and punch biopsies were generated using Militex sterile disposable 8mm punch biopsy tool (Thermo Fisher Scientific Inc., Oakwood Village, OH). Biopsies were placed into 48-well plates (Corning Inc., Corning, NY) with the epidermis exposed. From each skin section, one biopsy was used for each isolate screened and one biopsy treated with uninoculated EpiLife medium and used as a control to detect contamination.

For each isolate, an overnight culture in TSB was used to inoculate 5mL of TSB and grown to logarithmic growth phase. The cultures were diluted to an OD_{600} ranging from 0.50-0.59 and 500µL was inoculated into 4.5mL of supplemented EpiLife media generating an average MRSA inoculum concentrations of $4.18x10^6$ CFU/mL. The cultures were mixed well and 25µL was inoculated onto each skin biopsy resulting in an average inoculum distribution of $8.32x10^4$ MRSA/mm^2. After each skin biopsy in the

plate was inoculated, the plate was incubated for 1 hour at 37ºC with 5% CO_2. The skin biopsies were then washed four times with PBS to remove non-adherent bacteria and 50µL of trypsin 0.1% was added to each skin biopsy and they were incubated for 15 minutes at 37ºC to dislodge adhered bacteria. After trypsinization, the skin biopsies were moved to centrifuge tubes with 950µL of supplemented EpiLife media and vortexed to suspend the bacteria.

Serial dilutions were made of the inoculum and the bacteria retrieved from the skin biopsies after trypsinization. Dilutions were plated onto Trypticase Soy Agar plates containing 1µg/mL oxacillin. Plates were incubated for 48 hours at 37ºC, colonies were counted and used to determine CFU/mL for the inoculum and skin biopsies. Each isolate was tested with at least three biological replicates consisting of the average of three technical replicates. For each biological replicate, the CFU/mm^2 surface area of the skin biopsy was calculated.

Statistical analysis

All statistics were completed using GraphPad Prism 7.01 (GraphPad Software, Inc., La Jolla, CA). Mann Whitney tests were used to compare the CFU per HEK and the CFU per mm^2 between LA-MRSA ST5 isolates and clinical MRSA ST5 isolates from humans with no swine contact. Fisher's exact tests were used to compare the prevalence of individual genes between the subsets of isolates.

Accession Numbers

The draft genome sequences for all isolates were deposited in DDBJ/ENA/GenBank with the following accession numbers: LKVI00000000-LKWJ00000000, LKWY00000000-LKYQ00000000, LKYS00000000-LLBD00000000,

LLBG00000000-LLBI00000000, and LLBK00000000-LLBW00000000. Individual isolate accession numbers are listed in Appendix A [376-381].

Results

Adherence to human epidermal keratinocytes

Adherence assays were used to determine the capacity of swine associated LA-MRSA ST5 and MRSA ST5 from humans with no swine contact to colonize human skin. LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact adhered to the HEK cell line equivalently (Figure 6.1). There was no significant difference in the CFU adhered per HEK ($P = 0.74$) when isolates were compared as subsets (Figure 6.1-A). There was considerable varaibilityin the ability of individual isolates to adhere to HEK cells (Figure 6.2-A). For example, the average CFU per HEK for UCI27 was higher than the average CFU per HEK adhered for ISU980 (Figure 6.2-A). In some cases, there were also wide ranges in the CFU per HEK adhered between biological replicates of an individual isolate, such as that seen for ISU978 (Figure 6.2-A).

Adherence to swine skin biopsy

To assess the capacity of the selected isolates to colonize swine epithelial tissue, adherence assays were completed using swine skin biopsies. Swine associated LA-MRSA ST5 isolates adhered in greater CFU per mm^2 than MRSA ST5 isolates from humans with no swine contact ($P < 0.0001$) (Figure 6.1-B). Similar to adherence patterns for the HEK cells, differences were noted in the adherence capacity for individual isolates as well as variation between biological replicate (Figure 6.2-B).

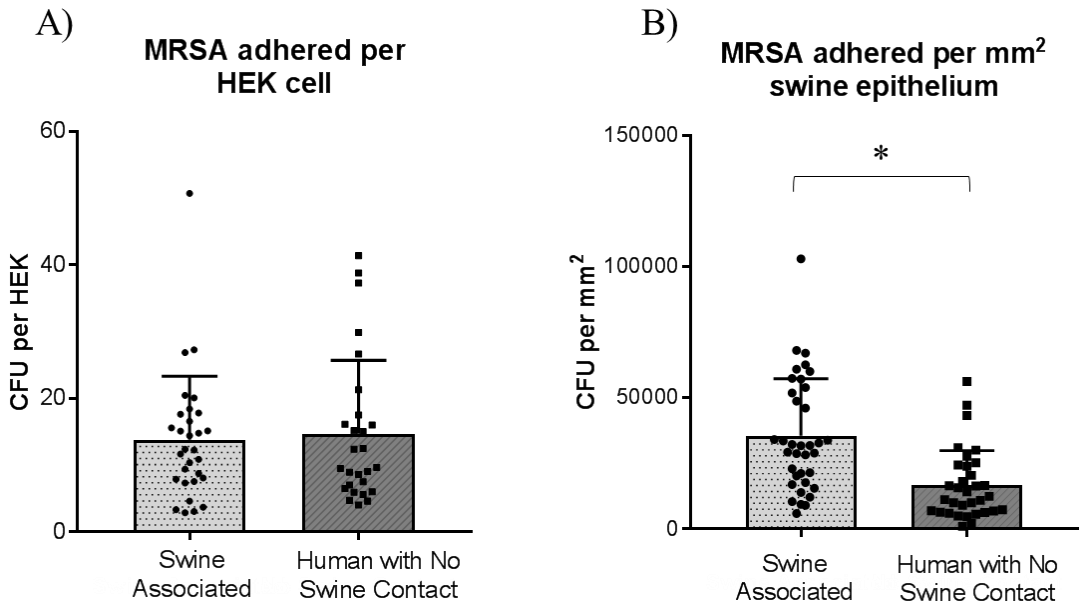

Figure 6.1: Adherence capacity of swine associated LA-MRSA ST5 isolates and clinical MRSA ST5 isolate from humans with no swine contact to human keratinocytes and swine skin biopsies. Figure 1A represents the CFU adhered per HEK for LA-MRSA ST5 isolates and clinical MRSA ST5 isolates with each point representing the average of three technical replicates. Figure 1B represents the MRSA CFU adhered per mm^2 of the swine skin biopsies for LA-MRSA ST5 and clinical MRSA ST5 isolates. (*) denotes statistical significance of $P < 0.05$.

Comparison of adherence genes

To examine genomic differences that may influence how MRSA ST5 isolates interact with their host and environment, we compared the nucleotide sequences of 22 genes encoding factors associated with adherence and skin colonization [69]. The percent identity for each of the adherence-associated genes in LA-MRSA ST5 and MRSA ST5 isolates from humans with no swine contact was determined for each isolate relative to Mu3 (Figure 6.3 and Figure 6.4). This analysis indicated the adherence genes harbored by both subsets of isolates showed a high degree of nucleotide identity. Specifically, a nucleotide percent identity greater than 90 relative to the reference isolate Mu3 was observed for all evaluated genes (Table 6.2). The gene displaying the greatest sequence divergence was

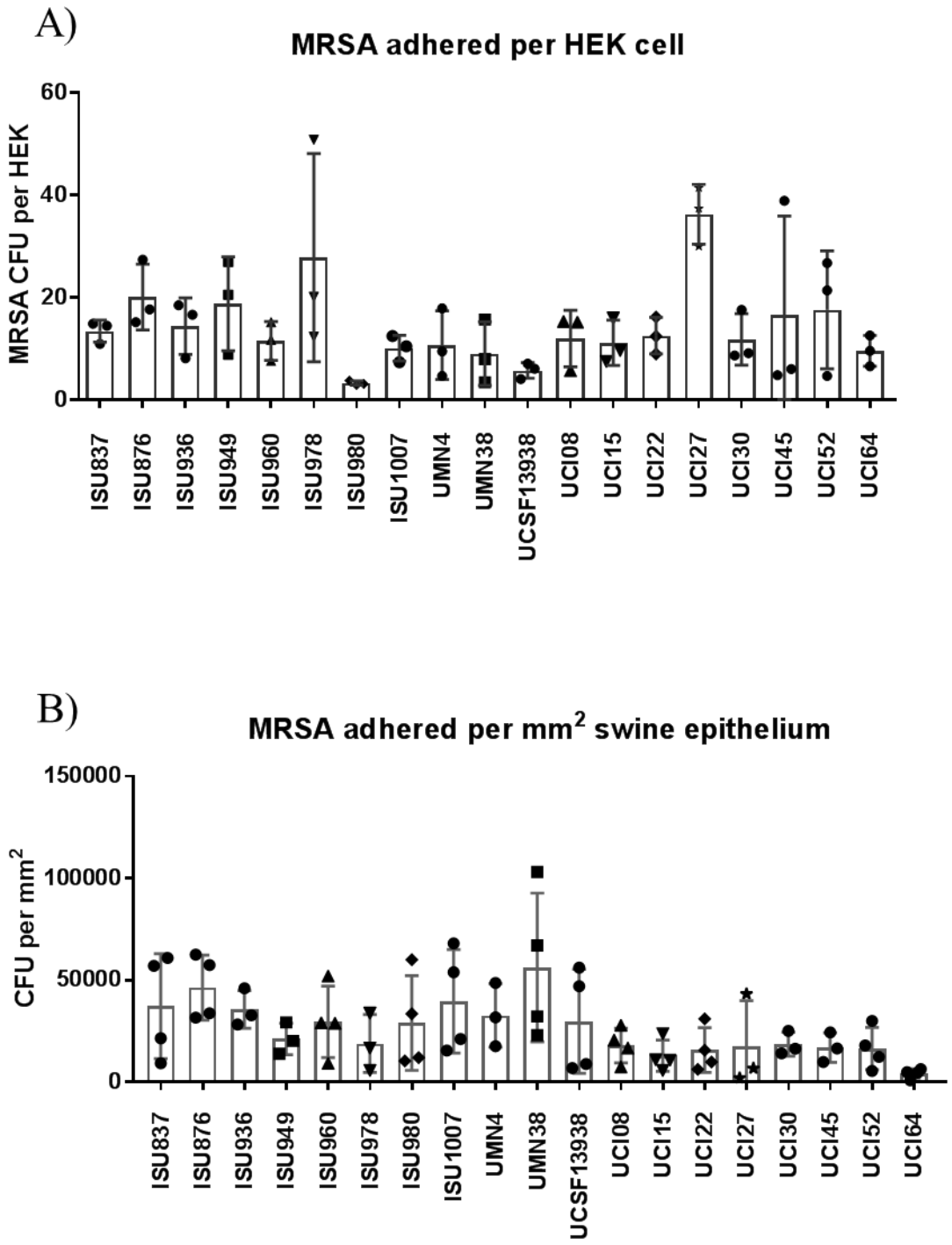

Figure 6.2: Adherence patterns of individual isolates to HEKs and swine skin biopsies. Differential adherence patterns were noted between individual isolates in adherence to HEKs (A) and swine skin biopsies (B). There were also differences noted in the adherence pattern between biological replicates of an individual isolate, such as CFU per HEK for ISU978 (A) or CFU per mm^2 for UMN38 (B).

clfA (Figure 6.3 and Figure 6.4); however, it was intact in all the isolates and variation was associated with single nucleotide polymorphisms, insertions, and deletions that did not result in a premature stop codon or removal of large segments of the *clfA* gene. The greatest variation in *clfA* was present in LA-MRSA ST5 isolates and was due to an insertion of 162 bp at position 2,125 and a deletion of 24 bp at position 2,799 detected in the *clfA* gene of 19 of isolates (Figure 6.5). There were isolates that lacked sequence associated with specific adherence genes (Figure 6.3 and Figure 6.4 indicated in black). This may be associated with gaps in the draft genome sequence rather than a true absence of the gene of interest.

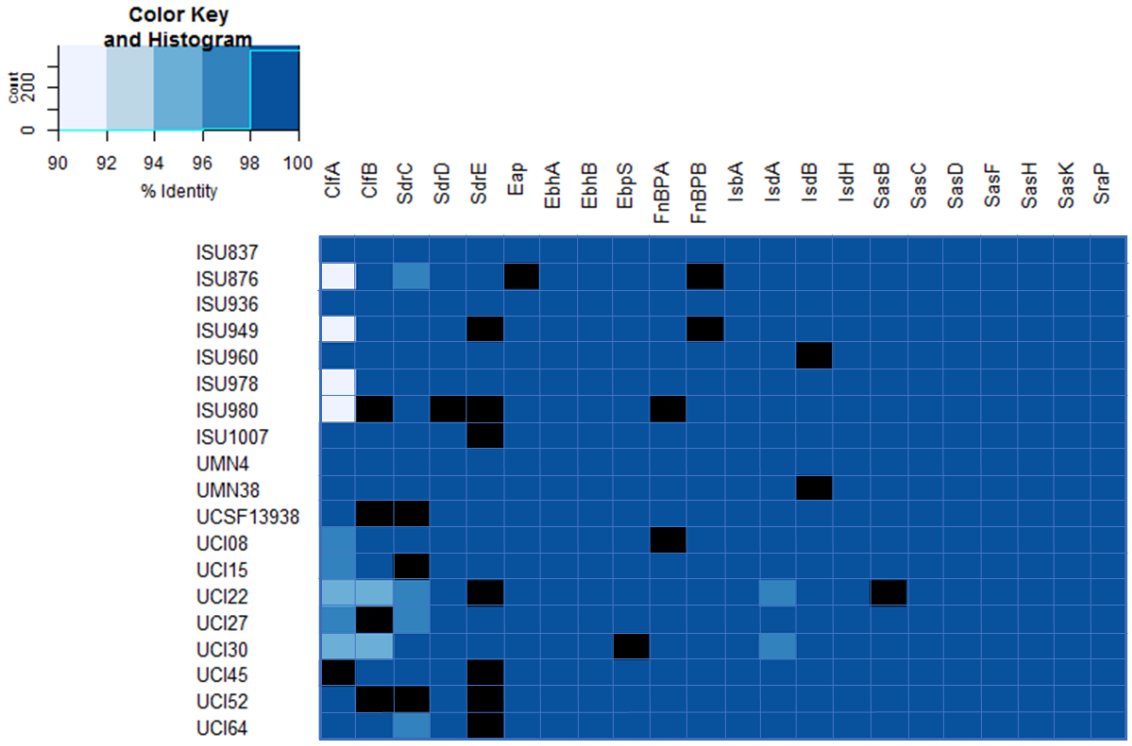

Figure 6.3: Percent identity of adherence associated genes for isolates evaluated with phenotypic assay. Genes were compared to the gene in the reference isolate, Mu3. The percent identity of all screened genes was greater than 90%. The greatest variation was seen in the *clfA* gene. This was true for both LA-MRSA ST5 isolates and clinical MRSA ST5 isolates; however, a subset of LA-MRSA ST5 isolates (ISU876, ISU949, ISU978, and ISU980) showed added variation due to nucleotide insertions and deletions. Genes not found in the draft genomes are depicted in black.

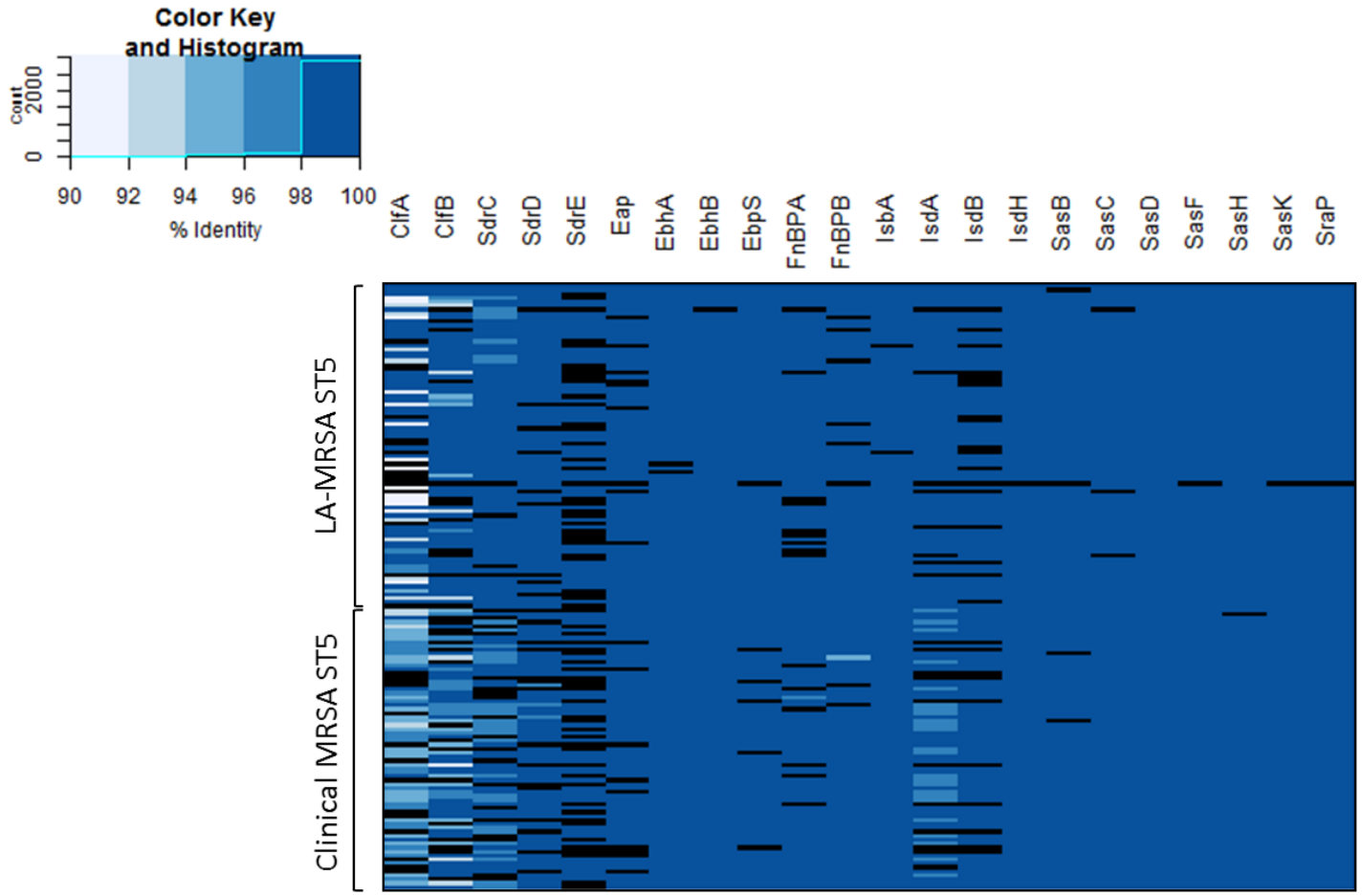

Figure 6.4: Percent identity of adherence associated genes in 82 LA-MRSA ST5 isolates and 71 clinical MRSA ST5 isolates. Percent identity of adherence genes as compared to the reference isolate Mu3 is depicted. All screened genes showed a high percent identity (greater than 90%) to the reference gene. Similar to that noted in the phenotypically screened isolates, the *clfA* gene had the greatest variation. Genes depicted in black were absent from the draft genome sequence.

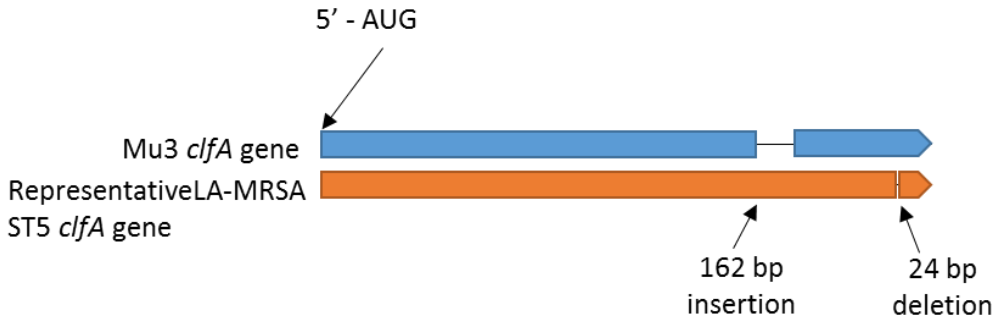

Figure 6.5: Representative image of *clfA* alignment from 19 LA-MRSA ST5 and Mu3. There were 19 LA-MRSA ST5 isolates in which the *clfA* gene showed a reduced percent nucleotide identity to Mu3. The *clfA* genes in LA-MRSA ST5 isolates with a reduced identity possessed an insertion of 162 bp at position 2,125 and a deletion of 24 bp at 2,799. The insertion and deletion did not result in a frameshift mutation and the *clfA* gene in these isolates remained intact.

Table 6.2: Percent nucleotide identity for adherence factors in LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact as compared to MRSA ST5 Mu3

Adherence Factor	LA-MRSA ST5 [a,b]	MRSA ST5 from Humans with No Swine Contact [a,c]
clfA	90-100 (97)	93-100 (96)
clfB	93-100 (99)	91-100 (97)
sdrC	97-100 (99)	96-100 (98)
sdrD	99-100 (100)	97-100 (99)
sdrE	98-100 (100)	99-100 (100)
eap	100-100 (100)	100-100 (100)
ebhA	100-100 (100)	99-100 (100)
ebhB	100-100 (100)	100-100 (100)
ebpS	100-100 (100)	100-100 (100)
fnbpA	98-100 (100)	97-100 (100)
fnbpB	100-100 (100)	95-100 (100)
isbA	100-100 (100)	100-100 (100)
isdA	99-100 (100)	97-100 (99)
isdB	100-100 (100)	100-100 (100)
isdH	100-100 (100)	100-100 (100)
sasB	98-100 (100)	100-100 (100)
sasC	100-100 (100)	100-100 (100)
sasD	100-100 (100)	100-100 (100)
sasF	99-100 (100)	100-100 (100)
sasH	100-100 (100)	100-100 (100)
sasK	100-100 (100)	100-100 (100)
sraP	99-100 (100)	100-100 (100)

[a] minimum identity-maximum identity (average identity)
[b] includes 82 swine associated LA-MRSA ST5 isolates
[c] includes 72 human clinical *S. aureus* ST5 isolates from humans with no swine contact

Several adherence genes were not conducive to sequence identity analysis due to variable repeat regions (*sasG*) or the absence of the gene in the reference genome (ACME and *speG*). Therefore isolate subsets were compared based on the presence or absence of these adherence genes (Table 6.3). The adherence gene *sasG* was found in equal prevalence in both subsets of isolates (P = 0.26). There were significantly more MRSA ST5 isolates from humans with no swine contact harboring the ACME *arc* gene cluster as well as *speG* (P = 0.02 and P < 0.0001). The adherence-associated genes *cna* and *sasX* were not found in ST5 isolates obtained from swine-associated sources or from humans with no known swine contact.

Table 6.3: Prevalence of adherence genes in LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact

Adherence Factor	LA-MRSA ST5	MRSA ST5 from Humans with No Swine Contact
sasG	67/82 (81.7%)	64/72 (88.9%)
ACME – arc gene cluster	0/82 (0%)	5/72 (6.9%)*
ACME – speG	0/82 (0%)	14/72 (19.4%)*
cna	0/82 (0%)	0/72 (0%)
sasX	0/82 (0%)	0/72 (0%)

* denotes significance of P < 0.05 as compared to LA-MRSA ST5

The *agr* genes encode a two-component sensor system that functions as a global regulator and a pivotal regulator of virulence factors and adherence genes [461, 462]. Swine associated LA-MRSA ST5 and MRSA ST5 from humans with no swine contact were evaluated for *agr* type. All isolates with detectable *agr* genes harbored a type II *agr*, including 78/82 of LA-MRSA ST5 isolates (95.1%) and 70/72 of clinical MRSA ST5 isolates (97.2%). No difference in in *agr* type was observed between isolate subsets. The isolates in which an *agr* system was not identified are likely missing the *agr* type due to gaps in the draft genome sequences.

Discussion

Reports of the colonization capacity of LA-MRSA ST398 indicate these isolates possess a reduced capacity for adherence to human epithelium [344], which results in transient colonization with ST398 isolates and reduced transmissibility of these isolates between humans in both the hospital and community settings [336, 338-342]. The reduction in adherence to human keratinocytes is suspected to be due to the absence of genes known to be involved in adherence, such as *sdrE*, and mutations and truncations in adherence genes, such as those noted in *clfA* and *clfB* [344]. These changes are hypothesized to contribute to the adaption of LA-MRSA ST398 isolates to livestock species [344]. Although adherence capacity and transmissibility has been reported for LA-MRSA ST398 isolates, there are no reports to date addressing these concerns in LA-MRSA ST5 isolates.

In this study, we investigated the ability of LA-MRSA ST5 isolates and human clinical MRSA ST5 to adhere to human keratinocytes and swine skin biopsies to better understand the potential for these isolates to colonize humans and pigs. We found no difference between the human keratinocyte adherence capacity of LA-MRSA ST5 isolates and MRSA ST5 isolates from humans with no swine contact. We further determined the keratinocytes adherence patterns exhibited by both subsets of isolates were consistent with the adherence-related genes the isolates harbored. Notably, adherence genes reported to be absent or truncated in LA-MRSA ST398 were present and intact in LA-MRSA ST5 isolates, including *clfA, clfB, sdrC, sdrE,* and *fnbB* [344]. In the case of LA-MRSA ST5 isolates, there was a high proportion of nucleotide identity with the specific gene in reference genome (Mu3), which was also seen for the human clinical

MRSA ST5 isolates (Table 6.2, Figure 6.3, and Figure 6.4). This indicates an equivalent genetic capacity for adherence that was confirmed with *in vitro* testing. Although adherence to human keratinocytes was equivalent, the LA-MRSA ST5 isolates did show significantly greater adherence to swine epithelium *in vitro* (Figure 6.1-B). This difference may reflect the adaptation of swine associated LA-MRSA ST5 isolates to colonizing swine epithelium through unidentified mechanisms.

Evaluation of multiple isolates of LA-MRSA ST5 and MRSA ST5 from humans with no swine contact indicated that there is a large amount of individual isolate variation as well as variation between test replicates for an individual isolate (Figure 6.2). Variability was anticipated between different isolates; however, the large variability between test replicates for the same isolate was not expected. The variation in replicates may be associated with differences in adherence gene expression during an individual test and is controlled for by screening *in vivo* adherence for each isolate multiple times.

Overall, we conclude that, unlike LA-MRSA ST398 isolates, LA-MRSA ST5 isolates do not have a reduced capacity to adhere to human keratinocytes. This was seen both *in vitro* and through *in silico* analysis of adherence genes, which showed greater than 90% identity in all isolates. While the adherence capacity of LA-MRSA ST5 isolates was not different from MRSA ST5 isolates from humans with no swine contact, this does not directly indicate their ability to colonize and cause disease. Colonization is a complex interaction between the host, the bacterium, and the microbiota, which is difficult to replicate in an *in vitro* setting. It is also important to note that adhesion alone is not sufficient to cause disease and many virulence factors that contribute to colonization and mediate disease in *S. aureus* were not found in LA-MRSA ST5 isolates [398]. This

includes ACME, which was found only in clinical MRSA ST5 isolates in this study and contribute to the persistence of *S. aureus* on the skin of hosts. We have also previously shown immune evasion genes, such as those found in the β-hemolysin converting bacteriophage, are absent in the LA-MRSA ST5 isolates evaluated in this study [398]. Collectively, this study indicates that LA-MRSA ST5 isolates are able to adhere to human keratinocytes equivalently to clinical MRSA ST5 isolates. This may result in humans that contact livestock harboring LA-MRSA ST5 isolates becoming colonized with those isolates; however, LA-MRSA ST5 isolates are suspected to be less virulent than clinical MRSA ST5 isolates and less capable of causing disease in humans.

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CHAPTER 7. SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

Summary and Conclusions

The association of LA-MRSA and swine has drawn concerns within the public health community surrounding the risk of LA-MRSA dissemination outside of the agricultural setting and the potential for livestock species to serve as the largest source of MRSA outside of the hospital setting [333]. The prototypical and most prevalent European lineage of LA-MRSA is ST398 [312], which has been shown to lack human specific virulence factors and a reduced capacity to colonize humans [12, 323, 330, 340-342, 344]. Outside of Europe, LA-MRSA shows regional diversity with Asian swine being colonized with predominantly ST9 isolates and swine in the United States harboring ST398, ST9, and ST5 isolates [335, 354, 357, 360-362]. Public health concerns elevated with the discovery of LA-MRSA ST5, because unlike MRSA ST398 and MRSA ST9, which are considered animal adapted [12, 251, 323, 370, 417], the MRSA ST5 lineage is a widespread and successful MRSA lineage [12]. While LA-MRSA ST398 isolates have been well studied, there have been no investigations addressing the concerns surrounding LA-MRSA ST5 isolates. The body of work presented in this dissertation addresses the concerns associated with LA-MRSA ST5 isolates obtained from swine associated sources.

The primary objective of this dissertation was to evaluate the genetic relatedness and pathogenic potential of LA-MRSA ST5 isolates as compared to human clinical MRSA ST5 isolates from humans with no swine contact. This was accomplished with the generation of draft genomes using the Illumina MiSeq platform and sequence analysis comparing chromosomal genes and mobile genetic elements. This began with SNP

detection and phylogenetic analysis of the core genomes, which were presented in Chapter 2. This study revealed LA-MRSA ST5 isolates are distinct from human clinical MRSA ST5 isolates. The degree of genetic relatedness among LA-MRSA ST5 isolates and the clonality of isolates from individual farms or production systems indicates there have been limited introductions of MRSA ST5 into the U.S. swine population. The clonality within farms was also sufficient to allow the traceback of isolates in humans with recent swine contact to the farm of origin.

Phylogenetic analysis was then paired with screening of mobile genetic elements (MGEs) to detect potential transfer of virulence factors or antimicrobial resistance genes between LA-MRSA ST5 isolates and human clinical MRSA ST5 isolates from humans with no swine contact. The prevalence of the immune evasion genes harbored by the β-hemolysin converting bacteriophage were evaluated in Chapter 3. The *sak*, *chp*, *scn*, and *sep* genes were found only in human clinical MRSA ST5 isolates and function to thwart the host immune response. The loss of immune evasion genes harbored by the β-hemolysin converting bacteriophage may represent an adaption of LA-MRSA ST5 isolates to colonizing swine, because *sak*, *chp*, and *scn* have been shown to have a high degree of specificity for human proteins and minimal reactivity in other mammalian species [165, 168, 408].

Additionally, the analyses found in Chapter 4 and 5 evaluate the distribution of antimicrobial resistance genes and the contribution of selective pressures to the development and acquisition of antimicrobial resistance in LA-MRSA ST5 and humanclinical MRSA ST5 isolates. In Chapter 4, the prevalence of *czrC* and zinc-chloride resistance was presented. While *czrC* and zinc resistance is widespread in LA-

MRSA ST398 and is thought to have had a significant contribution to the dissemination of LA-MRSA ST398 [348, 349, 426, 463], it was absent from LA-MRSA ST5 isolates. The absence of *czrC* in LA-MRSA ST5 isolates indicates that there may be a lineage association that enabled LA-MRSA ST398 isolates to acquire *czrC* and benefit from this acquisition, while *czrC* was not an essential gene for LA-MRSA ST5 isolates to become prevalent in United States swine herds. The report in Chapter 4 also evaluated the SCC*mec* type of LA-MRSA ST5 and human clinical MRSA ST5 isolates, which revealed LA-MRSA ST5 isolates harbored types III, IV, and untypable SCC*mec* elements and human clinical MRSA ST5 isolates harbored predominantly type II SCC*mec* elements. The difference in SCC*mec* elements may represent selection pressures in swine associated isolates against the larger SCC*mec* elements (type I-III) [183, 432]. It also identified that the SCC*mec* elements are different between the two subsets, which argues against the transfer of this resistance cassette between the subsets of isolates evaluated here.

In Chapter 5, LA-MRSA ST5 and human clinical MRSA ST5 isolates were evaluated for genes conferring antibiotic resistance and phenotypic resistance patterns. Antibiotic resistance profiles and genes conferring antibiotic resistance were found to differ between LA-MRSA ST5 and human clinical MRSA ST5 isolates. The antibiotic resistance harbored by each subset of isolates tended to represent selective pressures found in the environment from which the isolate was obtained, such that LA-MRSA ST5 isolates possessed resistance to antibiotics heavily used in animal agriculture (tetracycline antibiotics) and human clinical MRSA ST5 isolates possessed resistance to antibiotics widely used in a hospital setting (fluoroquinolone antibiotics). The differences noted in

antibiotic resistance elements, similar to the difference in SCC*mec* elements, provides further evidence against the transfer of mobile genetic elements harboring antibiotic resistance genes between LA-MRSA ST5 isolates and human clinical MRSA ST5 isolates.

Finally, to address the potential of LA-MRSA ST5 isolates to colonize human skin, Chapter 6 provides a genetic analysis of adherence factors in LA-MRSA ST5 isolates and human clinical MRSA ST5 isolates and phenotypic screening of a subset of LA-MRSA ST5 isolates and human clinical MRSA ST5 isolates for the capacity to adhere to human keratinocytes. The adherence factors harbored by LA-MRSA ST5 and human clinical MRSA ST5 isolates had a high percent identity to the reference gene in Mu3 and adhered equivalently to human keratinocytes *in vitro*. Human clinical MRSA ST5 were noted to harbor the arginine catabolic mobile element and *speG*, genes harbored on MGEs that are involved in survival and persistence on human skin, which were absent in LA-MRSA ST5 isolates. The absence of the arginine catabolic mobile element and *speG* is further support indicating a reduced virulence of LA-MRSA ST5 isolates as compared to their clinical counterparts and contributes additional evidence that transfer of MGEs between LA-MRSA ST5 and human clinical MRSA ST5 isolates is not occurring.

Overall, the work contained herein indicates that the LA-MRSA ST5 isolates screened in this study are genetically distinct from the human clinical MRSA ST5 isolates screened. This distinction was evident from the phylogenetic analysis conducted using identified SNPs and confirmed through the screening of MGEs. MGE analysis revealed few shared genes harbored by both subsets of isolates, which indicates MRSA

ST5 isolates may be adapting to their environment and their host species through the acquisition or loss of host specific virulence factors and acquiring antibiotic resistance genes that contribute to their fitness in the agricultural or clinical setting. The major pitfall to this investigation is that the human clinical MRSA ST5 isolates were obtained from university hospitals in urban areas of California. The hospitals selected for this analysis were chosen due to the availability of isolates and the ability to ensure the selected isolates were obtained from humans with no swine contact. The concern with the clinical isolates used in this study is due to the geographic isolation of the populations harboring the LA-MRSA ST5 and the human clinical MRSA ST5 isolates, which makes contact between the subsets of isolates unlikely and limits the physical interaction required for transfer of MGEs.

Future directions

As discussed, the experimental design of this study ensured that no clinical isolates were obtained from humans with swine contact and also caused the geographic separation of the LA-MRSA ST5 and human clinical MRSA ST5 isolates analyzed here. The results of this study indicate there are genetic differences between LA-MRSA ST5 isolates and human clinical MRSA ST5 isolates; however, because of the study design, these differences have the potential to represent regional variation in MRSA ST5 isolates rather than a difference in clinical and agricultural ST5 isolates. To better address this, future research should investigate human clinical MRSA ST5 isolates from regions of swine production and determine their genetic relationship with the isolates investigated here. If human clinical MRSA ST5 isolates from regions of swine production are more closely related to the clinical MRSA ST5 isolates from humans with no swine contact, it

indicates a difference in the capacity of LA-MRSA ST5 and human clinical MRSA ST5 to cause disease and provides evidence that LA-MRSA ST5 isolates have adapted to colonizing swine. Alternatively, if human clinical MRSA ST5 isolates from regions of swine production are more genetically similar to LA-MRSA ST5 isolates, it may indicate several conclusions: there are regional differences in MRSA ST5 populations, there may be transmission of LA-MRSA ST5 isolates outside of the livestock setting, or humans in regions of swine production are acting as a source of MRSA ST5 isolates in pigs. The information obtained from an analysis of human clinical MRSA ST5 isolates from regions of swine production when paired with patient history will be able to provide information on the capacity of these isolates to cause disease.

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APPENDIX A. ISOLATE INFORMATION

Isolate Name	Isolate Source	GenBank Accession Number	*Spa* Type	SCC*mec* Type	β-Hemolysin Converting Bacteriophage	*czrC* gene	Tetracycline Resistance Genes	*erm* Resistance Gene	ACME genes
ISU837	Environment	LKVQ00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU839	Environment	LKVR00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU842	Environment	LKVS00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU871	Pig	LKVT00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU872	Pig	LKVU00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU873	Pig	LKVV00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU874	Pig	LKVW00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU875	Pig	LKVX00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU876	Pig	LKVY00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU877	Pig	LKVZ00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU878	Pig	LKWA00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU879	Pig	LKWB00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU880	Pig	LKWC00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU881	Pig	LKWD00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU882	Pig	LKWE00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU883	Environment	LKWF00000000	t002	III	Absent	Absent	tetL, tetT	ermC	Absent
ISU886	Human - ST	LKWG00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU887	Human - ST	LKWH00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU888	Human - ST	LKWI00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU889	Human - ST	LKWJ00000000	t002	III	Absent	Absent	Absent	ermC	Absent
ISU928	Human - ST	LKWY00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU930	Human - ST	LKWZ00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU931	Human - ST	LKXA00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU933	Pig	LKXB00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU934	Pig	LKXC00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU935	Pig	LKXD00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU936	Pig	LKXE00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU939	Pig	LKXF00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU940	Environment	LKXG00000000	t002	IV	Absent	Absent	Absent	Absent	Absent

Appendix A Continued

Isolate Name	Isolate Source	GenBank Accession Number	*Spa* Type	SCC*mec* Type	β-Hemolysin Converting Bacteriophage	*czrC* gene	Tetracycline Resistance Genes	*erm* Resistance Gene	ACME genes
ISU941	Pig	LKXH00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU943	Environment	LKXI00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU944	Environment	LKXJ00000000	t002	IV	Absent	Absent	tetL	Absent	Absent
ISU946	Environment	LKXK00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU947	Environment	LKXL00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU948	Environment	LKXM00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU949	Pig	LKXN00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU951	Pig	LKXO00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU952	Pig	LKXP00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU953	Pig	LKXQ00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU954	Pig	LKXR00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU956	Pig	LKXS00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU960	Environment	LKXT00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU961	Environment	LKXU00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU962	Environment	LKXV00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU963	Environment	LKXW00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU964	Environment	LKXX00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU968	Pig	LKXY00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU969	Pig	LKXZ00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU970	Pig	LKYA00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU971	Pig	LKYB00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU972	Environment	LKYC00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU973	Environment	LKYD00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU976	Pig	LKYE00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU978	Pig	LKYF00000000	t002	IV	Absent	Absent	tetL, tetT	ermC	Absent
ISU979	Pig	LKYG00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU980	Environment	LKYH00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU981	Environment	LKYI00000000	t002	IV	Absent	Absent	Absent	Absent	Absent
ISU982	Environment	LKYJ00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU983	Environment	LKYK00000000	t002	IV	Absent	Absent	tetL, tetT	Absent	Absent
ISU992	Pig	LKYL00000000	t548	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU993	Environment	LKYM00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent

Appendix A Continued

Isolate Name	Isolate Source	GenBank Accession Number	*Spa* Type	SCC*mec* Type	β-Hemolysin Converting Bacteriophage	*czrC* gene	Tetracycline Resistance Genes	*erm* Resistance Gene	ACME genes
ISU994	Environment	LKYN00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU995	Environment	LKYO00000000	t002	untypable	Absent	Absent	tetL, tetT	Absent	Absent
ISU996	Environment	LKYP00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU998	Environment	LKYQ00000000	t002	untypable	Absent	Absent	tetL, tetT	Absent	Absent
ISU1000	Pig	LKVI00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1001	Pig	LKVJ00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1002	Pig	LKVK00000000	t548	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1004	Human - ST	LKVL00000000	t548	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1007	Human - ST	LKVM00000000	t1107	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1008	Pig	LKVN00000000	t548	untypable	Absent	Absent	tetL, tetT	ermC	Absent
ISU1009	Pig	LKVO00000000	t002	untypable	Absent	Absent	tetL, tetT	Absent	Absent
ISU1010	Pig	LKVP00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN1	Human - LT	LLBO00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN2	Human - LT	LLBP00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN3	Human - LT	LLBQ00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN4	Human - LT	LLBS00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN5	Human - LT	LLBT00000000	t002	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UMN6	Human - LT	LLBV00000000	t002	untypable	Absent	Absent	Absent	Absent	Absent
UMN7	Human - LT	LLBW00000000	t002	untypable	Absent	Absent	Absent	Absent	Absent
UMN38	Human - LT	LLBR00000000	t242	untypable	Absent	Absent	Absent	ermC	Absent
UMN50	Human - LT	LLBU00000000	t2049	untypable	Absent	Absent	tetL, tetT	ermC	Absent
UCSF 13502	Human - NSC	LLBG00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCSF 13582	Human - NSC	LLBH00000000	t845	II	Present	Absent	Absent	ermA	Absent
UCSF 13938	Human - NSC	LLBI00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCSF 14054	Human - NSC	LLBJ00000000	t14590	n/a	Present	Absent	Absent	Absent	Absent
UCSF 14262	Human - NSC	LLBK00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCSF 14436	Human - NSC	LLBL00000000	t002	IV	Present	Absent	Absent	Absent	Absent
UCSF 14655	Human - NSC	LLBM00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCSF 14811	Human - NSC	LLBN00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 01	Human - NSC	LKYS00000000	t045	II	Present	Absent	Absent	ermA	Absent
UCI 02	Human - NSC	LKYT00000000	t045	II	Present	Absent	Absent	ermA	Absent
UCI 03	Human - NSC	LKYU00000000	t002	II	Present	Present	Absent	Absent	*speG*

Appendix A Continued

Isolate Name	Isolate Source	GenBank Accession Number	*Spa* Type	SCC*mec* Type	β-Hemolysin Converting Bacteriophage	*czrC* gene	Tetracycline Resistance Genes	*erm* Resistance Gene	ACME genes
UCI 04	Human - NSC	LKYV00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 05	Human - NSC	LKYW00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 06	Human - NSC	LKYX00000000	t002	IV	Present	Absent	Absent	Absent	Absent
UCI 07	Human - NSC	LKYY00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 08	Human - NSC	LKYZ00000000	t045	II	Present	Absent	Absent	ermA	Absent
UCI 09	Human - NSC	LKZA00000000	t045	II	Present	Present	Absent	ermA	*speG*
UCI 10	Human - NSC	LKZB00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 11	Human - NSC	LKZC00000000	t242	II	Present	Present	Absent	ermA	*speG, arc* genes
UCI 12	Human - NSC	LKZD00000000	t242	II	intact	Absent	Absent	ermA	Absent
UCI 13	Human - NSC	LKZE00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 14	Human - NSC	LKZF00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 15	Human - NSC	LKZG00000000	t242	II	Absent	Absent	Absent	ermA	Absent
UCI 16	Human - NSC	LKZH00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 17	Human - NSC	LKZI00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 18	Human - NSC	LKZJ00000000	t002	II	Present	Absent	Absent	Absent	Absent
UCI 19	Human - NSC	LKZK00000000	t002	II	Present	Present	Absent	ermA	*speG*
UCI 20	Human - NSC	LKZL00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 21	Human - NSC	LKZM00000000	t002	II	Present	Present	Absent	ermA	*speG*
UCI 22	Human - NSC	LKZN00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 23	Human - NSC	LKZO00000000	t002	II	Absent	Absent	Absent	ermA	Absent
UCI 24	Human - NSC	LKZP00000000	t002	II	Present	Present	Absent	Absent	*speG*
UCI 25	Human - NSC	LKZQ00000000	t045	II	Present	Absent	Absent	ermA	Absent
UCI 26	Human - NSC	LKZR00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 27	Human - NSC	LKZS00000000	t242	II	Present	Present	Absent	ermA	*speG, arc* genes
UCI 28	Human - NSC	LKZT00000000	t242	II	Present	Present	Absent	ermA	*speG*
UCI 29	Human - NSC	LKZU00000000	t002	II	Present	Absent	Absent	Absent	Absent
UCI 30	Human - NSC	LKZV00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 31	Human - NSC	LKZW00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 32	Human - NSC	LKZX00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 33	Human - NSC	LKZY00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 34	Human - NSC	LKZZ00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 35	Human - NSC	LLAA00000000	t045	II	Present	Absent	Absent	ermA	Absent

Appendix A Continued

Isolate Name	Isolate Source	GenBank Accession Number	*Spa* Type	SCC*mec* Type	β-Hemolysin Converting Bacteriophage	*czrC* gene	Tetracycline Resistance Genes	*erm* Resistance Gene	ACME genes
UCI 36	Human - NSC	LLAB00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 37	Human - NSC	LLAC00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 38	Human - NSC	LLAD00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 39	Human - NSC	LLAE00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 40	Human - NSC	LLAF00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 41	Human - NSC	LLAG00000000	t002	II	Absent	Absent	Absent	ermA	Absent
UCI 42	Human - NSC	LLAH00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 43	Human - NSC	LLAI00000000	t242	II	Present	Present	Absent	ermA	*speG*
UCI 44	Human - NSC	LLAJ00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI 45	Human - NSC	LLAK00000000	t242	II	Present	Present	Absent	ermA	*speG*
UCI 46	Human - NSC	LLAL00000000	t242	II	Present	Present	Absent	ermA	Absent
UCI 47	Human - NSC	LLAM00000000	t045	II	Absent	Absent	Absent	ermA	Absent
UCI 48	Human - NSC	LLAN00000000	t242	II	Present	Present	Absent	ermA	*speG*, *arc* genes
UCI 49	Human - NSC	LLAO00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI 50	Human - NSC	LLAP00000000	t045	II	Present	Absent	Absent	ermA	Absent
UCI 51	Human - NSC	LLAQ00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI52	Human - NSC	LLAR00000000	t002	II	Present	Present	Absent	ermA	*speG*
UCI53	Human - NSC	LLAS00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI54	Human - NSC	LLAT00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI55	Human - NSC	LLAU00000000	t045	II	Absent	Absent	Absent	ermA	Absent
UCI56	Human - NSC	LLAV00000000	t242	II	Present	Present	Absent	ermA	Absent
UCI57	Human - NSC	LLAW00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI58	Human - NSC	LLAX00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI59	Human - NSC	LLAY00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI60	Human - NSC	LLAZ00000000	t242	II	Present	Absent	Absent	ermA	Absent
UCI61	Human - NSC	LLBA00000000	t002	II	Present	Absent	Absent	ermA	Absent
UCI62	Human - NSC	LLBB00000000	t242	II	Present	Present	Absent	ermA	*speG*, *arc* genes
UCI63	Human - NSC	LLBC00000000	t045	II	Absent	Absent	Absent	ermA	Absent
UCI64	Human - NSC	LLBD00000000	t242	II	Present	Present	Absent	ermA	*speG*, *arc* genes

APPENDIX B. COMPLETE GENOME SEQUENCES OF TWO *STAPHYLOCOCCUS AUREUS* SEQUENCE TYPE 5 ISOLATES FROM CALIFORNIA, USA

Modified from a paper published in *Genome Announcements*

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Abstract

Staphylococcus aureus causes a variety of human diseases ranging in severity. The pathogenicity of *S. aureus* can be partially attributed to the acquisition of mobile genetic elements. In this report, we provide two complete genome sequences from human clinical *S. aureus* isolates.

Genome Announcement

Staphylococcus aureus is a commensal of the skin and nasopharynx of various animals, including humans. It is also pathogenic in humans, causing disease that ranges in severity from mild skin infections to severe invasive infections [464]. Methicillin-resistant *S. aureus* (MRSA) isolates are categorized epidemiologically into three

categories: hospital-acquired MRSA (HA-MRSA), community-acquired MRSA (CA-MRSA), or livestock-associated MRSA (LA-MRSA). They are further characterized through multilocus sequence typing into sequence types (STs), which indicate the genetic lineage and characteristics of the isolates. ST5 isolates are widely distributed and known to readily acquire mobile genetic elements containing virulence factors or antibiotic resistance elements [12].

We sequenced two clinical ST5 isolates from the University of California, Irvine (UCI28 and UCI62) [375]. They were obtained from patients with MRSA-related disease who had no known exposure to livestock. Because a full patient history was not obtained, HA- and CA-MRSA could not be differentiated. Each isolate was grown in Trypticase soy broth (BD Biosciences, Sparks, MD), and total genomic DNA was extracted using the High Pure PCR template preparation kit (Roche Applied Science, Indianapolis, IN).

Whole-genome sequencing was performed on both the PacBio and Illumina MiSeq platforms. Library preparation for PacBio sequencing was performed according to the PacBio 10-kb insert library preparation protocol available at http://www.pacb.com/wp-content/uploads/2015/09/Procedure-Checklist-10-kb-Template-Preparation-and-Sequencing.pdf. The 10-kb library was sequenced using the PacBio RSII platform, with one single-molecule real-time (SMRT) cell for each isolate. Indexed libraries for the MiSeq protocol were generated with the Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA), pooled, and sequenced using the MiSeq version 2 500-cycle reagent kit, yielding 2 × 250-bp paired-end reads on the Illumina MiSeq platform (Illumina).

Whole-genome assemblies were generated using PacBio SMRT Analysis version 2.3.0 and the CANU version 1.3 software. The average PacBio coverages for the assembled genomes were 306× for UCI28 and 297× for UCI62. After assembling the PacBio data, any overlapping sequence was trimmed and the genomes oriented to start at the *dnaA* gene. The genomes were polished and error corrected using the Broad Institute's Pilon program version 1.18, with Illumina data at 75× and 112× average coverage for UCI28 and UCI62, respectively.

Accession numbers

The whole-genome sequences for these isolates were deposited in DDBJ/ENA/GenBank with the accession numbers CP018768 and CP018769 for UCI28 and CP018766 and CP018767 for UCI62.

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APPENDIX C. DRAFT GENOME SEQUENCES OF 14 *STAPHYLOCOCCUS AUREUS* SEQUENCE TYPE 5 ISOLATES FROM CALIFORNIA, USA

Modified from a paper published in *Genome Announcements*

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Abstract

Staphylococcus aureus is part of the human epithelial microbiota; however, it is also a pathogen. The acquisition of mobile genetic elements plays a role in the virulence of *S. aureus* isolates and contributes to treatment failures. This report details the draft genome sequences of 14 clinical *S. aureus* isolates.

Genome Announcement

Staphylococcus aureus is part of the microbiota found on the skin of humans and other animals. It is also a known pathogen that causes mild to severe infections, as well as toxin-mediated diseases [464]. Methicillin-resistant *S. aureus* (MRSA) is a growing concern in the health industry, and MRSA isolates are categorized based on each isolate's source as either hospital-acquired MRSA (HA-MRSA), community-acquired MRSA

(CA-MRSA), or livestock-associated MRSA (LA-MRSA). Multilocus sequence typing is employed to determine an isolate's sequence type (ST), which indicates the genetic background and denotes the characteristics of the isolate common to the group. The ST5 lineage is globally distributed and well known for acquisition of virulence factors and antibiotic resistance genes contained on mobile genetic elements [12].

We sequenced 14 ST5 isolates from patients with clinical disease caused by MRSA. These isolates were obtained from the University of California, Irvine (UCI3, UCI9, UCI11, UCI19, UCI21, UCI24, UCI27, UCI43, UCI45, UCI46, UCI48, UCI52, UCI56, and UCI64) [375]. The patients had no known livestock exposure; however, because full patient histories were not provided, it could not be determined whether the isolates were HA- or CA-MRSA. Isolates were grown in Trypticase soy broth (BD Biosciences, Sparks, MD), and total genomic DNA was extracted using the High Pure PCR template preparation kit (Roche Applied Science, Indianapolis, IN).

Draft genome sequences were generated using an Illumina MiSeq instrument. Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA) were used to generate indexed libraries. Libraries were pooled and sequenced using the MiSeq version 2 500-cycle reagent kit, yielding 2 × 250-bp paired-end reads (Illumina).

Draft genome assemblies were generated using MIRA version 4.0.2 (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html), resulting in the average coverages indicated here for each isolate: UCI3, 56×; UCI9, 82×; UCI11, 72×; UCI19, 47×; UCI21, 66×; UCI24, 62×; UCI27, 40×; UCI43, 46×; UCI45, 53×; UCI46, 73×; UCI48, 46×; UCI52, 33×; UCI56, 36×; and UCI64, 52×. Following assembly, only contigs >1,500 bp in length having a coverage of >66% of the average genome coverage

were retained. Additionally, when the assembly tool indicated that a contig was part of a potentially repetitive element, the contig was required to be >2,000 bp for inclusion in the assembly.

Accession numbers

The draft genome sequences obtained for these isolates were entered into DDBJ/ENA/GenBank with accession numbers as follows: UCI3, LKYU00000000; UCI9, LKZA00000000; UCI11, LKZC00000000; UCI19, LKZK00000000; UCI21, LKZM00000000; UCI24, LKZP00000000; UCI27, LKZS00000000; UCI43, LLAI00000000; UCI45, LLAK00000000; UCI46, LLAL00000000; UCI48, LLAN00000000; UCI52, LLAR00000000; UCI56, LLAV00000000; and UCI64, LLBD00000000.

Acknowledgements

The research herein was funded by the United States Department of Agriculture, Agricultural Research Service, and by the National Pork Board. S.J.H. is supported by the Iowa State University, Veterinary Diagnostic Laboratory. The funding sources had no role in the study design, data collection or analysis, decision to publish, or manuscript preparation.

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APPENDIX D. DRAFT GENOME SEQUENCES OF 50 MRSA ST5 ISOLATES OBTAINED FROM A U.S. HOSPITAL

Modified from a paper published in *Genome Announcements*

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Abstract

Methicillin resistant *Staphylococcus aureus* (MRSA) can be a commensal or pathogen in humans. Pathogenicity and disease are related to the acquisition of mobile genetic elements encoding virulence and antimicrobial resistance genes. Here, we report draft genome sequences for 50 clinical MRSA isolates from humans with MRSA related disease.

Genome Announcement

Methicillin resistant *Staphylococcus aureus* (MRSA) was first isolated in 1961 [246]. MRSA rapidly became widespread in the hospital setting and remained contained there until the late 1990s, when isolates began infecting patients with no known risk factors for hospital acquired MRSA (HA-MRSA) [370]. This subset of MRSA isolates

was termed community acquired MRSA (CA-MRSA). The third group of isolates are acquired by contact with livestock species and are called livestock associated MRSA (LA-MRSA). Specific lineages of *S. aureus* predominate within each subset and these lineages are defined by their multi-locus sequence type (ST). *S. aureus* lineages are defined by differing characteristics that allow them to possess distinct niches. The ST5 lineage, specifically, is a widespread and successful lineage of HA-MRSA [12]. This is primarily attributed to the capacity of this lineage to acquire mobile genetic elements encoding virulence factors and antimicrobial resistance genes [12].

Here, we report the generation of 48 draft genome sequences from MRSA ST5 isolates obtained from the hospital at the University of California, Irvine [375]. Isolates sequenced, listed in Table 1, were from patients who had MRSA related disease and no known livestock exposure. Minimal patient history was available, including source information, making HA- and CA-MRSA indistinguishable. The isolates were grown in Trypticase Soy Broth (BD Biosciences, Sparks, MD) and the High Pure Template Preparation Kit (Roche Applied Science, Indianapolis, IN) was used to isolate total genomic DNA.

Draft genome sequences were produced using the Illumina MiSeq platform. Indexed libraries were produced with the Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA). Sequencing employed the MiSeq v2 500 Cycle reagent kit (Illumina, San Diego, CA) and generated 2 × 250-bp paired-end reads.

Sequence reads were assembled into draft genomes using MIRA v.4.0.2 (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html). The average coverage for each isolate is listed in Table 1. For retention in the assembly, the contigs

were required to be >1500bp and have a coverage with at least 2/3 the average coverage of the genome. When repetitive elements were identified during assembly, the contig was required to be >2000bp to be included in the assembly.

Accession numbers

The assembled draft genome sequences obtained from this project were deposited into DDBJ/ENA/GenBank with the accession numbers listed in Table C.1.

Table C.1. Isolate name and genome information for 50 clinical MRSA ST5 isolates.

Isolate Name	Average Coverage	Number of Contigs	NCBI Accession Number
UCI 1	73.13×	89	LKYS00000000
UCI 2	51.15×	162	LKYT00000000
UCI 4	69.93×	137	LKYV00000000
UCI 5	83.47×	105	LKYW00000000
UCI 6	59.19×	111	LKYX00000000
UCI 7	51.04×	158	LKYY00000000
UCI 8	53.59×	120	LKYZ00000000
UCI 10	50.26×	125	LKZB00000000
UCI 12	61.68×	167	LKZD00000000
UCI 13	46.01×	144	LKZE00000000
UCI 14	43.15×	183	LKZF00000000
UCI 15	46.20×	123	LKZG00000000
UCI 16	83.09×	117	LKZH00000000
UCI 17	83.32×	140	LKZI00000000
UCI 18	55.44×	205	LKZJ00000000
UCI 20	55.04×	201	LKZL00000000
UCI 22	59.19×	148	LKZN00000000
UCI 23	38.77×	311	LKZO00000000
UCI 25	80.63×	125	LKZQ00000000
UCI 26	71.95×	114	LKZR00000000
UCI 28	48.98×	145	LKZT00000000
UCI 29	65.78×	247	LKZU00000000
UCI 30	66.07×	148	LKZV00000000
UCI 31	56.88×	219	LKZW00000000
UCI 32	43.65×	269	LKZX00000000
UCI 33	43.35×	242	LKZY00000000
UCI 34	49.12×	224	LKZZ00000000
UCI 35	46.42×	111	LLAA00000000
UCI 36	46.00×	246	LLAB00000000
UCI 37	37.53×	264	LLAC00000000

Table C.1 continued

Isolate Name	Average Coverage	Number of Contigs	NCBI Accession Number
UCI 38	53.12×	227	LLAD00000000
UCI 39	83.79×	107	LLAE00000000
UCI 40	44.79×	171	LLAF00000000
UCI 41	50.09×	175	LLAG00000000
UCI 42	54.66×	160	LLAH00000000
UCI 44	60.57×	140	LLAJ00000000
UCI 47	65.17×	191	LLAM00000000
UCI 49	57.85×	237	LLAO00000000
UCI 50	50.05×	174	LLAP00000000
UCI 51	41.44×	184	LLAQ00000000
UCI 53	69.80×	97	LLAS00000000
UCI 54	49.38×	138	LLAT00000000
UCI 55	80.91×	156	LLAU00000000
UCI 57	80.99×	87	LLAW00000000
UCI 58	79.17×	112	LLAX00000000
UCI 59	82.31×	132	LLAY00000000
UCI 60	49.91×	231	LLAZ00000000
UCI 61	66.52×	178	LLBA00000000
UCI 62	61.44×	131	LLBB00000000
UCI 63	54.37×	96	LLBC00000000

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APPENDIX E. DRAFT GENOME OF 1 MSSA AND 7 MRSA ST5 ISOLATES OBTAINED FROM CALIFORNIA

Modified from a paper published in *Genome Announcements*

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Abstract

Staphylococcus aureus is a commensal of humans that can cause a spectrum of diseases. An isolate's capacity to cause disease is partially attributed to the acquisition of novel mobile genetic elements. This report provides the draft genome sequence of one methicillin susceptible and seven methicillin resistant clinical human *S. aureus* isolates.

Genome Announcement

Staphylococcus aureus is a component of the microbiota of normal humans but is also able to cause a wide range of diseases, from mild skin and soft tissue infections to systemic or toxin mediated disease. In 1961, the first isolates of methicillin resistant *S. aureus* (MRSA) were reported in the hospital setting [246]. More recently, MRSA isolates have been found outside of the hospital setting and isolates are now classified

based on epidemiologic characteristics into the following categories: hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock associated (LA-MRSA). Multi-locus sequence typing is used to define lineages of *S. aureus* into sequence types (ST). Isolates within a given ST possess attributes specific to that group that cause them to behave similarly. For example, MRSA ST5 as well as methicillin sensitive (MSSA) isolates compose a lineage that is widespread and highly pathogenic [12]. These isolates are readily able to acquire virulence genes and antimicrobial resistance genes by horizontal gene transfer [12], which allows them to cause more severe disease and treatment failures.

In this report, we present draft genome sequences for one methicillin sensitive *S. aureus* ST5 (BD14054) and seven MRSA ST5 isolates (BD13502, BD13582, BD13938, BD14262, BD14436, BD14655, and BD14811). The isolates were obtained from the University of California San Francisco where they were isolated from patients with *S. aureus* related disease. Full patient histories were not available, so we were unable to determine whether isolates were HA- or CA- in origin; however, the urban location of the hospital ruled out LA-MRSA. Isolates were grown in Trypticase Soy Broth (BD Biosciences, Sparks, MD) and total genomic DNA was isolated with the High Pure Template Preparation Kit (Roche Applied Science, Indianapolis, IN).

The draft genome sequences were generated with the Illumina MiSeq platform. The Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA) was used to generate indexed libraries that were pooled and sequenced with the MiSeq v2 500 Cycle reagent kit (Illumina, San Diego, CA). This generated 2 × 250-bp paired-end reads that were assembled into draft genomes using MIRA v.4.0.2 (http://mira-

assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html). Average coverage for each isolate was the following: BD13502 (74.95×), BD13582 (58.07×), BD13938 (47.09×), BD14054 (42.02×), BD14262 (79.08×), BD14436 (55.28×), BD14655 (57.68×), and BD14811 (73.00×). To be retained in the assembly, contigs had to be >1500bp and have a coverage at least 2/3 the average coverage of the genome. When the assembly tool identified repetitive elements, it required the contig to be >2000bp for inclusion.

Accession number(s).

The draft genome assemblies generated in this project can be found in DDBJ/ENA/GenBank with the accession numbers as follows: BD13502 (LLBG00000000), BD13582 (LLBH00000000), BD13938 (LLBI00000000), BD14054 (LLBJ00000000), BD14262 (LLBK00000000), BD14436 (LLBL00000000), BD14655 (LLBM00000000), and BD14811 (LLBN00000000).

Acknowledgements.

Funding for this research was provided by the USDA Agricultural Research Service and the National Pork Board. SJH is supported by the Veterinary Diagnostic Laboratory at Iowa State University. Funding sources did not impact study design, data collection, data analysis, decisions on publication, or preparation of the manuscript.

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APPENDIX F. DRAFT GENOME SEQUENCES OF 64 SWINE ASSOCIATED LA-MRSA ST5 ISOLATES FROM THE USA

Modified from a paper published in *Genome Announcements*

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Abstract

Methicillin resistant *Staphylococcus aureus* colonizes humans and other animals such as swine. LA-MRSA sequence type (ST) 5 isolates are a public concern due to their pathogenicity and ability to acquire mobile genetic elements. This report presents draft genome sequences for 64 LA-MRSA ST5 isolates in the US.

Genome Announcement

Livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) was first isolated in association with the swine industry in 2004 [248]. Healthy pigs were found to harbor LA-MRSA as a component of their normal microbiota, although there are reports of swine disease attributed to LA-MRSA [251, 465, 466]. The most prevalent multi-locus sequence type (ST) of LA-MRSA in swine varies based on geography, with

ST398 and ST9 predominant in Europe and Asia, respectively [312, 357]. In the United States, LA-MRSA isolates are more diverse with ST398, ST9, and ST5 being found [335]. Although ST398 and ST9 are considered to be livestock adapted lineages, ST5 isolates comprise a widespread and highly pathogenic lineage [12]. This has been attributed to the capacity of these isolates to acquire mobile genetic elements containing antimicrobial resistance genes or virulence factors [12]. LA-MRSA ST5 isolates raise concerns due to their potential to cause disease or disseminate antimicrobial resistance elements, which can be further investigated through genome sequence analysis.

In this report, we present the draft genome sequences of 64 LA-MRSA ST5 isolates obtained by an Iowa State University study evaluating the presence and prevalence of LA-MRSA in the US swine herd [335]. Swabs were taken from healthy pigs and the environment of eight high density livestock operations. All isolates and related information are listed in Table 1. Each isolate was grown in Trypticase Soy Broth (BD Biosciences, Sparks, MD) and total genomic DNA was extracted using the High Pure Template Preparation Kit (Roche Applied Science, Indianapolis, IN).

Draft genome sequence data was produced using an Illumina MiSeq platform (Illumina, San Diego, CA). An indexed library was generated for each isolate using the Nextera XT DNA sample preparation and index kit (Illumina, San Diego, CA). They were then sequenced using MiSeq v2 500 Cycle reagent kit to generate 2 × 250-bp paired-end reads (Illumina, San Diego, CA).

Assemblies were generated using MIRA v.4.0.2, http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html. Average coverage for each isolate is listed in Table 1. Contigs were retained in the assembly if they were

>1500bp in length and had a coverage of >66% of the average coverage for the genome. Repetitive elements identified by the assembly tool were required to be >2000bp for inclusion in the assembly.

Accession numbers

The draft genome sequences generated in this study were deposited into DDBJ/ENA/GenBank with the accession numbers listed in Table E.1.

Table E.1. Isolate name, source, and genome information for 64 swine associated LA-MRSA ST5 isolates

Isolate Name	Isolate Source	Farm Number	Average Coverage	Number of Contigs	NCBI Accession Number
ISU 837	Environment	10	52.86×	60	LKVQ00000000
ISU 839	Environment	10	40.15×	138	LKVR00000000
ISU 842	Environment	10	77.53×	115	LKVS00000000
ISU 871	Pig	24	62.61×	137	LKVT00000000
ISU 872	Pig	24	64.16×	107	LKVU00000000
ISU 873	Pig	24	57.95×	110	LKVV00000000
ISU 874	Pig	24	58.50×	175	LKVW00000000
ISU 875	Pig	24	50.01×	107	LKVX00000000
ISU 876	Pig	24	35.52×	198	LKVY00000000
ISU 877	Pig	24	25.39×	168	LKVZ00000000
ISU 878	Pig	24	28.30×	142	LKWA00000000
ISU 879	Pig	24	59.75×	174	LKWB00000000
ISU 880	Pig	24	50.06×	173	LKWC00000000
ISU 881	Pig	24	26.98×	141	LKWD00000000
ISU 882	Pig	24	59.74×	102	LKWE00000000
ISU 883	Environment	24	21.31×	231	LKWF00000000
ISU 933	Pig	38	43.18×	163	LKXB00000000
ISU 934	Pig	38	26.69×	140	LKXC00000000
ISU 935	Pig	38	18.71×	192	LKXD00000000
ISU 936	Pig	39	33.15×	105	LKXE00000000
ISU 939	Pig	41	31.89×	154	LKXF00000000
ISU 940	Environment	41	52.49×	98	LKXG00000000
ISU 941	Pig	39	63.97×	134	LKXH00000000
ISU 943	Environment	40	54.66×	145	LKXI00000000
ISU 944	Environment	42	14.52×	264	LKXJ00000000
ISU 946	Environment	41	36.83×	80	LKXK00000000
ISU 947	Environment	42	39.23×	164	LKXL00000000
ISU 948	Environment	42	18.98×	172	LKXM00000000
ISU 949	Pig	42	52.56×	126	LKXN00000000

Table E.1 Continued

Isolate Name	Isolate Source	Farm Number	Average Coverage	Number of Contigs	NCBI Accession Number
ISU 951	Pig	42	35.47×	131	LKXO00000000
ISU 952	Pig	42	34.31×	153	LKXP00000000
ISU 953	Pig	42	27.51×	115	LKXQ00000000
ISU 954	Pig	42	31.65×	114	LKXR00000000
ISU 956	Pig	42	27.32×	198	LKXS00000000
ISU 960	Environment	38	22.56×	160	LKXT00000000
ISU 961	Environment	38	12.46×	392	LKXU00000000
ISU 962	Environment	39	40.02×	122	LKXV00000000
ISU 963	Environment	39	44.82×	118	LKXW00000000
ISU 964	Environment	39	36.13×	125	LKXX00000000
ISU 968	Pig	41	48.31×	155	LKXY00000000
ISU 969	Pig	41	18.26×	172	LKXZ00000000
ISU 970	Pig	41	30.09×	91	LKYA00000000
ISU 971	Pig	39	21.67×	187	LKYB00000000
ISU 972	Environment	42	17.93×	91	LKYC00000000
ISU 973	Environment	42	52.18×	200	LKYD00000000
ISU 976	Pig	39	16.94×	261	LKYE00000000
ISU 978	Pig	39	53.16×	188	LKYF00000000
ISU 979	Pig	41	51.52×	176	LKYG00000000
ISU 980	Environment	41	68.97×	126	LKYH00000000
ISU 981	Environment	39	78.78×	142	LKYI00000000
ISU 982	Environment	42	46.30×	272	LKYJ00000000
ISU 983	Environment	42	49.20×	85	LKYK00000000
ISU 992	Pig	46	32.47×	259	LKYL00000000
ISU 993	Environment	46	38.92×	221	LKYM00000000
ISU 994	Environment	46	20.49×	199	LKYN00000000
ISU 995	Environment	46	43.37×	149	LKYO00000000
ISU 996	Environment	46	17.77×	185	LKYP00000000
ISU 998	Environment	46	51.18×	102	LKYQ00000000
ISU 1000	Pig	46	31.07×	243	LKVI00000000
ISU 1001	Pig	46	22.23×	157	LKVJ00000000
ISU 1002	Pig	46	29.08×	157	LKVK00000000
ISU 1008	Pig	46	25.44×	111	LKVN00000000
ISU 1009	Pig	46	55.49×	69	LKVO00000000
ISU 1010	Pig	46	54.23×	91	LKVP00000000

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Laboratory at Iowa State University. Funding sources did not impact study design, data collection, data analysis, decisions on publication, or preparation of the manuscript.

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APPENDIX G. DRAFT GENOME SEQUENCES OF 9 LA-MRSA ST5 ISOLATES OBTAINED FROM HUMANS AFTER SHORT TERM SWINE CONTACT

Modified from a paper published in *Genome Announcements*

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Abstract

Livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) sequence type 5 have raised concerns surrounding the potential for these isolates to colonize or cause disease in humans with swine contact. Here, we report draft genome sequences for 9 LA-MRSA ST5 isolates obtained from humans after short term swine contact.

Genome Announcement

Methicillin resistant *Staphylococcus aureus* (MRSA) was first isolated in 1961 [246]. Isolates have since been categorized based on epidemiologic characteristics into hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock

associated (LA-MRSA). Humans can be colonized by all categories of MRSA isolates; however, LA-MRSA isolates have been considered less pathogenic and livestock adapted when compared to HA- and CA-MRSA isolates [369, 370]. The predominant multi-locus sequence type (ST) found in European swine is ST398, while Asian swine harbor ST9 [312, 357]. In the United States, swine carry a more diverse population of isolates including ST398, ST9, and ST5 isolates [335]. LA-MRSA ST5 isolates are concerning due to the widespread and pathogenic nature of MRSA ST5 isolates in the hospital and community setting [12]. This has been attributed to the ability of this lineage to acquire mobile genetic elements encoding virulence factors and antimicrobial resistance genes [12], which are found rarely in LA-MRSA ST398 and ST9 isolates. Genome sequence data can be used to further evaluate the capacity of LA-MRSA ST5 to colonize and cause disease in humans.

Here, we report the draft genome sequences of 9 LA-MRSA ST5 isolates obtained from humans after short term contact with swine (ISU 886, ISU 887, ISU 888, ISU 889, ISU 928, ISU 930, ISU 931, ISU 1004, and ISU 1007). Each isolate was obtained by Iowa State University from nasal swabs taken from veterinary students after visiting high density swine operations [335]. To obtain genomic DNA, isolates were grown in Trypticase Soy Broth (BD Biosciences, Sparks, MD) and total genomic DNA was extracted utilizing the High Pure PCR Template Preparation Kit (Roche Applied Science, Indianapolis, IN).

The Illumina MiSeq platform (Illumina, San Diego, CA) was employed to generate draft genome data. Indexed libraries were produced using the Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA), pooled, and sequenced

using the MiSeq v2 500 Cycle reagent kit (Illumina, San Diego, CA) to generate 2 × 250-bp paired-end reads. The data was then assembled using MIRA v. 4.0.2 software (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html) leading to the following average coverage: ISU 886 (82.26×), ISU 887 (41.07×), ISU 888 (42.62×), ISU 889 (39.08×), ISU 928 (29.49×), ISU 930 (37.43×), ISU931 (36.54×), ISU 1004 (31.37×), and ISU 1007 (54.60×). For inclusion in the assembly, contigs were filtered allowing only those with a length greater than 1500bp and coverage over 2/3 the average coverage of the genome. When potentially repetitive elements were identified, the contig was required to be greater than 2000bp for inclusion in the assembly.

Accession numbers

The draft genome sequences produced in this study were deposited into DDBJ/ENA/GenBank with the following accession numbers: ISU 886 (LKWG00000000), ISU 887 (LKWH00000000), ISU 888 (LKWI00000000), ISU 889 (LKWJ00000000), ISU 928 (LKWY00000000), ISU 930 (LKWZ00000000), ISU 931 (LKXA00000000), ISU 1004 (LKVL00000000), and ISU 1007 (LKVM00000000).

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APPENDIX H. DRAFT GENOME SEQUENCES OF 9 LA-MRSA ST5 ISOLATES FROM HUMANS WITH LONG TERM SWINE CONTACT

Modified from a paper published in *Genome Announcements*

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Abstract

Humans have been found to harbor livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) isolates. LA-MRSA are considered adapted to colonizing livestock and less pathogenic in humans than their hospital- and community-acquired counterparts. Here, we present 9 LA-MRSA ST5 isolates from veterinarians with long-term swine contact.

Genome Announcement

Methicillin resistant *Staphylococcus aureus* (MRSA) is found as a component of the nasal microbiota of 1.5% of the U.S. population [42], and colonization can be transient or persistent [339]. MRSA isolates are classified based on source into hospital acquired (HA-MRSA), community acquired (CA-MRSA), and livestock associated (LA-MRSA). LA-MRSA isolates are considered less pathogenic than HA- or CA-MRSA isolates and are thought to be adapted to colonize livestock species [369]; however, LA-MRSA has been isolated from humans with swine contact and there are reports of disease associated with multi-locus sequence type (ST) 398 isolates [441]. Although ST398 MRSA is the most prevalent genotype in European swine [312], the ST5 and ST9 genotypes also occur in swine in the United States [335]. Potential animal reservoirs of LA-MRSA ST5 raise particular concerns due to the prominence and pathogenicity of this lineage among hospital and community acquired infections globally [12]. The clinical significance of MRSA ST5 is primarily attributed to the capacity of these isolates to acquire mobile genetic elements encoding virulence factors and resistance to antibiotics [12]. The evaluation of genome sequence data from LA-MRSA ST5 isolates obtained from humans is important to determining the capacity of LA-MRSA ST5 isolates to colonize and cause disease in people.

This report presents the draft genome sequence of 9 LA-MRSA ST5 isolates obtained from the University of Minnesota (MN 1, MN 2, MN 3, MN 4, MN 5, MN 6, MN 7, MN 38, and MN 50). They were isolated from swine veterinarians who had long-term contact with pigs. Total genomic DNA was extracted with the High Pure Template

Preparation Kit (Roche Applied Science, Indianapolis, IN) after overnight growth in Trypticase Soy Broth (BD Biosciences, Sparks, MD).

DNA sequencing was performed on the Illumina MiSeq platform (Illumina, San Diego, CA). Indexed libraries were produced using the Nextera XT DNA sample preparation and index kits (Illumina, San Diego, CA). The libraries were pooled and the MiSeq v2 500 Cycle reagent kit was used with an Illumina MiSeq instrument (Illumina, San Diego, CA) generating 2 × 250-bp paired-end reads.

Assembly was completed with the software MIRA v. 4.0.2 (http://mira-assembler.sourceforge.net/docs/DefinitiveGuideToMIRA.html). This resulted in the average coverage for each isolate as follows: MN 1 (27.44×), MN 2 (47.54×), MN 3 (57.94×), MN 4 (21.15×), MN 5 (37.41×), MN 6 (64.26×), MN 7 (59.56×), MN 38 (28.24×), and MN 50 (24.95×). For contigs to be included in the assembly, they had to be >1500bp in length and the coverage had to be >2/3 the average coverage of the genome. Repetitive elements identified by the assembler were only included in the assembly if the contig was >2000bp in length.

Accession numbers

Draft genome sequences data from this study has been deposited into DDBJ/ENA/GenBank with the following accession numbers: MN 1 (LLBO00000000), MN 2 (LLBP00000000), MN 3 (LLBQ00000000), MN 4 (LLBS00000000), MN 5 (LLBT00000000), MN 6 (LLBV00000000), MN 7 (LLBW00000000), MN 38 (LLBR00000000), and MN 50 (LLBU00000000).

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