

**Differential Expression Profiling of Proteomes of Pathogenic and Commensal
Strains of *Staphylococcus Aureus* Using SILAC**

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Keywords: SILAC, *Staphylococcus aureus*, differential protein expression

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ABSTRACT

Staphylococcus aureus (*S. aureus*) is the etiological agent of food-borne diseases, skin infections in humans and mastitis in bovines. *S. aureus* is also known to exist as a commensal on skin, nose and other mucosal surfaces of the host. This symbiotic association is a result of immune dampening or tolerance induced in the host by this pathogen. We proposed the variation in protein expression by commensal and pathogenic strain as an important factor behind the difference in pathogenicity. The identification of differentially expressed proteins was carried out using a quantitative mass spectrometry (MS)-based proteomic approach, known as stable isotope labeling of amino acids in cell culture (SILAC). Four commensal and pathogenic strains each were grown in the SILAC minimal media (RPMI 1640), containing light (^{12}C) and heavy (^{13}C) form of lysine, respectively, until early stationary growth phase. Various protein fractions, including cell wall, membrane and secreted, were extracted from the bacterial cultures and mixed in a 1:1 ratio. The relative abundance of proteins present in light and heavy labeled samples was determined using MS analysis. From a total of 151 differentially expressed proteins, 58 were found to be upregulated in the pathogenic strains. These proteins are involved in a variety of cellular functions, including immune modulation, iron-binding, cellular transport, redox reactions, and metabolic enzymes. The differentially expressed proteins can serve as putative candidates to improve current approach towards development of a vaccine against *S. aureus*.

Keywords: SILAC, *Staphylococcus aureus*, differential protein expression

DEDICATION

Dedicated to my parents, Mr. S. Manickam and Mrs. Usha Manickam

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List of Abbreviations

2-D fluorescent difference gel electrophoresis	2-D DIGE
Antigen-presenting cell	APC
Chemotaxis inhibitory protein of <i>Staphylococcus aureus</i>	CHIPS
Clonal clusters	CC
Clumping factor	Cif
Colony forming unit	CFU
Core-variable	CV
False Discovery Rate	FDR
Iron surface determinant B	IsdB
Isotope-coded affinity tag	ICAT
Lipopolysaccharide	LPS
Lipotechoic acid	LTA
Maldi-assisted laser desorption/ionization –time of flight	MALDI-TOF
Mass spectrometry	MS
Methicillin-resistant <i>Staphylococcus aureus</i>	MRSA
Microbial Surface Components Recognizing Adhesive Matrix Molecules	MSCRAMMs
Mobile genetic elements	MGE
Neutrophil extracellular trap	NET
Panton-Valentine leukocidin	PVL
Peptidoglycan	PGN
Polymorphonuclear neutrophil	PMN
Somatic cell count	SCC
Stable Isotope Labeling of Amino acids in Cell culture	SILAC
Staphylococcal cassette chromosomes	scc
Staphylococcal enterotoxins	SE
<i>Staphylococcus aureus</i>	<i>S. aureus</i>
Superantigen	SAg
Toll-like receptor	TLR
Toxic shock syndrome	TSS

Chapter 1. Introduction

Staphylococcus aureus (*S. aureus*) accounts for nearly 50% of nosocomial infections in the United States [1]. The incidence of *S. aureus* infections is increasing steadily, with antibiotic resistant strains, such as methicillin-resistant *S. aureus* (MRSA), accountable for more than one-half of cases [2]. The high prevalence of such infections is a threat to human society, and responsible for huge economic losses to the agriculture industry due to mastitis. Despite the availability of common treatments, such as infusion of antibiotics, the cure rates of mastitis vary from 20 to 75% [3]. The development of a vaccine against *S. aureus* is of great importance due to inefficiency of control measures and treatments. So far, no vaccine has been able to provide complete protection against *S. aureus* infections. One major reason is the incomplete knowledge of the huge repertoire of virulence factors produced by this pathogen.

S. aureus causes staphylococcal food poisoning, a prevalent foodborne intoxication worldwide [4]. The staphylococcal enterotoxins (SE) are extremely heat-stable and hence, resistant to processing such as pasteurization. The animals colonized by *S. aureus* on their skin and mucosal surfaces are reservoirs and therefore, a potential risk factor for food contamination [5]. During slaughter, contamination of carcasses occurs and ingestion of such meat causes nausea, emesis, and diarrhea [6]. A recent study found that 40% of the 120 retail meat samples examined contained *S. aureus*, with 45.6% prevalence in pork and 20% in beef [7].

The transmission of antibiotic resistant *S. aureus* may occur from animals to humans, and vice versa due to physical contact, or ingestion of food products of animal

origin. The transfer of resistant genes from ingested *S. aureus* to the host's commensal microflora may make it difficult to treat disease. In immunocompetent people, the risk of infection due to commensal *S. aureus* is minimal or the condition remains asymptomatic. This genetic plasticity or alteration is responsible for evolution of various drug-resistant and virulent strains of *S. aureus* [8]. The commensal and pathogenic strains have genetically different profiles, which implies that the proteome composition differs between these two strain types [9]. The differential protein expression in the commensal and pathogenic strains may reason the bias for variation in the pathogenicity of the strains.

With the advent of mass spectrometry based proteomic techniques, chemical or metabolic labeling approaches are now used for comparison of protein expression. Stable Isotope Labeling of Amino acids in Cell culture (SILAC) is a simple, robust approach in the field of MS - based quantitative proteomics. SILAC is a metabolic labeling strategy for the identification and quantification of relative protein expression levels between two or more cell states. SILAC enables incorporation of the 'heavy' ($^{13}\text{C}_6$, $^{15}\text{N}_7$) or 'light' ($^{12}\text{C}_6$, $^{14}\text{N}_7$) labeled form of the amino acid into the cellular proteomes [10]. This powerful strategy finds application in various biological systems, including yeast, bacteria, and mammalian cell lines. SILAC can be used for identification of differentially expressed proteins among two different systems. Here, we are reporting the identification of the proteins, which are differentially expressed by pathogenic and commensal *S. aureus* strains using SILAC, revealing their role in disease progression.

Chapter 2. Review of Literature

STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a remarkably versatile pathogen with immune evasive mechanisms to address the challenges posed by the host immune system. The name staphylococcus is derived from the greek term ‘staphlyé’ which means a bunch of grapes, based on microscopic appearance. *S. aureus* is a gram positive, non-motile, non-sporulating, catalase-positive, coagulase-positive, and facultative anerobic cocci. *S. aureus* (*aureus* means golden in latin) colonies appear golden yellow due to the presence of a pigment called staphyloxanthin [11]. The commensal strains of *S. aureus* exist harmlessly, inhabiting the skin or mucosal membranes of nearly 20-50% of animal and human population, along with various other microbes, collectively referred to as the “microbiome”; however, only a percent of them cause infection [12, 13]. *S. aureus* is a well-known causative agent for a wide variety of infections in humans and animals. In humans, the infection can range from being cutaneous or mucosal to visceral (endocarditis, bacteremia, pneumonia) or bone related (osteomyelitis) [14, 15]. The antibiotic resistant strains like methicillin-resistant *S. aureus* (MRSA) are a frequent cause of nosocomial infections [14, 16]. In domestic animals, *S. aureus* is a prevalent pathogen responsible for 41% of clinical cases of mastitis, defined as inflammation of mammary glands in lactating mammals [17]. The condition generally remains subclinical and leads to changes in milk composition, thereby affecting the quality of the milk [18]. In summary, *S. aureus* poses a great threat to health care, food, and agricultural industry.

Genome Composition

Whole genome sequences of various *S. aureus* strains have been determined and published. All staphylococcal genomes are approximately 2.8Mbp in size and comprised of a single chromosome, with an occasional presence of plasmids [19]. The genome encodes for nearly 2600 to 2700 proteins, including 1000 proteins with unknown functions [20]. Comparative analysis revealed that nearly 75% of *S. aureus* genomes consist of core components which are conserved across all strains and include genes required for growth and survival [9, 21]. This core genome can be subdivided into “core-stable” and “core-variable” (CV) regions. CV genes account for 10% of genome and are comprised of virulence genes specific to certain *S. aureus* strains, such as cell surface adhesion proteins, toxins, secreted enzymes, superantigens (SAGs), and biofilm production genes [21, 22]. The remaining 25% of genome consists of variable genes which encode non-essential functions, such as antibiotic resistance, or virulence. The variable genes are present on mobile genetic elements (MGEs). MGEs consist of plasmids, transposons, bacteriophages, pathogenicity islands or staphylococcal cassette chromosomes (scc) [23, 24]. The variable elements are acquired through horizontal gene transfer and integration into the genome [25, 26]. Gene transfer facilitates acquisition of genes encoding virulence factors, toxins which may contribute to the pathogenicity of the bacteria. In general, the CV and variable regions together largely determine the pathogenicity or invasiveness of a given strain. The genetic diversity of this pathogen contributes to the phenotypic changes and in turn, the wide spectrum of disease manifestations [8].

Bacterial colonization

S. aureus is an opportunistic pathogen with the ability to adapt to ecological niches. Although ubiquitous in nature, *S. aureus* is a frequent colonizer of skin, nose, and other mucosal surfaces of human and animal population [27, 28]. Bacterial pathogens support their life cycle by utilizing host cells for adherence, replication, and nutrition. Colonization serves as a reservoir from which bacteria may enter the host system, in case of a breach in skin or host immune suppression and turn infectious [29]. In order to successfully establish colonization, the bacteria must first adhere to host cells by evading the host anatomical defenses, such as ciliary movement in the upper respiratory tract, acidic environment of stomach, etc. [30].

S. aureus adheres to extracellular matrix substrates and host epithelial cells via surface proteins or adhesins. These surface molecules, covalently attached to the cell wall peptidoglycan, are collectively termed as MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules). MSCRAMMs are identified by their ability to bind extracellular matrix proteins like collagen (Cna), fibrinogen (ClfA, ClfB), and fibronectin (FnBPA, FnBPB) [31, 32]. Studies have demonstrated that ClfB plays a vital role in the ability of *S. aureus* to invade host epithelial cells *in vivo* in order to establish nasal colonization [33, 34]. FnBP lacking mutant strains have reduced ability to persist intracellularly in the host epithelial cells and human keratinocytes [35, 36]. *S. aureus* strains lacking teichoic acid, a major surface antigen, are unable to bind endothelial cells and are attenuated in the ability to induce endovascular infections [37]. The adhesion may involve non-specific physiochemical interactions and/or specific interactions between bacterial cell wall-associated ligands and

host receptors [38]. Surface hydrophobicity of this bacterium favors its fixation to the host cells non-specifically [39].

S. aureus relies on its extensive and highly regulated pathogenic components including surface adhesins, extracellular enzymes, toxins, and polysaccharides to survive and establish infection [40, 41]. The hydrolyzing enzymes such as lipases, proteases, nucleases, and metalloproteases enable evasion and destruction of host cells for nutrition [41-44]. The *isdI* (iron-regulated surface determinant locus) enzyme produced by *S. aureus* degrades the heme molecules of host hemoproteins for subsequent iron release as a nutrient source [45, 46]. High-affinity iron-chelators, known as siderophores, are also secreted by *S. aureus* and sequester free iron into the cytoplasm [47]. The staphylococcal cysteine protease staphopain B (SspB) possess the ability to induce degradation of the host neutrophils and monocytes [48]. These enzymes and toxins may contribute to the pathogenicity, and consequently the pathogen may transition from colonization to infection stage.

Besides producing a wide array of virulence factors, the variation in the transcriptional regulation, expression of proteins and post-transcription modifications also introduces additional dimensions in the heterogeneity at proteome level. A recent study indicated that exoproteome expression is extremely variable with only 11% of the exoproteins shared among all 25 clinical *S. aureus* isolates used in the study, while each strain expressed its own secretome [20]. The diversity was also determined in the surface proteins of four strains, where only five out of 190 proteins identified were common among the four strains [49, 50].

HOST IMMUNE RESPONSE TO *S. aureus*

The host immune system is programmed to discriminate between self and non-self molecules. The innate immune system, or the first line of defense, comprised of skin, mucous surface, phagocytes, etc., can non-specifically recognize foreign pathogens. The epithelial cells have intrinsic ability to sense invasion by pathogens through a set of membrane bound structures known as toll-like receptors (TLRs) [51]. TLRs are genome-encoded receptors, belonging to a class of pattern recognition receptors (PRRs), which can recognize various conserved components of microbes [52]. These microbial structures are known as pathogen-associated molecular patterns (PAMPs), and include a variety of ligands like lipopolysaccharide (LPS) – an endotoxin on cell membrane of gram negative bacteria, lipoteichoic acid (LTA) and peptidoglycan (PGN) – major cell wall constituents of gram positive bacteria, etc. [53]. TLR2 plays a crucial role in host defense against *S. aureus* by binding to its ligand, PGN. Initial studies proved that mice deficient in TLR2 are susceptible to *S. aureus* infections [54]. However, there is conflicting evidence that murine macrophages lacking TLR2 or TLR4 still respond to *S. aureus* using another class of cytoplasmic sensor, known as nucleotide-binding oligomerization domain 2 (NOD2) [55, 56]. The binding of TLR with its specific ligand activates a cascade of signal transduction, such as NF- κ B pathway which leads to production of cytokines, chemokines, antimicrobial peptides, etc [57, 58]. TLR signaling also regulates T-cell responses through activation of dendritic cells (DCs), a type of antigen presenting cell (APC).

The evasion of host tissues by *S. aureus* evokes resident macrophages and often epithelial cells, which act as APCs, to recognize the pathogen and release

chemokines [59]. These chemical messengers, including interleukin-8 (IL-8), chemokine C-X-C ligands 1 (CXCL1), and 5 (CXCL5), trigger the migration of leukocytes, primarily polymorphonuclear neutrophils (PMN) into the site of infection from the blood stream [60-62]. The infiltration of phagocytic PMN is crucial to the host defense against bacterial infection. PMN and macrophages are phagocytes responsible for internalizing the pathogen to form an internal phagosomes [63]. The fusion of phagosomes with hydrolytic enzymes-containing lysosomes results in formation of “phagolysosomes” and eventually microbial degradation [64]. Complement system in conjunction with antibodies facilitates the process of opsonizing or “flagging” the bacteria, thereby targeting the pathogen to the phagocytes [65]. Deposition of complement protein, C3b on *S. aureus* promotes PMN activity [66]. The surface components of *S. aureus*, mainly PGN induces the production of C5a, a potent chemotactic agent for PMN [60]. Phagocytes along with complement proteins act as the first line of defense against bacterial invasion.

The phagocytic activity of PMN and macrophage leads to production of chemokines leading to infiltration of other leukocytes which specifically recognize the pathogen, mounting a stronger immune response. The peripheral blood mononuclear cells (PBMC), including monocyte, lymphocyte, and macrophage produce proinflammatory cytokines, such as IL-6, tumor necrosis factor alpha (TNF- α), IL-1 β upon stimulation by pathogens. Staphylococcal enterotoxin A (SEA) elicits a strong T-helper 1 (Th1) type response, concomitant with the production of TNF- α and macrophage inflammatory protein-1 α (MIP-1 α) [67]. Another staphylococcal toxin, alpha-toxin upregulates the production of IFN- γ in CD4⁺ T cells leading to Th1-biased immune response [68]. Host

systems with dysfunctional CD4⁺ T cells, or patients with Human Immunodeficiency Virus (HIV) infection having low CD4⁺ T cell counts, specifically Th17 cells, are predisposed to cutaneous *S. aureus* infections [69, 70]. Furthermore, studies have demonstrated the inability of a $\gamma\delta$ T cell deficient mice to clear *S. aureus* cutaneous infection along with impaired neutrophil recruitment due to decreased levels of IL-17 (produced by $\gamma\delta$ T cell) [71]. Another subset of CD4⁺ cells, T regulatory (Treg) cells which possess anti-inflammatory properties and suppress proinflammatory CD4⁺ effector T cell, also play a role in *S. aureus* infections. Treg cells modulate the SEB-induced T cell activation in order to prevent mucosal inflammation [72]. However, there is also evidence that SEB, a prototypic superantigen, is responsible for inhibiting the activity of Treg cells to suppress the T-effector cell proliferation [73]. The inflammation inducing ability is not only restricted to SAgs. Staphylococcal DNA has also proved to induce a strong inflammatory response in mice upon cutaneous injection [74]. The host relies on both innate and adaptive components of immune system to mount a strong response, in order to clear the infection.

IMMUNOMODULATION BY *S. aureus*

S. aureus counteracts the host immune defense with the help of a vast arsenal of specific immune-modulating proteins. The pathogenic bacterial species constantly evolve in order to evade the host immune response. Nevertheless *S. aureus* has co-evolved to adapt well inside the host and escape recognition. The initiation of bacterial colonization induces an immune response with the proliferation and differentiation of specific cell lineages of the immune system. However, *S. aureus* has developed various

counteractive mechanisms to evade the host immune response. The genomic flexibility, acquisition of antibiotic resistant traits, biofilm formation, and escape from phagocytosis are just a few of the evolved techniques for immunomodulation. Virulence factors like protein A prevent opsonization, by binding to the F_c (constant) region of antibodies [75, 76]. Pantan-Valentine leukocidin (PVL) is a toxin secreted by *S. aureus* that forms pores on the surface of leukocytes, thereby promoting cell lysis [77]. Most pathogenic strains of *S. aureus* are resistant to the bactericidal action of lysozyme due to activity of peptidoglycan-specific O-acetyltransferase (OatA) that modifies the C6 hydroxyl group of muramic acid. For example, a strain mutant in OatA was shown to be sensitive to lysozyme, whereas complementation with OatA restored acetylation and lysozyme resistance [78].

PMNs produce reactive oxygen species (ROS) among other lethal agents to kill bacteria [79]. *S. aureus* however, respond by producing substances, including staphyloxanthin to detoxify the ROS [80]. *S. aureus* deploys an array of enzymes and cytotoxins to lyse PMNs. Even upon lysis, the PMNs carry out bactericidal activity by releasing ROS and DNA which form a network of traps, known as neutrophil extracellular traps (NETs), to entrap bacteria [81]. Nevertheless, *S. aureus* defends by secreting nucleases to degrade the DNA [82]. About 60% of *S. aureus* strains secrete chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS) which inhibit chemotaxis of PMN and monocyte by binding to C5a and formylated peptides [83]. Another interesting mechanism for modification of host complement activity is the binding of ClfA to complement control protein factor I, in order to promote degradation of C3b to inactive C3b (iC3b), resulting in diminished phagocytosis by PMN [84, 85].

The resistance of *S. aureus* is further enhanced by the secretion of staphylokinase, which induces the production of defensins from PMNs only to neutralize their bactericidal effect. Staphylokinase binds to α -defensins preventing adhesion of the latter to bacterial surface and thereby facilitate infection [86, 87]. All the above mentioned immunomodulatory mechanisms permit the escape of *S. aureus* from the innate immune cells.

S. aureus expresses on the surface, as well as secretes into the extracellular milieu of the host, a wide array of immunomodulatory proteins. Extracellular adherence protein (Eap), an adhesion factor secreted by *S. aureus*, contains tandem repeat domains and mediates immune evasion by not stimulating proliferation of PBMCs [88]. Surface components such as PGN, LTA, and teichoic acid down-regulate TLR2 dependent-proinflammatory responses required by the host to clear the pathogen [89]. Another cell wall-anchored protein, plasmin-sensitive protein (Pls) utilizes steric hindrance as a mechanism to reduce cellular invasiveness [90]. Upon phagocytosis by PMNs, *S. aureus* exhibits differential regulation of nearly 40% of the genes to promote intracellular survival [82]. Aureolysin, a metalloprotease contributes to the intracellular persistence of this pathogen inside host macrophages [91]. *S. aureus* strains with both invasive and hemolytic phenotypes are capable of inducing apoptosis in human endothelial cells in a caspase-dependent manner [92]. The nature of the immunomodulatory molecules may vary from strain to strain, immunocompetent host to immunocompromised host; depend on tissue location and the effector response of the immune cell mediating immunomodulation [93].

Among the presence of infectious bacteria that trigger strong immune response, there is resident microflora that occurs persistently with low pathogenic potential. An intriguing question is how the commensal bacteria escape immune surveillance or how immune system remains ignorant of these bacteria. Various mechanisms have been proposed and demonstrated so far, but the exact mechanism remains unknown. The inflammatory response is induced by the bacteria when present below the dermis, but not when on epidermal surface [94]. The apical surfaces of epithelial cells showed absence of TLRs, thereby contributing to hypo-responsiveness to the existing microflora [95]. The normal microflora is required to maintain host immune homeostasis and minimize the unintended inflammation yet rapidly respond to infection.

The symbiotic relationship shared between the commensal bacteria and its host plays a very vital role in immune tolerance. The commensal bacterial strains differ genetically and/or physiologically from the corresponding pathogenic isolates [29, 96]. The immune dampening by these strains can be due to the secretion of various effector molecules by the bacteria into external milieu with the help of type III/ IV secretion systems [97, 98]. Such microbial products promote IL-10 (an immunosuppressant) producing DCs which induce Treg cells by inhibiting Th1 cells [99]. Studies have shown that these carrier strains possess the ability to delay recognition through TLR2 on host nasal epithelial cells, as compared to pathogenic non-carrier strains of *S. aureus* [100]. Staphylococcal products like LTA inhibit unintended inflammation triggered through TLR3 signaling during wound repair, by acting selectively on keratinocytes using a TLR2-dependent mechanism. [94]. Furthermore, PGN-embedded molecules of *S. aureus* act as TLR2 ligands, inhibiting the IL-2 response against SAgS. This TLR2/TLR6

signaling induces IL-10 production by monocytes causing apoptosis and decreased T cell activation, thereby preventing SAg induced-toxic shock syndrome (TSS) [101]. The importance of immune dampening by the commensal strains relies on the fact that it prevents undesirable inflammation, thereby producing a state of tolerance in the host [12]. But at the same time, a systemic inflammatory response is necessary during an infection by the pathogenic strains. Hence, the balance must be maintained for the immune modulation providing necessary protection to the host.

CURRENT TREATMENTS & VACCINES

S. aureus has emerged as a major pathogen in community, affecting hosts without predisposing risk factors [102]. The increasing rate of *S. aureus* infections has sparked the interest of researchers across the globe in the development of a vaccine for individuals at high risk of such infections. Even a broadly protective vaccine against this pathogen must be designed based on its virulence factors, nutritional requirement, and other survival techniques [103].

Antibiotic therapy is a traditional way of treating lactating cows with clinical mastitis and also used at drying-off to prevent *S. aureus* infection. Antibiotics like cephalosporin, penicillin-streptomycin, penicillin-novobiocin, tilmicosin, and cephalonium have similar antimicrobial activity [104-106]. A combinatorial use of these drugs shows synergistic effects. The prepartum antibiotic treatment of heifers resulted in a lower incidence of clinical mastitis throughout lactation, compared to untreated heifers [107, 108]. The ability of the bacteria to form biofilm, abscess, and survive intracellularly

within host epithelial cells and macrophages, thereby resisting antibiotics renders the treatment ineffective in some cases [109, 110]. Also, due to the inefficacy of treatments, antibiotic-resistant pathogenic strains of *S. aureus* like MRSA pose a serious threat to the host.

The limitations of antibiotic treatment have led to the development of vaccines as a promising preventive measure to control *S. aureus* infections. This gained the interest of researchers world-wide and numerous vaccine strategies and targets were studied, although none have passed the Phase III clinical trials [111, 112]. Human passive vaccines are often designed based on pre-clinical evaluations with *S. aureus* antigens selected as targets. Tefibazumab (Aurexis®) is one such passive vaccine containing human monoclonal antibody against ClfA, a fibrinogen binding adhesin. The antibodies bind ClfA with high affinity to prevent adherence of *S. aureus* to fibrinogen and thereby reducing incidence of bacteremia. Altastaph® is a polyclonal human IgG with high levels of antibodies to capsular polysaccharide type 5 and 8 (CP 5 and CP8) [113]. Approximately 85% of the clinical isolates of *S. aureus* are known to express CP 5 and CP8. However, during the phase II clinical trial, the vaccine proved to be inefficient in reducing *S. aureus* infections [114]. Similarly, the phase II clinical trials of INH-A21 (Veronate®), a donor selective intravenous polyclonal antistaphylococcal human immunoglobulin, showed no significant protection against nosocomial *S. aureus* infections [115-117]. An efficient passive vaccine may prove to be beneficial for immunodeficient individuals or provide protection during immediate risk of infection.

In order to overcome the drawbacks of short-lived passive antibodies, an active vaccine conferring broad protection is desired. StaphVAX® is an active, bivalent

vaccine consisting of CP5 and CP8 components bound to the mutant non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A. However, in a Phase III clinical trial, the vaccine was unsuccessful at preventing bacteremia in end-stage renal disease patients. The vaccine failed during another Phase III trial involving hemodialysis patients [111, 112, 118]. Merck-V710® is another active vaccine containing the conserved *S. aureus* iron surface determinant B (IsdB), which is yet to clear the clinical trials [119]. The Phase III clinical trials completed so far have failed to deliver a vaccine against, *S. aureus* suggesting the multicomponent vaccines as an alternative strategy.

The differences in virulence factor production across strains require the bacterin-based vaccines to originate from more than one strain. However, the autogenous bacterin vaccine proved to be inefficient at reducing the prevalence of mastitis in the dairy cattle [120]. Vaccine targeting epitopes offer an alternative approach to control mastitis, focusing on a specific immune response. A multiepitope vaccine candidate consisting of fusion proteins – ClfA of *S. aureus* and surface immunogenic protein (SIP) of *Streptococcus agalactiae*, created based on their B-cell epitopes proved to provide protection against both the pathogens by inducing a humoral response. The vaccine induced serum IgG1 production significantly in mouse trials, thereby increasing the opsonization ability and promoting ingestion of the bacteria by PMNs [121]. The inability of existing vaccines to induce both humoral and cellular immune response was challenged by a DNA-based vaccine consisting of the epitopes of FnBP and ClfA of *S. aureus* used as antigenic targets [122]. Partial protection was attained in dairy cows by this vaccine with a decrease in bacteria shedding, stress, and inflammation.

Almost every active or passive *S. aureus* vaccine tested seemed to confer protection in mice, but none worked in humans [123]. One explanation could be that *S. aureus* is less well adapted to mouse when compared to natural human host. Various immune evasion factors of *S. aureus* are species specific and functional only at high concentrations in mouse. Secondly, the antigenic diversity of this pathogen renders the single component vaccine ineffective, as all *S. aureus* strains don't confront the host with the same antigen that the latter has been vaccinated for. Thirdly, humans are somewhat pre-immunized for *S. aureus* since birth due to constant exposure and genetic predisposition in some cases, while the experimental animals are comparatively naïve [124]. The assessment of the efficiency of these vaccine candidates is difficult because of differences in their retrospective methodologies (preparation of vaccines, routes, doses, and experimental group, etc.), discrepant parameters, and criteria (e.g., SCC or bacterial shedding) used to conclude a vaccine effective [110]. Hence, in summary an ideal vaccine must be able to induce appropriate immune response and exclude antigens which may drive side effects like hypersensitivity, inflammation, etc.

SILAC: AN INTRODUCTION

The post-genomic era paved way for a proteomics age focused on improving our understanding of the dynamic cellular protein network. With the introduction of protein structure prediction techniques, a huge repertoire of macromolecular structural data was made available. However, the knowledge was limited to qualitative information and lacked quantitative data for the protein of interest. The

application of mass spectrometry (MS) techniques provide a tool for the quantification of proteins with attomolar sensitivity [125].

Comparative MS-based quantitative proteomic techniques aim to determine the relative abundance of proteins under different biological states [126, 127]. Two-dimensional polyacrylamide gel electrophoresis (2-DE) has long served as the initial primary tool for comparative proteomic analysis. The detection of protein differences between two samples rely on comparison of at least two different gels, and often use computational methods to merge the image of one gel onto another [128]. However, the major drawback of this technique is the low reproducibility and limited sensitivity towards hydrophobic and low abundance proteins [129]. Presently, 2-DE coupled with MS analysis provides semi-quantitation, relying on intensity of gel spots for difference in abundance and MS analysis for protein identification [125].

An improved version of 2-DE, namely 2-D fluorescence difference gel electrophoresis (2-D DIGE) has circumvented some of the limitations of conventional 2-DE [130]. This technique involves pre-labeling protein samples with one of three spectrally distinct fluorescent CyDyes; cyanine 3 (Cy3), cyanine 5 (Cy5), and cyanine 2 (Cy2); a covalent modification that does not affect their relative migrations in 2-DE gels [131]. DIGE allows the labeled samples to be combined and run in a single 2-D gel, minimizing the gel-to-gel variation. However, recent studies have shown that comigration and partial comigration of multiple proteins into a single spot renders comparative quantification rather inaccurate [132]. Another limitation of DIGE is the separation capabilities of 2-DE, which does not efficiently resolve large proteins or hydrophobic membrane proteins [133, 134].

Isotope labeling strategies serve the purpose of quantification of ‘relative’ protein expression levels in two samples or states [135]. Stable isotope labeling methods can use either *in vitro* chemical modifications of proteins (e.g. ICAT/iTRAQ), or *in vivo* metabolic labeling which requires live cells [136]. Chemical labeling strategies rely on a derivatization reagent for chemical modifications by introducing a mass tag. This technique can be applied to any type of sample and at both peptide and protein stage [137]. One such method, isotope-coded affinity tag (ICAT) utilizes a reagent containing a biotin tag and a reactive group that binds specifically to the sulfhydryl groups of the cysteine residue. Each sample is derivatized by the light and heavy isotopic reagent and further combined and cleaved enzymatically. The tagged peptides are separated using avidin affinity chromatography. The isolated peptides are then analyzed by LC-MS/MS [138]. However, ICAT targets only cysteine-containing peptides which account of ~85% of the proteome in most model organisms [139]. This limits the quantitation coverage of the peptides. Moreover, the enrichment steps require extra processing of samples, accounting for losses during handling, resulting in quantitation errors [140, 141]. Metabolic labeling methods overcome this bias problem by mixing the samples and processing them together during the workflow.

Stable Isotope Labeling of Amino acids in Cell culture (SILAC) is a metabolic labeling strategy that utilizes stable isotope labeled amino acids in the growth medium which will be incorporated in the proteome of the organism *in vivo*. In order to ensure the complete incorporation of the amino acid, the organism or cell type being studied must be auxotrophic for that amino acid. In other words, the amino acid chosen must be an ‘essential’ amino acid for the organism of interest [10].

The workflow involves growing the cells in two groups in a culture medium that is identical except that one version contains a light (non-labeled) form of the essential amino acid, while the other one contains the heavy (stable isotope labeled) form. The cells are cultured for enough cell doublings to ensure that ~95% of the proteins incorporate labeled amino acid. With the process of cell doubling and protein synthesis taking place, the heavy amino acid replaces the light form. The incorporation of each labeled amino acid brings about a mass shift in the peptide. The proteins extracted from the cells grown in heavy and light medium are mixed together, digested with trypsin and analyzed by MS. The signal intensity from the light peptide and its corresponding heavy peptide allow comparison of their relative abundances in the two groups [136, 142].

The simple and straightforward approach of SILAC has led to its wide spread applicability. SILAC has been extensively applied to the study of post-translational modification, such as protein phosphorylation [143], comparing tissue proteome expression [144], [136], investigating complex network of signal transduction pathways [145]. However, SILAC is not just restricted to these applications, and researchers across the globe are implementing it to their studies. This technique can be extended to a variety of systems, including yeast [146], bacteria [147], plants [148], and mammalian cell lines [136].

This approach was first demonstrated for relative quantitation of changes in protein abundances during the course of myoblast differentiation in mouse C2C12 cells [136]. The study was aimed at analyzing the protein expression changes as the myoblasts differentiate into myotubes. Using deuterated leucine (Leu-d3) as the isotopically labeled amino acid in their media, they identified nine proteins whose expression levels varied

during the time course. In yeast, phosphoproteins that play a role in the pheromone signaling and mating were identified using SILAC [146]. Using a double auxotroph yeast strain, isotope labeling of proteome was achieved by incorporation of arginine- $^{13}\text{C}_6$ and lysine- $^{13}\text{C}_6$. With one population exposed to pheromones and the other isotopically labeled, the enriched phosphopeptides obtained from cell lysates were analyzed using LC-MS. One hundred-thirty nine proteins out of more than 700 phosphopeptides showed 2-fold change in response to pheromone stimulation. These proteins were mapped to be belonging to pheromone-signaling pathway, transcriptional regulators, and receptors. SILAC coupled with MS techniques can produce vast amount of data that can be analyzed through the use of various automated quantitation software.

Two recent studies have focused on labeling of *Caenorhabditis elegans* with either heavy lysine alone or both heavy lysine and arginine by feeding the nematodes with heavy isotope labeled *Escherichia coli* grown in their respective heavy amino acid counterparts. One of the studies characterized the heat shock response in the nematodes, while also providing a generic solution to the arginine to proline conversation by using RNAi [149]. Another study led to the identification of various proteins that are regulated in response to loss or RNAi-mediated knockdown of the nuclear hormone receptor 49 in *C. elegans* [150]. In summary, MS-based approaches in combination with quantitative proteomics provide solutions to analysis on a global-scale for the study of cellular changes and/or protein expression in systems biology.

Recent studies have applied various proteomic approaches in the comparison of the proteomes of different strains of bacteria. The comparative proteomic analysis between the invasive and commensal strains of *S. epidermidis* using 2-DE and

matrix-assisted laser desorption/ionization –time of flight (MALDI-TOF) MS revealed 64 differentially expressed proteins involved in carbohydrate metabolism, lipid degradation and amino acid binding. The results demonstrated 3.5 –fold more expression of two proteins (RNAIII activating protein, accumulation-associated protein) involved in biofilm formation, by the invasive strain than the counterpart, suggesting the contribution of the proteins to virulence and biofilm formation [151]. However, 2-DE imaging analysis is limited in the ability to produce good quantitative data and hence the need for analytical techniques coupled with MS, such as SILAC. In another study, the surface proteomes of the enterotoxigenic and commensal *E. coli* strains were compared using SILAC. Twenty-three differentially expressed outer membrane proteins were identified and upon bioinformatics evaluation for putative vaccine candidates only three of them were chosen [152]. In summary, SILAC proves to be applicable in various systems for the detection of differentially expressed proteins. In this study, we focus on the use of SILAC to identify proteins that are differentially expressed among the commensal and pathogenic strains of *S. aureus*. The identification of such proteins may help determine their role in pathogenesis, and potentially help improve the current approaches to vaccine development.

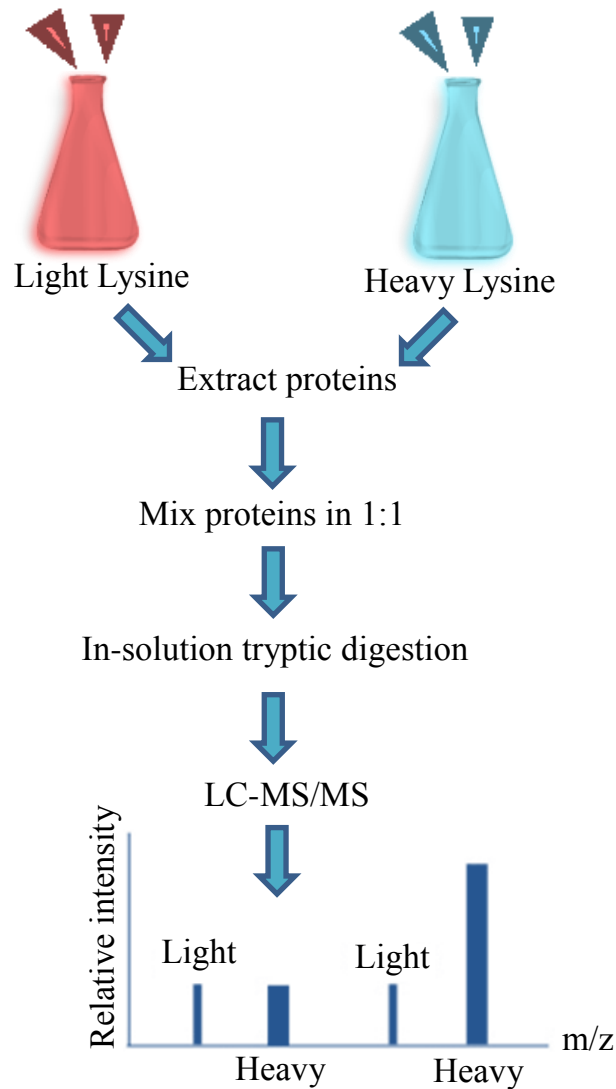


Figure 2-1: The workflow of SILAC experiment. The bacteria are grown in ‘light’ and ‘heavy’ SILAC medium containing light ($^{12}\text{C}_6$) lysine and heavy ($^{13}\text{C}_6$) lysine, respectively. The proteins are extracted and mixed in 1:1 ratio, followed by an in-solution tryptic digestion to obtain peptides that are analyzed by MS.

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Chapter 3. A study of growth patterns of pathogenic and commensal strains of *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a major animal and human pathogen and yet is carried asymptotically by a large proportion of both populations. Studies have characterized a wide array of genes expressed by *S. aureus*, revealing the complex regulatory circuits which promote the ecological fitness of this opportunistic pathogen. Bacterial colonization can lead to progressive invasion of the host in case of a breach or injury to the skin, providing access to the blood stream, resulting in an infection. The transition of bacteria from the commensal state to pathogenic state induces genotypic and physiological changes. In this study, four commensal strains and four pathogenic strains of *S. aureus* were used. The commensal isolates were obtained from the bovine's hock, teat, and nose, whereas the pathogenic strains were isolated from clinical cases of mastitis. The growth rate patterns of all the strains were determined in a minimal growth media. RPMI 1640 was chosen as the minimal media due to the flexibility in altering the concentrations of amino acids, a requirement for further studies. All the strains reached the early stationary growth phase at approximately around 11-13 hrs. The strains showed variation in growth rates which is consistent with the fact that genotypic differences among the strains lead to changes in the physiological adaptability to the environment.

INTRODUCTION

Staphylococcus aureus is a major pathogen responsible for a wide spectrum of diseases among humans and animals ranging from skin infections to osteomyelitis, endocarditis, bacteremia, or toxic shock syndrome. The pathogenic diversity of *S. aureus* is attributed to production of a diverse range of virulence factors, in the form of extracellular or surface proteins. The production of such virulence determinants occurs in a coordinately regulated growth-dependent manner, reflecting the ability of *S. aureus* to survive and adapt in different environmental niches [1]. Factors such as cell density, pH, nutritional availability, environmental signals, and host immune defenses also affect the production of virulence factors [2].

The regulation of virulence factors is controlled by a network of interacting regulons, including accessory gene regulator (*agr*) and staphylococcal accessory regulator (*sar*). The *agr* system is responsible for controlling the quorum-sensing driven regulation. This two-component system differentially regulates the expression of cell wall proteins and secreted exoproteins in response to cell density [3]. The *agr* operon enhances the production of exoproteins and represses the synthesis of cell wall proteins [4]. The *sar* locus is required for the activation of the *agr* locus, providing an additional level of regulation to production of virulence factors [5].

The expression of each regulon is modulated during different growth phases of the bacteria. The initiation of exponential phase involves activation of metabolic pathways required for growth and cell division, and also synthesis of surface proteins or adhesion proteins [6]. The *agr* system is activated after mid-exponential growth phase, which leads to down-regulation of the surface proteins [7]. In other words,

the early exponential phase involves the upregulation in expression of surface proteins and by the mid-exponential phase, the population density-sensor *agr* is activated leading to upregulation of the secreted proteins with a downregulation in several surface proteins [8, 9]. Hence, our study focused on the transition from late exponential phase to early stationary phase where bacteria express both the surface and secreted proteins, with minimal cell death.

The virulence gene expression profiles between commensal and pathogenic strains of *S. aureus* are generally not similar [10, 11]. The virulence genes have evolved to adapt according to bacterial signal transduction systems that respond to a wide variety of environmental cues, including quorum sensing, ecological niche, and host defense response [12, 13]. The production of virulence determinants, such as toxins, hemolysins, and most adhesion proteins is more frequently observed in pathogenic strains as compared to commensal strains [14]. The low pathogenicity index of the commensal strains allows the host to remain immunologically hyporesponsive [15]. Furthermore, the genotypic and phenotypic differences between the commensal and pathogenic strains are responsible for variations in growth rates, adhesion, virulence factors, and hence, pathogenicity. In this study, we focused on comparing the growth pattern of commensal and pathogenic strains of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains. The pathogenic isolates of *S. aureus* were obtained from the milk of mastitis affected cows. The commensal strains were isolated from the potential reservoirs of *S. aureus*, such as bovine's skin surface of hock, teat, and nose using sterile PBS dipped swabs [16]. A total of four commensal strains and five pathogenic strains, including ATCC 27217 were used for all the experiments. Frozen stocks were prepared for all strains for future use throughout the period of experimentation. Cultures were prepared by inoculating 5 mL of Tryptic Soy Broth (TSB) with a single colony from a streaked Tryptic Soy Agar (TSA) plates and growing it overnight at 37°C using a model 12400 Incubator Shaker (New Brunswick Scientific, USA) under gentle shaking (190 rpm). Finally, stocks were made by mixing 85% of the culture with 15% of sterile glycerol (100% pure) and stored in cryovials at -80°C.

***S. aureus* strain confirmation.** The commensal strains were confirmed as *S. aureus* using gram staining, catalase, and coagulase tests. Gram staining was performed on a glass slide using crystal violet stain. The catalase test was carried out by smearing a colony over a drop of hydrogen peroxide on a glass slide. The coagulase test was performed in a test tube containing rabbit plasma and inoculating a colony (from TSA plates) into it, followed by 4 hrs of incubation at 37°C. The strains were also streaked out on Esculin blood agar (EBA) plates to verify clear hemolysis. The confirmation was carried out using PCR for selected genes – 16S rRNA (gene specific to all *Staphylococcus* species), and ClfA (gene specific to *S. aureus*). The designed primers were purchased from IDT (Integrated DNA Technologies, Inc, USA). The primers for ClfA were forward: 5' GCAAAATCCAGCACAACAGGAAACGA 3', reverse: 5'

CTTGATCTCCAGCCATAATTGGTGG 3' and that for 16S rRNA were forward: 5' GTGAATACGTTCCCGGGTCTT 3', reverse: 5' CGGCTTCGGGTGTTACAAAC 3'. The primers were designed to yield amplification products of size 638 bp and 68 bp for ClfA and 16S rRNA respectively.

PCR analysis. DNA was extracted from all the commensal strains by a simple cell lysis procedure involving boiling at 105°C. All PCRs were performed in a total reaction volume of 55 µl containing 10 µl genomic DNA, 2.5 units of Taq polymerase (GoTaq® Flexi, Promega, Madison, WI), 10 µl PCR buffer 5×, 2 µl MgCl₂, 4 µl of 2.5 mM dNTPs and 2 µl of each primer (5µM). Thermal cycling conditions were as follows: initial denaturation step at 95°C for 3 min; 40 cycles of PCR consisting of 94°C for 45 sec, 45 sec at annealing temperature of 59°C and 72°C for 1 min; then a final extension step at 72°C for 10 min. The amplified PCR products were analyzed using standard agarose gel electrophoresis. The agarose gel was prepared using 2% agarose, 1X TBE (10.8 g Tris base, 5.5 g boric acid, and 4 mL of 0.5 M EDTA (pH 8.0); made upto 1L with distilled water) and 10 µl SYBR safe. Twenty µL of the PCR product was loaded onto the gel, which was run at 150 V until the dye reached the bottom of the gel. The gel was visualized using Chemidoc apparatus and Quantity One software (Bio-Rad).

SILAC minimal media determination. Dulbecco's Modified Eagle Medium-DMEM (Gibco, Carlsbad, CA), Minimal Essential Medium-MEM (Gibco, Carlsbad, CA), RPMI 1640 (Gibco, Carlsbad, CA), Mannitol Salt Broth (MSB), and TSB were used to determine the most suitable minimal media for SILAC. The strains were cultured at 37°C under gentle shaking (190 rpm) in 50 mL of each media and grown over a period of 24 hours. Aliquots were collected at various time points to determine the colony forming

unit (CFU) counts in the cultures. The growth rates were compared by plotting a graph of time points against CFU counts using GraphPad Prism 4.03 (Graphpad software, Inc.).

Growth curves. The commensal and pathogenic strains were routinely streaked from the frozen stock cultures onto EBA plates, and single colonies were used to inoculate 50 mL of RPMI 1640 (no L-glutamine). The cultures were grown at 37°C under gentle shaking (190 rpm) for 24 hours, with CFU counts and OD₆₀₀ readings determined at several time points (0, 3, 6, 9, 10, 11, 12, 13, 14, 15, 18, and 24 hrs).

RESULTS

***S. aureus* strain confirmation.** The pathogenic strains were strain typed and confirmed as *S. aureus*. The *Staphylococcal* protein A (spa) typing data (Table 3-1) revealed the presence of spa repeats and different enterotoxin genes in some strains. The cassettes found in the strains refer to the various spa repeat sequence. The commensal strains were strain typed at a later stage in the study and found to lack enterotoxin genes (Table 3-2); until then, they were tested and identified using Gram stain, coagulase, and catalase tests. The gram stained bacteria appeared as dark purple-colored, round shaped, and clustered together, suggesting gram positive cocci species. The inoculation of rabbit plasma with bacterial colonies, lead to the coagulation of plasma after 4 hours, suggesting the strains are coagulase positive *Staphylococcal* species. During the catalase test, the bacterial colonies upon contact with hydrogen peroxide produced bubbles, suggesting the release of oxygen, a reaction catalyzed by catalase enzyme produced by *Staphylococci*. The PCR products were analyzed using agarose gel electrophoresis, to separate them based on their molecular mass. The ClfA and 16S rRNA gene PCR products were sized to 638 bp and 68 bp (based on standard ladder), confirming the presence and amplification of the desired target in the strains (Fig. 3-1). All the commensal strains were confirmed to not only belong to the genus *Staphylococcus* but specifically *S. aureus* species, based on the presence of 16S rRNA and ClfA gene, respectively. The *Streptococcus* and water samples showed faint bands for 16S rRNA gene, which could be attributed to the cross-reactivity of the primers to the pervasiveness of this prokaryotic gene or possible contamination.

Selecting minimal media. The pathogenic and commensal strains had variable growth rates in the culture media used. The growth rate of the representative pathogenic strain was the maximum in TSB media, followed by that in RPMI 1640, when compared to the other mediums (Fig. 3-2). The commensal strains showed similar growth pattern in RPMI 1640 (result not shown here). A requirement for SILAC is that amino acids must be controllable in the minimal medium. Since, it is not possible to modulate the amount of amino acids in TSB, RPMI 1640 was chosen as the minimal media based on the growth rate of the isolates which was comparable growth in TSB.

Growth curves comparison between pathogenic and commensal strains. The growth of the strains was monitored to focus on the transition from exponential to stationary phase, or when the expression of secreted proteins is upregulated. The variation in the growth rates among the different isolates of pathogenic strains, as well as commensal strains in RPMI 1640 was established (Fig. 3-3). The pathogenic strains, #4065 and #4338 grew faster compared to the other two strains, with a doubling time of 1.79 hrs and 1.96 hrs, respectively. Among the commensal strains, #4277 exhibited highest growth rate, with a doubling time of 1.94 hrs, compared with 2.69 hrs for the slowest growing strain #4483. The doubling time of all the *S. aureus* strains used was comparable to the growth rate in iron-depleted media, as reported before [17]. Also, the transition from the exponential to stationary phase occurred at about the same time for all the strains, at around 11-13 hrs after inoculation. Nevertheless, the pathogenic strain #4170, a slow growing strain, never reached stationary phase until 24 hrs. Each strain varies in the ability to adapt to the environmental conditions due to alterations in genotype or

production of proteins necessary for survival. Since all the strains were provided the same physiological conditions, such as temperature, medium, pH, and agitation, the variability in growth can be attributed to the genotypic and phenotypic differences among the isolates [18].

DISCUSSION

Bacterial species express genetic information in a coordinated manner. The factors which are not essential for growth, but are required for the survival of the bacteria within the host in a non-symbiotic manner can be termed as virulence factors, and hence are not constitutively produced. *S. aureus* has evolved control machinery which coordinates regulation of gene expression by detecting environmental changes. This growth-dependent expression of virulence factors is regulated by two major “virulons”, namely *agr* and *sar*. Generally, the target virulence gene can be under the influence of more than one regulator that “cross-talk” in order to ensure expression of the virulence factor under specific conditions [19]. For instance, studies have demonstrated that *sarS* binds to *spa* promoter, which encodes staphylococcal protein A (Spa), and activates its expression, whereas *agr* negatively regulates the production of Spa by suppressing the expression of its activator, *sarS* [20].

Strain-to-strain variation can occur in both gene content and expression levels. Any sequence variation in a *S. aureus* strain could result in a change in genotypic and phenotypic characteristics, including adhesion, virulence, expression of toxins, and/or surface proteins [21]. This variation explains the differential pathogenicity of various clonal lineages of *S. aureus* strains. Studies have shown that *agr* or *sarA* mutants are attenuated for virulence, however, *agr-sarA* double mutant have complete loss of virulence [22-24]. The role of *agr* and *sarA* was demonstrated in an *agr* and *sarA* mutant strain which failed to escape phagocytosis by inducing apoptosis in a culture of bovine mammary epithelial cells [25].

The genetic variation between the commensal and pathogenic strains supports the observed disparities in their potential to induce inflammatory responses in the host [26]. The reduced pathogenicity of the commensal strains may be due to their impaired adherence or production of toxins and surface antigenic proteins [27]. The commensal strains used in the study lacked any enterotoxin gene, shown by the spa-typing results (Table 3-2). Multilocus sequence typing (MLST) has revealed that genetic alterations (recombination and mutation events) causes clonal diversification, having pleiotropic effects on virulence and colonization ability. This is consistent with the variation in invasiveness of different strains of *S. aureus*. However, it can't be ruled out that a commensal strain can turn invasive. Aggressive colonization leads to production of genetic factors which may contribute to local tissue damage, providing an access to the blood stream, hence causing an invasive disease. This is supported by the fact that fimbriae, an attachment factor produced by *Escherichia coli* for colonization is associated with urinary tract infection [28]. Studies have shown that induction of oxidative stress can lead to emergence of antibiotic resistant strains of *Pseudomonas aeruginosa* [29].

The variations observed in growth rates represent different biotypes that may have clinical relevance [30]. The fast growing strains may have an edge at *in vivo* growth, survival, and maintenance of infection. Previous studies indicate that growth rate of a *S. aureus* strain can be considered as a marker for virulence, which is also related to expression of adhesins [31]. Therefore, differences can be expected in the growth rates of commensal and pathogenic strains. However, in our study, the doubling times of the pathogenic strains studied here were comparable to that of the commensals. Growth rates of two commensal strains (#4277, #4257) were similar to that of the two fastest growing

pathogenic strains (#4065, # 4338) , but faster than two slow growing pathogenic strains (#4170, #4131) (Fig. 3-3). Few strains used in the study depict similar spa-types (#4131/#4338, and #4396/#4483), but the growth rate and protein expression varied among them. However, more details about lineages of the strains need to be obtained by using MLST technique. The growth rate difference can potentially be attributed to the fact that each strain has a different strategy to utilize the nutrients in conjunction with diverse regulatory controls [32].

The strains reached early stationary phase approximately around 11-13 hrs of growth. There was no significant variation observed in the growth rate between the two strain types; growth rates varied widely among strains grown in RPMI 1640. RPMI 1640 lacks iron, an important factor in *S. aureus* growth, and hence, differs from other growth media and *in vivo* conditions inside host. However, the comparison cannot be truly determined, since the *in vitro* conditions lack the host immune cells, which play an important role in modulating the bacterial gene and protein expression.

Table 3-1. Pathogenic strains used in the study. The table lists the four pathogenic bovine isolates used in the study (strain ID or cow number), species (spp), number of spa types (spa), spa repeat sequence (cassettes) and different enterotoxin gene profile.

S. No.	Strain ID	spp	spa	Cassettes	Enterotoxin genes
1	4065	aureus	92	ujgfmbbpb	C, A
2	4131	aureus	105	u.new.gfmbbpb	
3	4170	aureus	309	tjmbmdm	B, D
4	4338	aureus	105	ujgfmbbpb	C

Table 3-2. Commensal strains used in the study. The table lists the four commensal bovine isolates used in the study (strain ID or cow number), source of isolates (source), species (spp), number of spa types (spa), spa repeat sequence (cassettes) and different enterotoxin gene profile.

S. No.	Strain ID	Source	spp	spa	Cassettes	Enterotoxin genes
1	4257	Teat	aureus	529	zb	-
2	4277	Nose	aureus	267	ujgfmbbbpb	-
3	4396	Hock	aureus	237	ubbbp	-
4	4483	Nose	aureus	237	ubbbp	-

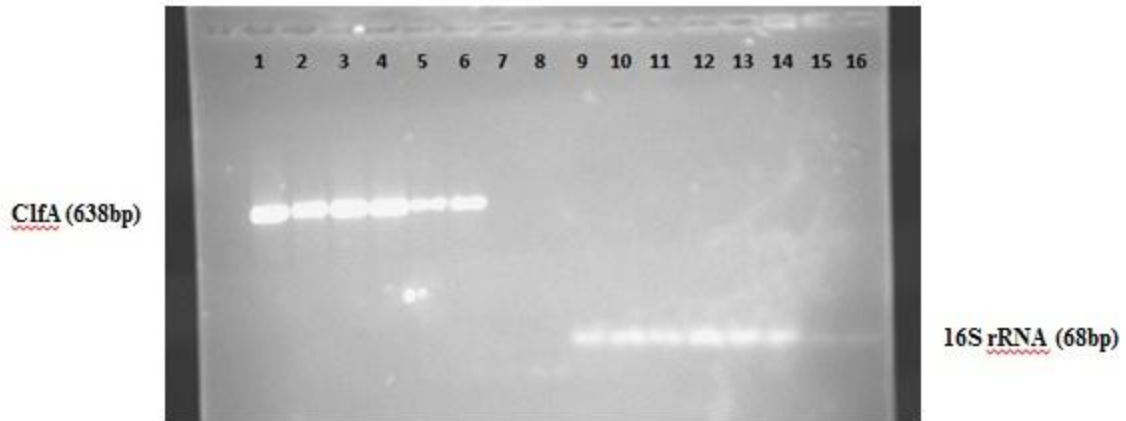


Figure 3-1: Confirmation of *S. aureus* strains. PCR products of ClfA (638 bp) and 16S rRNA (68 bp) gene were run on a 2% agarose gel. Lane 1 to 5 and 9 to 13 represents the amplified products from five different commensal strains; lane 6 and 14 from ATCC 27217 (positive control); lane 7 and 15 from *Streptococcal* species (negative control); lane 8 and 16 from water (technical control).

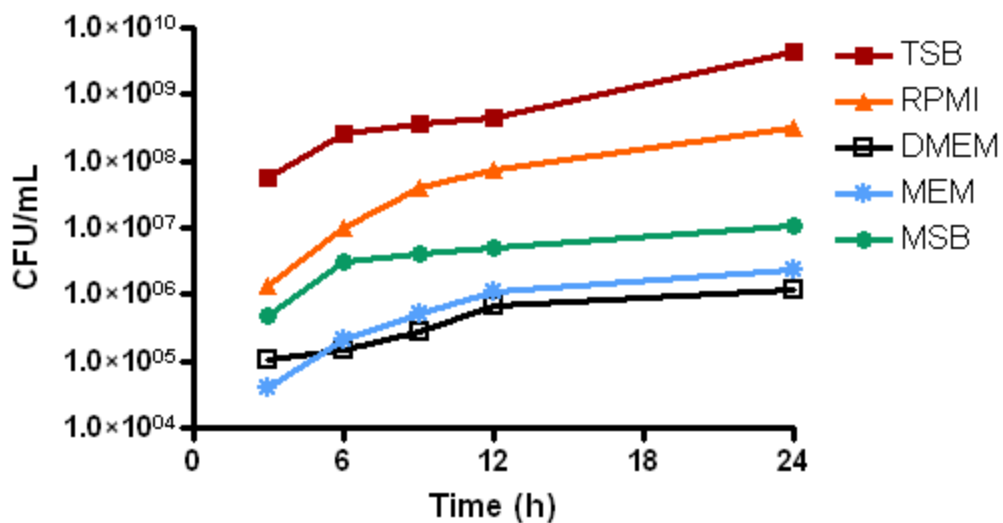


Figure 3-2: Comparison of bacterial growth in minimal media. The growth of a *S. aureus* pathogenic strain (#4065) was compared in TSB, RPMI 1640, DMEM, MEM and MSB over a time period of 24 hrs.

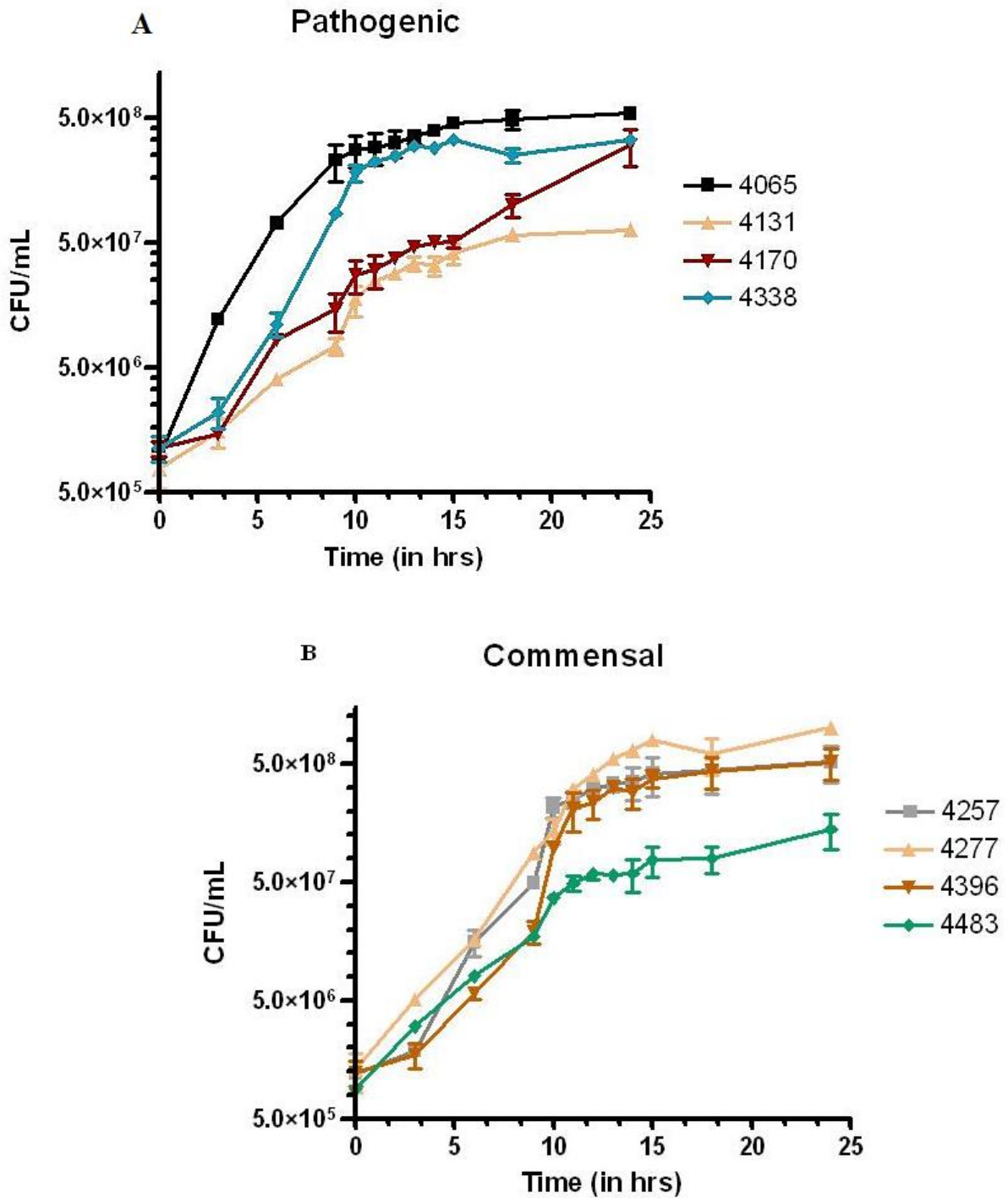


Figure 3-3: Growth curves of pathogenic and commensal *S. aureus* strains. The growth curves of pathogenic (A) and commensal (B) strains of *S. aureus* grown in RPMI 1640 over a time period of 24 hrs. Fifty mL of RPMI was inoculated with 10^6 CFU/mL at 0hr. Standard error is indicated (n=2).

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Chapter 4. Identification of proteins differentially expressed between pathogenic and commensal *Staphylococcus aureus* strains

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ABSTRACT

With the advent of advanced mass spectrometry based techniques, proteomics can now be used to comprehensively analyze and compare different proteomes providing quantitative protein information. Stable isotope labeling by amino acids in cell culture (SILAC) is a simple and robust technique to label the cellular proteome in metabolically active cells by incorporating stable isotope containing amino acids in the newly synthesized proteins. In this study, we compared the differential proteome expression of pathogenic and commensal strains of *Staphylococcus aureus* using SILAC. Four isolates each of commensal and pathogenic strain types were grown in a customized minimal media, RPMI 1640 containing light (^{12}C) or heavy (^{13}C) lysine, respectively. The cell wall, membrane, and secreted proteins were extracted from the strains and analyzed using mass spectrometry (MS) analysis of tryptic peptides. Based on the relative intensity peaks of each isotopic peptide determined by MS, the relative abundance of the proteins in the pathogenic and commensal strains were quantified. The study aimed at characterizing proteins that are involved in the pathogenesis of *S. aureus* and screening them with respect to commensal strains as background. In total, 58 proteins were found to be upregulated in pathogenic strains and 93 proteins in the commensal strains during the early stationary growth phase. The dataset provides insight into disease progression by the pathogenic strains, and can aid the development of a vaccine against *S. aureus* infections.

INTRODUCTION

The commensal strains of *S. aureus* reside on the skin or mucosal surface of the host, without stimulating a strong immune response, whereas pathogenic strains are responsible for causing disease or infection in the host. The differential genomic expression between the commensal and pathogenic bacterial strains have been illustrated before as an evidence for reduced pathogenicity of commensals [1]. The genomic comparison of the nasal carriage and pathogenic strains of *S. aureus* using microarray failed to identify specific genes associated with invasiveness [2]. Nevertheless, the discrepancy in correlations between RNA and protein abundance levels is a major drawback of microarray technique, hence, the need for more specialized proteomic techniques to study the differential expression between two cell types.

MS-based approaches allow the determination of relative protein expression levels within a cellular system under different conditions, or even between two cells or tissue states [3]. This quantitative expression analysis is attained by introducing isotope labels into the proteins and examining the MS signal for each peptide. These approaches involve either chemical labeling of the peptides or biological incorporation of isotopic labels into living cells [4]. Stable isotope labeling by amino acids in cell culture (SILAC) is a metabolic labeling strategy which involves incorporation of stable isotopes into proteins by introduction of labeled amino acids in the growth medium. The two or more cell systems being studied are grown in mediums that contain either 'light' (normal) amino acid, or 'heavy' (isotope labeled) amino acid. The complete incorporation of stable isotopes can be ensured by using cells that are auxotrophic for the amino acid being used to label.

SILAC has found its application in identification of plasma membrane proteins that are differentially expressed in a MARCH9-expressing B-cell line [5]. Studies have proved that SILAC can be adapted to not only mammalian cell cultures, but also bacteria [6], yeast [7] and plants [8]. A study employing SILAC labeling in *Bacillus subtilis* lead to quantitation of more than 1500 proteins under two different physiological conditions – growth on succinate and under phosphate deprivation [6]. Differential quantitative profiling of proteomes of normal versus cancerous human kidney tissues using SILAC has revealed potential biomarkers for tumors [9].

In this study, we sought to identify the proteins that are differentially expressed among the pathogenic and commensal strains *Staphylococcus aureus* during the early stationary growth phase, using SILAC. The identified proteins would help illustrate their roles in disease progression by the pathogenic strains, or immune dampening by the commensals, and therefore can be targeted as potential vaccine candidates.

MATERIALS AND METHODS

SILAC “light” and “heavy” media. RPMI 1640 (without L-glutamine) deficient in arginine and lysine was custom synthesized from Athena ES, Inc., MD, USA. SILAC amino acid kit, containing light arginine, light and heavy forms of lysine, was purchased from Invitrogen (Life technologies, Carlsbad, CA, USA). Concentrated stock solutions of the amino acids were prepared in PBS and stored at -20°C until use. The ‘light’ and ‘heavy’ SILAC media were prepared using light (¹²C) lysine and heavy (¹³C) lysine, respectively. The lysine and arginine were supplemented to the medium in accordance with the concentrations in RPMI 1640 (Invitrogen, USA), i.e. 40 mg/L and 200 mg/L respectively. The media was filtered sterilized using a 0.22 µm vacuum filter flasks (Nalgene, Rochester, NY, USA).

Culturing *S. aureus* strains. The commensal and pathogenic strains were cultured in 5 mL of TSB by inoculating single colonies from a routinely streaked EBA plate and growing at 37°C for 5 hours, shaking at 190 rpm using 12400 Incubator Shaker (New Brunswick Scientific, USA). The CFU counts of the culture were determined by serial dilutions and drop plating the dilutions on TSA plates. The strains were sub-cultured by inoculating 10⁶ CFU/ mL into 100 mL of SILAC media. The pathogenic strains were cultured in ‘heavy’ SILAC media, and the commensal strains in ‘light’ SILAC media. The bacterial populations were grown in their respective SILAC media at 37°C under gentle shaking (190 rpm) until they reached the early stationary phase (OD₆₀₀=1.0). The above mentioned experiments were performed in biological replicates for all the strains.

Protein extraction. The bacterial cell cultures collected at post-exponential phase were centrifuged at 12,500 x g for 15 mins at 4°C to pellet the cells. The supernatant was used for extracting the exoproteins using the trichloroacetic acid (TCA) – acetone precipitation protocol [10]. The cell pellets were resuspended in 1 mL lysis buffer, containing lysostaphin (25 U) (Sigma, MO, USA), protease inhibitor cocktail (Thermo Scientific, Rockford, IL, USA) and 30% raffinose (Acros, NJ, USA), and incubated at 37°C without shaking for 90 mins. The cell lysates were centrifuged at 7,500 x g for 20 mins at 4°C to obtain cell wall proteins fractions in the supernatant. The pellet was resuspended in 500 µL lysis buffer and homogenized. The disrupted cells were centrifuged at 100,000 x g for 60 min, and the resulting supernatant containing cytoplasmic proteins was isolated. The pellet containing membrane fraction was resuspended in 500 µL lysis buffer. The protein concentrations were determined by Bradford assay using bovine serum albumin (BSA) (Calbiochem, Gibbstown, NJ, USA).

In solution digestion and sample fractionation. Portions of two protein fractions were combined so that each digest contained a 1:1 (w/w) ratio of protein from a heavy sample to protein from a light sample based on protein assay results. Proteins were precipitated by adding 4X methanol (LC/MS grade, Spectrum Chemical, Gardena, CA, USA) and incubating at -20°C for 30 mins. Precipitated protein was collected by centrifugation in 13,000 x g for 20 mins at RT. The supernatant was discarded and the protein pellet was resuspended in freshly prepared 8 M urea (Sigma, MO, USA) in 20 mM Tris-HCl, pH 8, at a protein concentration of 1 mg/ml. Samples were then brought to a final concentration of 4.5 mM using a freshly prepared stock solution of 45 mM dithiothreitol (DTT) (Sigma, MO, USA) in 20 mM Tris-HCl, pH8, and incubated for 1 hr at 37°C. Samples were then

brought to a final concentration of 10 mM iodoacetamide (Sigma, MO, USA) using a freshly prepared stock solution of 100 mM iodoacetamide in 20 mM Tris-HCl, pH8, and incubated for 30 minutes at RT in the dark. The remaining iodoacetamide was then inactivated by adding the molar equivalent of DTT and samples were diluted to 1.4 M urea using 20 mM Tris-HCl, pH 8. Trypsin (Sigma, MO, USA) was then added to each sample at a 1:50 (w/w) ratio to total protein and digests were incubated overnight at 37°C.

Following digestion, samples were brought to 0.1% trifluoroacetic acid (TFA) (Sigma, MO, USA) using a 5% TFA stock and then, if needed, the pH was adjusted to 3 or less using formic acid. Samples were then desalted utilizing either 1 ml solid phase extraction cartridges, Strata-X 33 μm polymeric reversed phase (Phenomenex, Torrence, CA, USA) or 100 μl reversed phase C18 OMIX tips (Varian, Lake Forest, CA, USA) depending on the digestion volume. After elution from the solid phase extraction material, samples were dried using a vacuum concentrator (CentriVap concentrator, Labconco, Kansas City, MO, USA) and then resuspended in 30 μl 98:2 water:acetonitrile (Spectrum Chemical, Gardena, CA, USA), supplemented with 0.1% TFA, by sonication in a water bath for 20 minutes.

A portion of each digest (10-30 μg of tryptic peptides), was loaded onto a reversed phase trap cartridge 150 micrometers ID x 10 millimeters Reprosil C18-AQ (SGE Analytical Science, Austin, Texas) and flushed with 300 μl 98:2 water:acetonitrile, supplemented with 0.1% TFA, at 15 $\mu\text{l}/\text{min}$ using an Eksigent (Dublin, CA) nanoLC-AS-2 autosampler and nanoLC-2D HPLC. The trap cartridge was then switched in-line with a 100x0.1 mm analytical column packed in-house using Magic C18AQ 100 \AA 5 μm

(Michrom Bioresources, Auburn, CA, USA). The analytical column had been equilibrated using 95:5 solvent A:solvent B delivered at 1 μ l/min. Solvent A was water supplemented with 0.1% TFA and solvent B was acetonitrile. Note that all water and acetonitrile utilized post-digestion was LC-MS grade. The elution gradient consisted of a 5 minute hold at 95% A followed by a 10 minute linear change to 84% A then a 90 minute linear change to 66% A and a 5 minute linear change to 30% A followed by a 20 minute hold at 30% A. Alternatively, for some samples, the elution gradient consisted of a 6 minute linear change to 82% A followed by a 60 minute linear change to 62% A. Column elutes were spotted onto a 384 Opti-TOF stainless steel MALDI target plate (ABSciex, Foster City, CA, USA) at 20 seconds per spot using an Eksigent Ekspot.

Mass spectrometry analysis. Spots were overlaid with 1 μ l freshly prepared matrix, 4 mg/ml α -cyano-4-hydroxycinnamic acid (CHCA) (Sigma, MO, USA) in 1:1 water:acetonitrile supplemented with 0.1% TFA, 0.5% formic acid and 10 mM ammonium chloride. A 4800 MALDI TOF/TOF analyzer (ABSciex, Foster City, CA, USA) was then used to obtain MS and MS/MS data, utilized for protein identification and quantitation. First, an MS spectrum for the m/z range of 800 to 4000 was collected utilizing reflector positive operating mode. Each spectrum was the sum of 1000 laser shots. An interpretation method was then utilized to build an MS/MS job wherein the top 10 peaks per spot with a signal to noise above 50 were chosen and MS/MS for each peak was performed only on the spot at which it reached its maximum intensity. Each MS/MS spectrum utilized the MS-MS 1 kV positive operating mode and was the sum of approximately 3000 laser shots.

Protein identification and quantitation. Protein identification and quantitation was performed using Protein Pilot v. 4.0.8085 and the Paragon algorithm 4.0.0.0 (ABSciex, Foster City, CA, USA). The Paragon algorithm requires no input of mass tolerance, and is determined based on the mass accuracy of the MS instrument used. Parameters used for identification and quantitation were as follows: sample type, SILAC (Lys+6); cysteine alkylation, iodoacetamide; digestion, trypsin; instrument, 4800; special factors, urea denaturation and gel-based ID (due to the increased possibility of oxidation of amino acid residues for extracellular proteins); a thorough ID search effort with quantitation and bias correction and an ID focus including biological modifications and amino acid substitutions. Peptide searches were performed against the whole genome sequence of Newman strain of *S. aureus* in UniProt knowledgebase (UniProtKB) database, downloaded to the local search engine. To compensate for some issues with calibration across the entire MALDI plate, MS tolerance was increased to 0.5 with a standard deviation of 0.09 and MSMS tolerance was increased to 0.7 with a standard deviation of 0.12. The ProteinPilot.exe.config file was also modified to ensure only peptides with a 95% or greater confidence level were used for quantitation. Protein abundance ratio was calculated from the average of ratios of individual peptides that had a confidence value above the 95% threshold. Only the proteins having at least two unique peptides identified were used for relative quantitation. Most of the reported protein ratios have p-value (statistical evaluation of the difference between observed ratio and unity) and EF (Error factor). However, the Protein Pilot™ software manual indicates that p-value may not be suited for every experiment to rely in terms of statistical significance. Protein Pilot™ allows normalization of ratios using the feature “bias correction” which fixes the

systemic errors by correcting the average median protein ratio to unity and applying the correction factor to rest of the ratios.

RESULTS

Sample processing and MS analysis. The four isolates each of the commensal and pathogenic *S. aureus* strains were grown in the minimal SILAC media, RPMI 1640, containing light (^{12}C) or heavy (^{13}C) lysine, respectively, in biological duplicates. The cells were harvested at the early stationary growth phase for each strain, where the expression of surface and secreted proteins is highest. The extraction of cell wall proteins was carried out using lysostaphin, an endopeptidase that cleaves pentaglycine bridges of peptidoglycan [11]. The membrane proteins were pelleted down using high-speed ultracentrifugation, while the exoproteins were precipitated using TCA-acetone protocol [12]. The protein concentrations were quantified using Bradford assay (BSA). There was slight disparity in the consistency of protein concentrations among the duplicate experiment (Table 4-1). This could also be attributed to the technical issues due to occurrence of *S. aureus* in clusters, leading to differences in CFU count and ultimately in protein yield. Also, the concentration of each protein type varied widely among the strains, suggesting differential protein abundance or production.

The strains were grouped together as SILAC pairs according to their growth rate, and their respective protein samples were mixed together to compare the protein expression among the paired strains. The heavy labeled protein samples were mixed with the light ones (according to the SILAC pairs) in a 1:1 ratio (by weight) based on protein concentrations. The mixed proteins (total 20 μg) were then digested with trypsin, a serine protease that hydrolyses proteins by cleaving at the carboxyl end of the lysine and arginine residues. The peptides were fractionated by gradient reverse phase chromatography and spotted directly onto MALDI plates. The resulting fractions were

subsequently analyzed using MALDI-TOF/TOF analyzer to obtain MS and MS/MS data, as MALDI provides better resolution. The incorporation of each heavy lysine residue in the protein brings about a mass shift of 6 Da, which can be detected by MS analysis. The relative intensities from the light and heavy samples provide a quantitative comparison of the abundance or expression levels of the protein in the mixed samples.

Identification and relative quantification of differentially expressed proteins. The ‘Search Effort’ through ‘Thorough ID’ provides extensive protein identification search, considering various modifications and cleavages. The peptides were searched against *S. aureus* Newman strain proteome at UniProtKB database for protein identification. The software also enlists parameters such as Unused (ProtScore), Peptides (95%) and Cov (95%), which contribute to the confidence in identification of protein. “Unused” is a measure of confidence in protein identification based on the confidence of peptides which were not completely used by high-scoring proteins. “Peptides (95%)” refers to the number of unique peptides with a 95% confidence level. Whereas, “Cov (95%)” depicts the percentage of matching amino acids of peptides identified with 95% or more confidence, to that of the total number of amino acids in the sequence. The EF implies that the actual ratio value lies between (reported ratio)/(EF) and (reported ratio) x (EF) with a 95% confidence.

The protein abundance ratios were calculated by the software as an average of the ratios of peptides used for quantitation. Therefore, among the stringently identified proteins, the following thresholds were applied for selecting proteins that would be considered to be differentially expressed among commensal and pathogenic strains: 1) at least two unique lysine containing peptides, identified with > 95%

confidence, are used for quantitation, and 2) the ratio must be above 1.5 (for upregulation) and 0.7 (for downregulation). Using this criterion, a list of proteins with an abundance ratio was generated (Table 4-2). Applying such stringent thresholds maintained the protein false discovery rate (FDR), to essentially less than 1%. FDR is estimated based on the number of hits from reversed (decoy) database. Moreover, normalizing the data improves the statistical analysis by removing the bias that was introduced by error in sample loading or quantitative measurements [13-15]. The normalized abundance ratios were used for further analysis of differential expression.

Further, the classification of proteins as differentially regulated resulted in identification of a total of 58 differentially upregulated proteins (Tables 4-3, 4-4, 4-5) and 93 downregulated proteins (Appendix table A-1, A-2, A-3) in pathogenic as compared to commensal strains. Applying such stringent thresholds ensured that only those specific proteins which are indeed being differentially expressed are identified. For example, out of a total of 641 cell wall proteins identified, only 12 were classified as differentially expressed in pathogenic strains (Table 4-2). The proteins identified were classified into different categories based on their functions, such as ability to modulate host immune response, stress-related, or enzymes involved in metabolism (Figure 4-1). The distribution of functions illustrated increased activity in pathogenic strains in terms of proteins involved in iron-binding or uptake, and transport of molecules but relatively less ribosomal proteins and metabolic enzymes.

DISCUSSION

Differential expression profiling to quantify changes in gene expression levels is carried out using microarrays. However, the discrepancies in the correlation between RNA levels and protein measurements render this approach inefficient, and opens avenue for suitable proteomic approaches. Quantitative proteomics, employing stable isotope labeling and high-throughput mass spectrometry technology, has gained popularity due to its ease of implementation, high accuracy and adaptability to a variety of biological systems [5, 16-18].

In this study, we sought to determine the proteins that are differentially expressed between commensal and pathogenic strains of *S. aureus*, which can serve as a potential vaccine candidate, using SILAC. *S. aureus* strains often depend on certain amino acids supplied in the growth medium, in spite of the ability to produce enzymes required to synthesize the amino acids. In our study, we tested labeling the *S. aureus* strains using ^{13}C labeled lysine and found the cells incorporating the isotope, suggesting their dependence on growth media for lysine. The rapid cell division and protein synthesis during the growth allows complete incorporation of the isotopic amino acids into the bacterial proteome.

A total of 58 proteins were identified with significant upregulation in pathogenic, of which 27 were exoproteins (Table 4-1), 19 cell wall (Table 4-2) and 12 membrane proteins (Table 4-3). These proteins could be classified into different categories based on their functions, i.e., iron regulation or uptake, metabolic enzymes, ribosomal, stress-related or immunomodulatory proteins (Figure 4-1). The functional distributions of differentially regulated proteins suggest increased iron uptake and

transport activity in the pathogenic strains, while the commensal strains significantly express more proteins that are involved in translation and metabolism. Previous studies have demonstrated the role of these iron-binding proteins as important virulence factors, and explain the increase observed in pathogenic strains [19].

S. aureus secretome includes an arsenal of proteins such as enzymes to degrade host cells for nutrition, or toxins that impair host immune response. Altogether the secreted proteins promote invasiveness, cytotoxicity, and pathogenicity of this pathogen. In our study, 27 exoproteins were identified to be significantly expressed in the pathogenic strains (Table 4-1). Among them, the virulence relevant proteins include IgG-binding protein sbi & protein A, MHC class II analog protein (MAP), iron-regulated surface determinant proteins -IsdA, IsdB, IsdE, and a less evident protein, N-acetylmuramoyl-L-alanine amidase domain protein (AM). The excreted IgG-binding protein sbi, not only binds to Fc portion of the IgG like its counterpart protein A, but also interacts with C3 complement protein to prevent the attachment of iC3b-opsonized bacteria to PMN or macrophages [20, 21]. A greater than 3-fold expression of protein sbi was evident in pathogenic strains as compared to the commensal. AM is a peptidoglycan hydrolase with catalytic activity secreted by *S. aureus* [22]. It is known to be involved in bacterial autolysis, cell wall turnover, and cell division [23]. It can be speculated that targeting this protein in a vaccine would arrest the bacterial growth and prevent spread of infection in host. This protein has been reported to be excreted extracellularly by bovine *S. aureus* isolates with clinical and subclinical relevance [24].

Isd system encodes cell-wall anchored proteins that are responsible for iron acquisition by binding to heme proteins from the host. IsdA protein provides

additional support in terms of adherence and colonization of *S. aureus* by binding to extracellular matrix components, such as fibrinogen and fibronectin [25]. IsdB, due to its role in heme uptake, is important for the growth and ultimately the spread of infection [26]. These proteins are expressed during iron-deprivation, and hence are produced in iron depleted growth medium, RPMI 1640. Interestingly, we found different Isd proteins to be present in the cell wall, membrane as well as secreted protein fractions of pathogenic strains. It could be reasoned that possible cell death or autolysis during the early stationary phase lead to the release of these surface-associated components into the growth medium. Glutamine synthase (GS) was another protein found to be upregulated in all the protein types, with specifically around 6.5 fold upregulation in the cell wall. This significant expression in cell wall can be explained by its requirement in the production of glutamine, which acts as an ammonium (NH_4^+) source during the synthesis of peptidoglycan [27]. It can also be justified by the need to synthesize glutamate, due to its absence in the minimal growth media used.

Among the proteins downregulated in pathogenic strains, most of them could be classified as metabolic enzymes. Elongation factor Tu was shown to be highly downregulated, and also present in all the protein fractions. Interestingly, certain proteins, such as IsdA, IsdB, were found to be significantly expressed in some pathogenic strains, while being downregulated in others. This discrepancy could be attributed to either the distinct genetic profiles of the strains or technical errors in the experimentation.

The proteins that are downregulated in the pathogenic strains, or in other words, highly expressed in commensal strains suggest their role in immune dampening response produced by the latter. Whereas, the proteins significantly upregulated in the

pathogenic strains when compared to commensals, could be the reason for pathogenicity of the former. Such proteins can be targeted as putative vaccine candidates against *S. aureus*. Further *in vivo* studies would confirm the role of these proteins in pathogenicity of *S. aureus*.

TABLES

Table 4-1. CFU counts and protein concentrations for *S. aureus* strains.

The table lists the CFU concentrations of each strain harvested at the early stationary phase for SILAC 1 (A) and 2 (B) (duplicate experiments), and also the protein concentrations of each fraction extracted.

A SILAC 1

			Protein Conc (mg/mL)		
	Strain	CFU/mL	Cell wall	Membrane	Exo proteins
Commensal strains	4257	3.60E+08	0.417	0.098	0.225
	4277	5.00E+08	0.514	0.288	0.411
	4396	1.50E+08	0.387	0.243	0.152
	4483	3.36E+08	0.536	0.255	0.292
Pathogenic strains	4065	4.50E+08	0.243	0.430	0.325
	4131	3.30E+08	0.346	0.365	0.168
	4170	2.80E+08	0.394	0.169	0.012
	4338	2.40E+08	0.403	0.288	0.213

B SILAC 2

			Protein Conc (mg/mL)		
	Strain	CFU/mL	Cell wall	Membrane	Exo proteins
Commensal strains	4257	4.00E+08	0.532	0.263	0.111
	4277	3.96E+08	0.397	1.260	0.128
	4396	2.10E+08	0.391	0.275	0.145
	4483	2.00E+08	0.212	0.458	0.175
Pathogenic strains	4065	5.20E+08	0.528	0.449	0.359
	4131	2.00E+08	0.306	1.374	0.170
	4170	3.50E+08	0.847	0.350	0.077
	4338	2.76E+08	0.417	0.774	0.212

Table 4-2. Total peptides and proteins quantified.

The table lists the total number of distinct peptide with 95% confidence, proteins and differentially expressed proteins for cell wall, membrane and exoproteins fractions from SILAC 1 and 2 (duplicate experiments).

Cell wall proteins						
SILAC Pair	Peptides (95%)		Total Proteins		Diff. proteins	
	SILAC 1	SILAC 2	SILAC 1	SILAC 2	SILAC 1	SILAC 2
4131-4483	641	433	179	85	12	6
4170-4396	668	459	141	92	10	8
4338-4257	193	423	64	82	2	3

Membrane proteins						
SILAC Pair	Peptides (95%)		Total Proteins		Diff. proteins	
	SILAC 1	SILAC 2	SILAC 1	SILAC 2	SILAC 1	SILAC 2
4131-4483	1222	110	258	33	5	1
4170-4396	345	326	93	83	6	1
4065-4277	262	339	82	77	Nil	1

Exo proteins						
SILAC Pair	Peptides (95%)		Total Proteins		Diff. proteins	
	SILAC 1	SILAC 2	SILAC 1	SILAC 2	SILAC 1	SILAC 2
4131-4483	1096	314	202	37	13	6
4338-4257	341	305	59	74	6	7
4065-4277	675	327	146	37	4	1

Table 4-3. Differentially upregulated exoproteins in pathogenic *S. aureus* strains.
The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID ^a	Protein	Remarks ^b	SILAC expt ^c	SILAC Pair ^d	Peptides (95%) ^f	H:L ^e	EF H:L _g	Unused ^h	%Cov (95) ⁱ
1	A6QKD3	N-acetylmuramoyl-L-alanine amidase domain protein	Catalytic domain of autolysin (PGN hydrolase)	1	4131-4483	11	3.13	> 2	21.16	27.95
				2	4131-4483	8	3.87	< 2	13.73	17.93
					4338-4257	7	3.05	> 2	12.06	12.28
2	A6QJQ7	Immunoglobulin-binding protein sbi	Bind IgG Fc fragment & complement; blood coagulation	1	4131-4483	8	3.11	< 2	10.08	18.35
				2	4131-4483	5	3.18	< 2	7.01	13.76
3	A6QG31	Iron-regulated surface determinant protein A	Iron acquisition by degrading heme	1	4338-4257	19	2.21	< 2	22.06	43.14
				2	4338-4257	7	2.50	> 2	10	16.00
4	A6QG30	Iron-regulated surface determinant protein B	Iron acquisition by degrading heme	1	4338-4257	18	2.71	> 2	28.23	27.6
				2	4065-4277	10	1.88	< 2	14.01	14.42
5	A6QDV2	Truncated triacylglycerol lipase	Catalysis of the reaction: triacylglycerol + H ₂ O = diacylglycerol + a fatty acid anion	1	4131-4483	23	1.57	< 2	31.1	27.33
				2	4131-4483	2	2.47	> 2	1.69	7.764
6	A6QFA0	Thermonuclease	Heat-stable endonuclease	1	4338-4257	10	1.69	> 2	15.09	31.58
				2	4131-4483	17	3.70	< 2	23.76	50
7	A6QES5	Putative uncharacterized protein	Unknown	1	4131-4483	9	3.83	< 2	10.24	47.62
				2	4131-4483	3	6.01	> 2	5.4	18.45
8	A6QG34	High-affinity heme uptake system protein isdE	Bind heme; iron acquisition	1	4131-4483	5	4.13	> 2	7.62	17.81
					4338-4257	3	3.15	> 2	6.04	10.96

S. No	Accession ID ^a	Protein	Remarks ^b	SILAC expt ^c	SILAC Pair ^d	Peptides (95%) ^f	H:L ^e	EF H:L _g	Unused ^h	%Cov (95) ⁱ
9	A6QF17	Putative lipoprotein	Lipoprotein	1	4131-4483	3	3.55	> 2	4	14.38
					4338-4257	3	2.87	> 2	5.14	20.55
10	A6QDB7	Superoxide dismutase	Stress resistance; starvation survival;	1	4065-4277	5	2.27	< 2	5.33	33.17
11	A6QGA ₂	Dihydroorotase	Zinc binding enzyme; synthesis uridine monophosphate	1	4065-4277	2	2.17	> 2	3.62	11.32
12	A6QGK ₇	Glutamine synthetase	Enzyme; condenses glutamate and ammonia	1	4065-4277	10	1.91	< 2	16.78	30.6
13	A6QIV7	Serine hydroxymethyltransferase	Catalyze conversion of serine and tetrahydrofolate into glycine and methylenetetrahydrofolate, respectively	1	4065-4277	2	1.61	> 2	2.49	7.767
14	A6QI23	Foldase protein prsA	Folding of secreted proteins	1	4131-4483	2	3.09	> 2	3.17	6.25
15	A6QF27	Lipoteichoic acid synthase	Synthesis of polyglycerophosphate of LTA	1	4131-4483	12	2.67	< 2	19.44	26.32
16	A6QFJ1	Truncated MHC class II analog protein	Map-secreted; binds ECM components; host immune modulation	1	4131-4483	2	2.38	> 2	2.07	14.58
17	A6QD95	Immunoglobulin G binding protein A (Protein A)	Binds Fc fragment of IgG	1	4131-4483	15	1.78	< 2	21.96	34.62

S. No	Accession ID ^a	Protein	Remarks ^b	SILAC expt ^c	SILAC Pair ^d	Peptides (95%) ^f	H:L ^e	EF H:L _g	Unused ^h	%Cov (95) ⁱ
18	A6QEF4	50S ribosomal protein L25	50S ribosomal subunit; Binds to the 5S rRNA	1	4131-4483	3	1.78	> 2	6.02	17.97
19	A6QEU1	Iron-repressed lipoprotein	Heavy metal ion transport	1	4131-4483	8	1.73	< 2	12	29.13
20	A6QJC5	Iron compound ABC transporter, iron compound-binding protein	ABC transport of ferric siderophores and metal ions	1	4131-4483	3	1.67	> 2	6	10.6
21	A6QG33	Iron-transporting membrane protein	Iron acquisition & transport	1	4338-4257	2	2.23	> 2	4	6.704
22	A6QK59	Probable transglycosylase isaA	Cleave peptidoglycan	2	4131-4483	18	1.82	< 2	8	20.17
23	A6QE62	Alkyl hydroperoxide reductase subunit C	Protects cell against DNA damage by alkyl hydroperoxides	2	4338-4257	6	3.84	> 2	10	42.86
24	A6QK93	Fructose-bisphosphate aldolase class 1	Enzyme in glycolysis	2	4338-4257	3	3.48	> 2	6.06	13.85
25	A6QEK0	Elongation factor Tu	Promote binding of aminoacyl-tRNA to the A-site of ribosomes during translation	2	4338-4257	16	3.38	> 2	26.13	39.34
26	A6QFV0	Pyruvate dehydrogenase E1 component, beta subunit	Acyl-transferring activity	2	4338-4257	3	1.94	> 2	6	14.15

S. No	Accession ID ^a	Protein	Remarks ^b	SILAC expt ^c	SILAC Pair ^d	Peptides (95%) ^f	H:L ^e	EF H:L ^g	Unused ^h	%Cov (95) ⁱ
27	A6QF81	Glyceraldehyde 3-phosphate dehydrogenase 1	Oxidoreductase enzyme in glycolysis	2	4338-4257	8	1.64	> 2	14	21.43

^a Uniprot accession ID for protein

^b Functional annotation of the protein

^c Represents SILAC replicate experiments (1 or 2)

^d Indicates the pathogenic-commensal strain SILAC pair as the protein source

^e Normalized relative abundance ratio (> 1.5 for upregulation)

^f The number of unique peptides identified with 95% confidence

^g Error factor calculated by Protein Pilot™ software is a measure of the error in calculating the average ratio

^h Unused (or ProtScore) refers to the measure of protein confidence based on the peptides “not already used” for identification

ⁱ Percentage of matching amino acids from peptides identified with more than 95% confidence, to that of the total amino acids in the protein sequence

Table 4-4. Differentially upregulated cell wall proteins in pathogenic *S. aureus* strains.

The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
1	A6QG31	Iron-regulated surface determinant protein A	Iron acquisition by degrading heme	1	4170-4396	5	7.73	> 2	6.750	6.750
					4338-4257	7	17.5	< 2	10.480	19.430
				2	4170-4396	11	10.7	< 2	14.800	36.000
					4338-4257	13	6.22	> 2	15.640	38.290
2	A6QG30	Iron-regulated surface determinant protein B	Iron acquisition by degrading heme	1	4170-4396	6	14.2	> 2	8.030	9.612
					4338-4257	8	31.7	< 2	10.000	11.320
				2	4170-4396	9	20.3	< 2	15.440	17.360
					4338-4257	9	7.59	> 2	9.950	11.940
3	A6QGK7	Glutamine synthetase	enzyme; condenses glutamate and ammonia	1	4170-4396	24	7.60	< 2	26.270	33.920
				2	4170-4396	17	5.42	< 2	22.060	28.820
4	A6QD95	Immunoglobulin G binding protein A (Protein A)	Binds Fc fragment of IgG	1	4131-4483	12	2.43	< 2	16.790	32.500
				2	4170-4396	5	2.03	< 2	7.380	15.000
5	A6QJ26	Alkaline shock protein 23	Alkaline pH tolerance	1	4131-4483	4	2.05	< 2	7.280	28.400
					4170-4396	3	2.21	> 2	6.000	6.000
				2	4131-4483	4	2.79	< 2	6.000	24.260
					4170-4396	7	2.81	< 2	8.110	31.360
6	A6QK93	Fructose-bisphosphate aldolase class 1	Enzyme in glycolysis	1	4170-4396	11	2.23	< 2	16.350	40.880
				2	4170-4396	9	2.40	< 2	14.940	28.040
7	A6QI27	UPF0342 protein NWMN_1737	Uncharacterized protein family	1	4170-4396	4	1.85	< 2	5.700	45.610
				2	4170-4396	5	1.69	< 2	8.380	60.530

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
8	A6QEJ2	50S ribosomal protein L7/L12	Binding site for factors in translation	1	4131-4483	2	3.49	> 2	4.000	23.770
9	A6QI36	Glucosamin e-6-phosphate isomerase	Isomerase enzyme	1	4131-4483	3	1.86	> 2	6.000	24.120
10	A6QE61	Alkyl hydroperoxide reductase subunit F	Protects cell against DNA damage by alkyl hydroperoxides	1	4170-4396	9	5.67	> 2	15.120	29.980
11	A6QE62	Alkyl hydroperoxide reductase subunit C	Protects cell against DNA damage by alkyl hydroperoxides	1	4170-4396	15	4.69	> 2	12.000	42.860
12	A6QFT1	Bifunctional purine biosynthesis protein PurH	Purine metabolism, synthesis	1	4170-4396	7	2.63	> 2	12.210	18.090
13	A6QGP4	Transketolase	Transferase enzyme in Calvin cycle	1	4170-4396	4	1.54	> 2	6.820	9.063
14	A6QGX3	Phosphotransferase system glucose-specific IIA component	Phosphoenolpyruvate-dependent sugar phosphotransferase system	2	4170-4396	2	2.21	> 2	2.000	6.627
15	A6QHF6	UPF0473 protein NWMN_1516	Uncharacterized protein family	2	4338-4257	2	2.23	> 2	4.000	10.780
16	A6QFC3	CsbD-like superfamily protein	Heat shock protein	2	4131-4483	4	6.64	> 2	4.410	39.060
17	A6QF81	Glyceraldehyde 3-phosphate dehydrogenase 1	Oxidoreductase enzyme in glycolysis	2	4131-4483	7	2.46	< 2	13.400	23.810

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
18	A6QG47	Thioredoxin	Protects against oxidative stress	2	4131-4483	2	1.79	> 2	4.000	24.040
19	A6QD54	50S ribosomal protein L9	Translational protein	2	4131-4483	2	1.62	> 2	4.000	14.860

Table 4-5. Differentially upregulated cell membrane proteins in pathogenic *S. aureus* strains.

The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
1	A6QJ26	Alkaline shock protein 23	alkaline pH tolerance	1	4131-4483	15	1.72	< 2	15.33	34.32
					4170-4396	3	2.76	> 2	6	13.61
				2	4131-4483	2	1.51	> 2	4.0	8.3
2	A6QI27	UPF0342 protein NWMN_1737	Uncharacterized protein family	1	4131-4483	7	1.67	< 2	9.11	34.21
3	A6QGV3	Major cold-shock protein CspA	Response to stress induced by cold shock	1	4131-4483	3	1.60	> 2	5.2	50
4	A6QGF7	Elongation factor Ts	Promote binding of aminoacyl-tRNA to the A-site of ribosomes during translation	1	4131-4483	5	1.59	> 2	9.14	18.43
5	A6QEK6	Branched-chain-amino-acid aminotransferase	Amino acid biosynthesis	1	4131-4483	4	1.50	> 2	8.06	14.53
6	A6QJC5	Iron compound ABC transporter, iron compound-binding protein	ABC transport of ferric siderophores and metal ions	1	4170-4396	4	3.25	> 2	6.76	21.19
7	A6QFR2	Bifunctional autolysin	peptidoglycan hydrolase & amidase activity	1	4170-4396	7	3.13	< 2	11.9	8.997

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
8	A6QJV4	Oligopeptide permease, peptide-binding protein	Belong to ABC transporter family	1	4170-4396	4	3.06	> 2	6.54	10.71
9	A6QFU9	Pyruvate dehydrogenase E1 component, alpha subunit	Catalytic site that binds magnesium ion and pyrophosphate fragment	1	4170-4396	10	2.29	> 2	10.05	24.05
10	A6QG30	Iron-regulated surface determinant protein B	Iron acquisition by degrading heme	1	4170-4396	7	1.54	> 2	10.03	11.16
11	A6QD99	Siderophore compound ABC transporter binding protein	Siderophore (iron-chelators) uptake	2	4065-4277	5	1.65	< 2	10.0	24.2
12	A6QGK7	Glutamine synthetase	enzyme; condenses glutamate and ammonia	2	4170-4396	16	2.15	< 2	18.3	25.9

Figure 4-1. Distribution of the protein functions. The proteins that were differentially upregulated (A) and downregulated (B) proteins in the pathogenic strains are grouped into categories based on their functions.

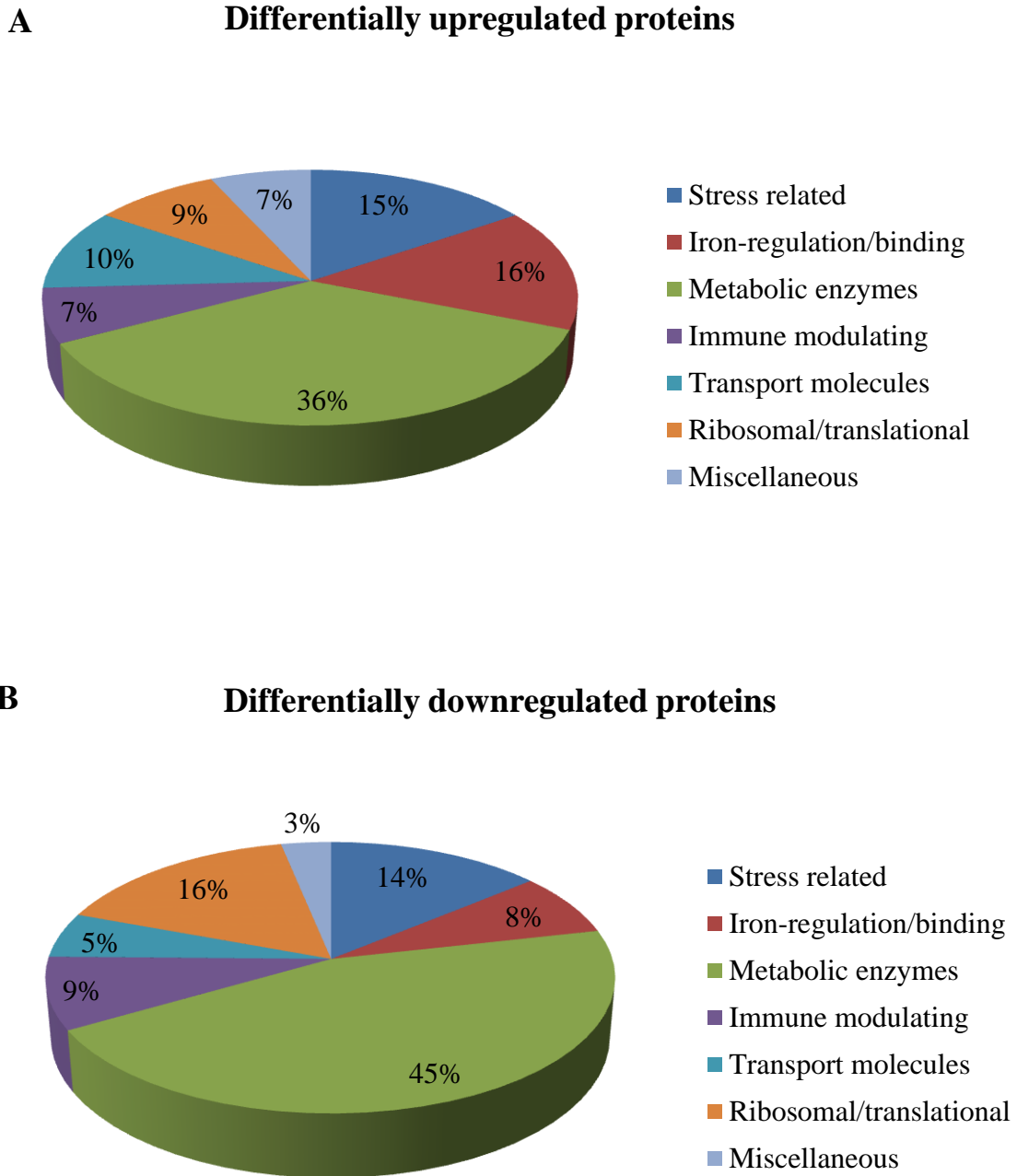


Figure 4-2. Relative intensities of light and heavy peptide showing upregulation in pathogenic *S. aureus* strains. The peak intensities of light (from commensal) and heavy (from pathogenic) forms of a representative peptide of alkaline shock protein 23, with a mass difference of 6 Da and relative abundance (H:L) of 2.6.

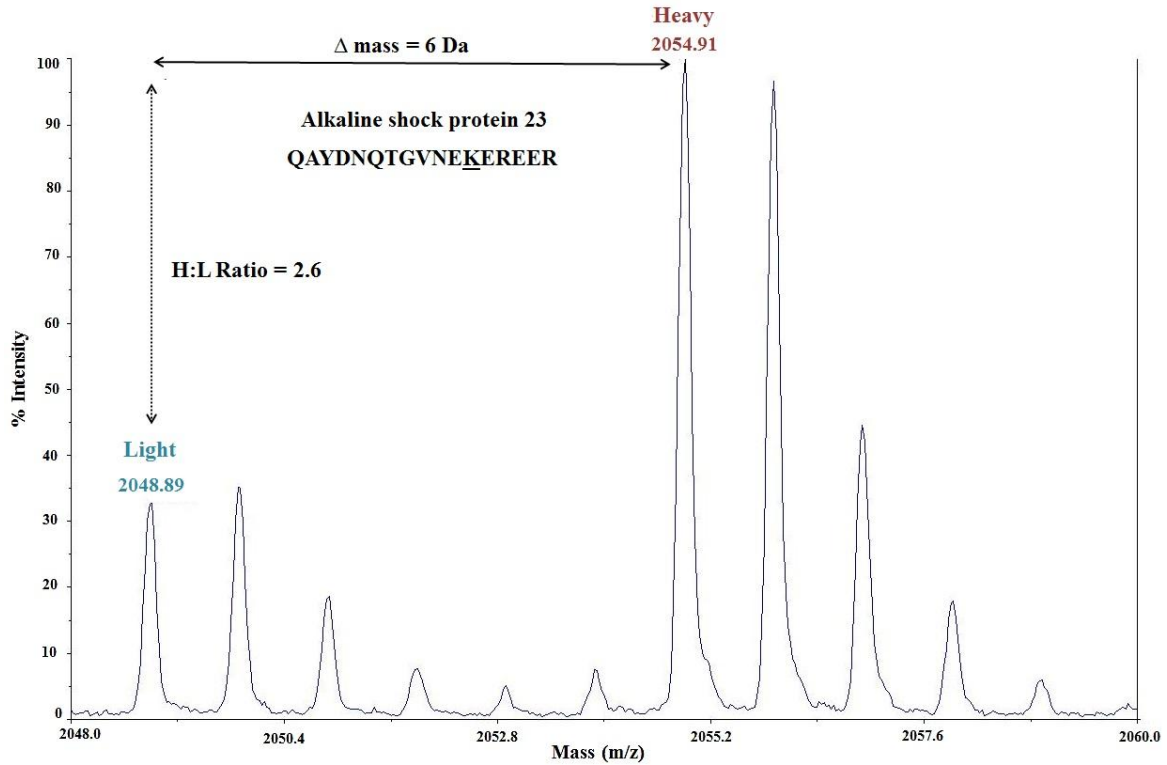
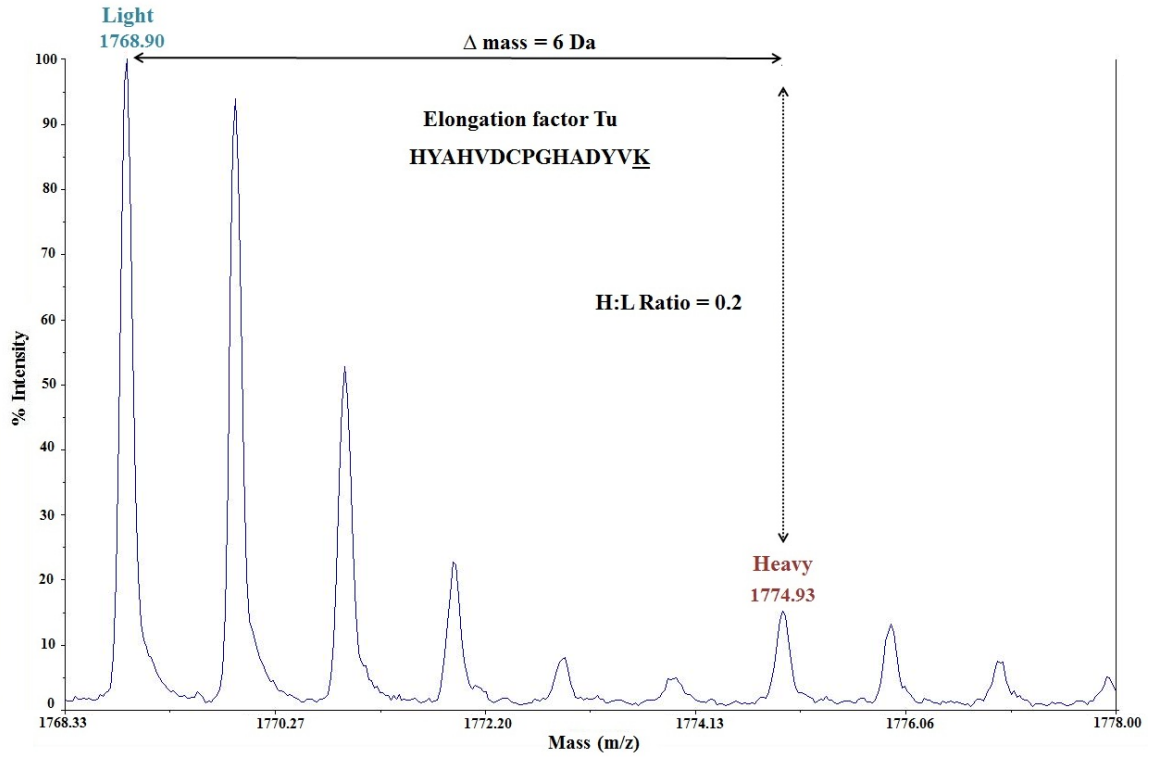


Figure 4-3. Relative intensities of light and heavy peptide showing downregulation in pathogenic *S. aureus* strains. The peak intensities of light (from commensal) and heavy (from pathogenic) forms of a representative peptide of the protein, elongation factor Tu, with a mass difference of 6 Da and relative abundance (H:L) of 0.2.



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Chapter 5. Conclusions

To our knowledge, this is the first study to use SILAC for comparing the proteomes of pathogenic and commensal *S. aureus* strains as an approach to identifying putative vaccine candidates. We proposed that the identification of proteins upregulated in the pathogenic strains would help us identify their role in the pathogenicity. In addition, the proteins highly expressed in the commensal strains would help define the observed immune dampening response in host. In this study, we identified potential virulent proteins that are involved in the pathogenesis of *S. aureus* and screened them with respect to commensal strains as background. Further, we observed no explicit variation in the growth rates of pathogenic and commensal *S. aureus* strains.

Adapting the cell line of interest to SILAC media, is one of the major drawbacks of this technique [1]. In our study, the SILAC media used, i.e., RPMI 1640, is essentially used for tissue culture, and hence limited the efficiency of culturing bacterial strains. Troubleshooting involved inoculating the media with different CFU concentrations and dilution methods. Certain limitations of the project were learnt after analyzing the data. The lysine terminated tryptic peptides do not tend to ionize well in MALDI-based instruments, as compared to the arginine containing ones. This issue could be resolved by labeling the cells with arginine as well as lysine. However, previous studies have reported concern over conversion of arginine to proline in some cell systems that may affect quantitation accuracy, and need to be dealt systematically [2]. Some of the identified proteins were either not differentially expressed or not identified at all in the duplicate experiments. Such discrepancies between biological or technical replicates are expected and have been previously reported [3, 4]. That is the reason why

incorporating such stringent thresholds proves to be necessary. Analyzing the differential protein expression of pathogenic and commensal strains by switching over the isotopic amino acids used in the media, would provide more statistical evidence. However, performing the MS analysis in replicates would have not only led to identification of more proteins, but also increase the confidence in the data. But, the large sample size of proteins and strains analyzed was a limitation for producing complementary data in this study. Besides, evaluating the protein expression at different growth phase would provide a broader aspect at examining differential expression.

Among the differentially expressed proteins identified in this work, N-acetylmuramoyl-L-alanine amidase domain protein (AM) and IgG-binding protein *sbi* could serve as potential targets for vaccine development. AM has been indicated to play a role in autolysis, cell-wall turnover and cell division [5, 6]. Inhibiting the activity of this protein could arrest cell replication and thereby, prevent the bacterial multiplication and spread in the host. IgG- binding protein *sbi* is expressed on the cell surface as well in secreted form. It is one of the potent virulent factors of *S. aureus*, with ability to inhibit activity of host IgG and complement proteins [7]. Targeting this protein, in order to suppress its expression would prevent the inhibition of host immune response. Further *in vivo* studies need to be conducted to elucidate the role of these proteins and their implications in promoting disease progression. However, it is important to verify the frequency of occurrence of these proteins in other *S. aureus* isolates, so that the vaccine can be targeted for various pathogenic strains.

The future of vaccine development against *S. aureus* rests in the engineering of a multivalent protein vaccine. In other words, the production of a vaccine

for this pathogen depends on the development of concerted multi-epitope components that provide protective immunity when administered, instead of a single antigenic target which may or may not be present in all the strain types. The significantly expressed cell wall proteins identified in the pathogenic strains can be targeted by the use of antibodies against them. Antibodies designed to identify various epitopes of the significantly expressed surface proteins identified in pathogenic strains, can help clear the invading bacteria. Immunizing the host with such antibodies would help elicit a strong immune response during bacterial invasion. However, for virulent factors that are being secreted, antibody cannot prove to be effective. In that case, targeted therapy, such as DNA vaccine could be an alternative, in order to elicit both cellular and humoral response in the host [8, 9]. Epitope vaccines can be designed by identification of specific immunodominant epitopes of the protein which elicit strong T-cell response. Previous studies have shown that immunizing the host with purified form of major exoproteins of *Mycobacterium tuberculosis* has proved to induce a strong cellular response and substantial immunity [10]. Therefore, subunit vaccine designed with components of the exoproteins capable of inducing a strong immune response could provide immunoprotection against *S. aureus*.

Proteomics techniques can help elucidate the global changes occurring in gene and protein expression in the pathogen during infection. Understanding the dynamics and interaction of pathogen with the host immune system using an immunoproteomics approach can make significant contributions of the development of vaccines. This data can help accelerate the progress of therapeutic drugs and vaccines to control and prevent infections.

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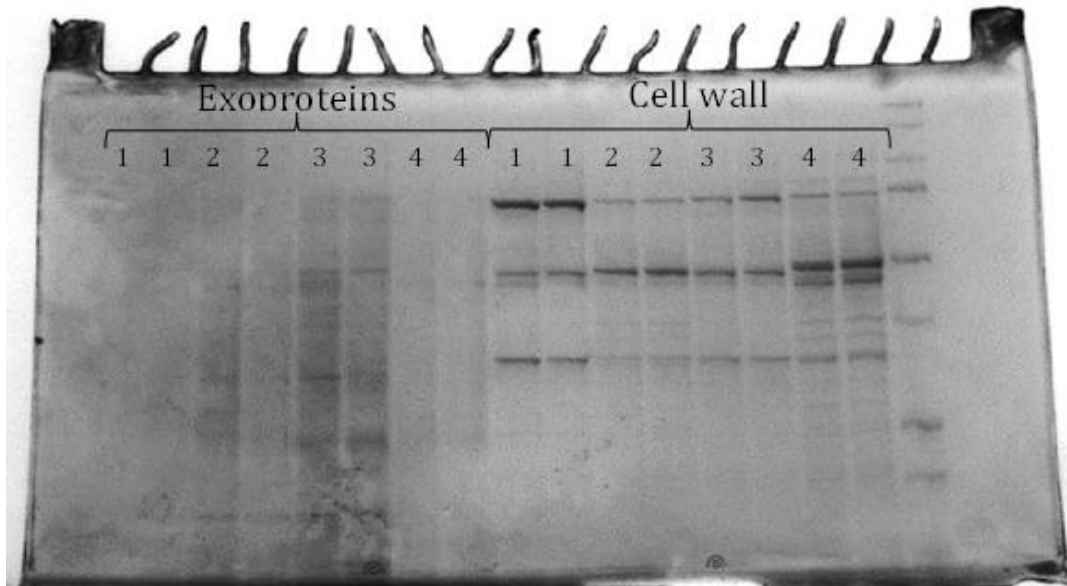
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Appendix A. Supporting Data

Figure A-1. Consistency in extraction of exo and cell wall proteins.

The exo, cell wall proteins (A), and membrane, cytoplasmic (B) were extracted from two strains, 4257 and 4170 in duplicate on two days, equally loaded across lanes and run on SDS-PAGE gel (4-12%). The lanes represented as follows: **1, 2:** proteins extracted on day (D) 1 from 4257 and 4170 resp.; **3, 4:** on D2 from 4257 and 4170 respectively.

A



B

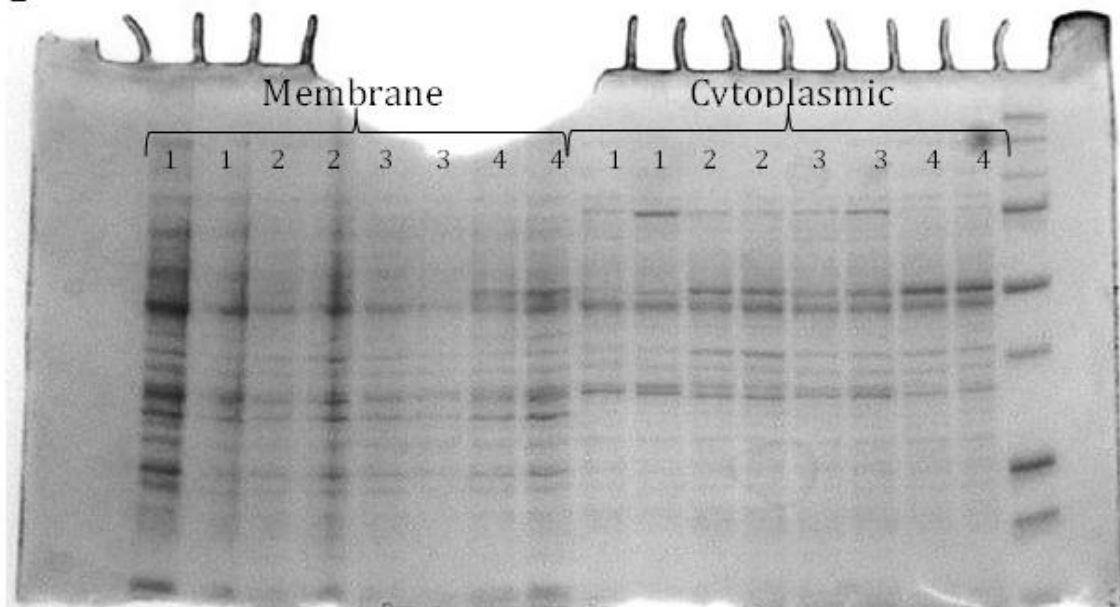


Figure A-2. Differential expression of cell wall, membrane and exo proteins among all strains.

The cell wall (A), membrane (B) and exo proteins (C) were extracted from all the commensal and pathogenic strains, equally loaded across lanes and run on SDS-PAGE gel (4-12%). Lane 1 to 4 depict proteins from pathogenic strains, lane 5 is from standard laboratory ATCC 27217 strain, lane 6 to 9 are proteins expressed by commensal strains, and lane 10 is the protein standard (ladder).

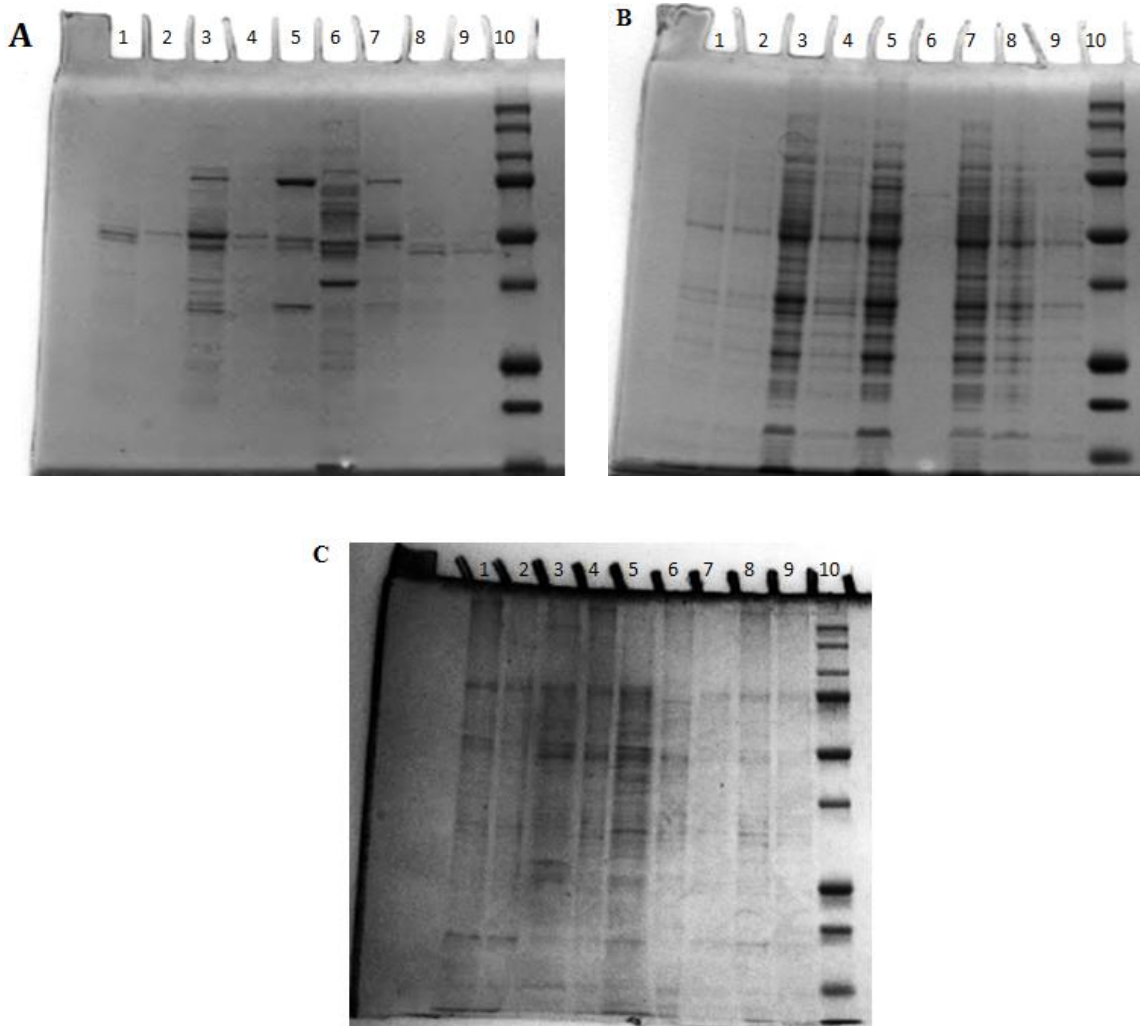


Table A-1. Significantly downregulated exoproteins in pathogenic *S. aureus* strains.
The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
1	A6QEK0	Elongation factor Tu	Promote binding of aminoacyl-tRNA to the A-site of ribosomes during translation	1	4131-4483	30	0.68	< 2	32.41	55.08
					4338-4257	8	0.17	> 2	14.74	30.96
				2	4065-4277	11	0.68	< 2	14.29	25.38
2	A6QG30	Iron-regulated surface determinant protein B	Iron acquisition by degrading heme	1	4131-4483	40	0.23	< 2	48.12	37.52
					4065-4277	26	0.47	> 2	26.86	26.36
				2	4131-4483	11	0.19	> 2	18.16	18.6
3	A6QG31	Iron-regulated surface determinant protein A	Iron acquisition by degrading heme	1	4131-4483	25	0.18	> 2	28	47.71
					4065-4277	28	0.33	< 2	25.06	50.29
				2	4131-4483	22	0.58	> 2	26.34	45.71
4	A6QGK7	Glutamine synthetase	Enzyme; condenses glutamate and ammonia	1	4131-4483	28	0.13	< 2	37.74	48.56
				2	4131-4483	8	0.33	< 2	11.19	16.63
5	A6QK93	Fructose-bisphosphate aldolase class 1	Enzyme in glycolysis	1	4065-4277	11	0.48	< 2	15.74	55.07
					4338-4257	4	0.17	> 2	7.03	14.19
				2	4131-4483	5	0.21	> 2	6.09	21.96
6	A6QJQ7	Immunoglobulin-binding protein sbi	Bind IgG Fc fragment & complement; blood coagulation	1	4065-4277	4	0.39	> 2	6.8	8.257
				2	4065-4277	5	0.50	< 2	9.98	14.68

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
7	A6QE62	Alkyl hydroperoxidase reductase subunit C	Protects cell against DNA damage by alkyl hydroperoxides	1	4065-4277	17	0.10	> 2	10.07	42.86
					4338-4257	13	0.11	> 2	10.93	39.15
				2	4131-4483	17	0.07	< 2	16.7	64.02
8	A6QEG5	Cysteine synthase	Cysteine biosynthesis	1	4131-4483	7	0.68	< 2	14	45.16
					4065-4277	2	0.44	> 2	4	14.84
9	A6QFU9	Pyruvate dehydrogenase E1 component, alpha subunit	Catalytic site that binds magnesium ion and pyrophosphate fragment	1	4131-4483	6	0.38	> 2	8.73	25.14
					4065-4277	4	0.56	> 2	4.43	12.97
10	A6QJQ5	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	Catalyzes interconversion of 2-phosphoglycerate and 3-phosphoglycerate; glycolysis	1	4131-4483	12	0.68	> 2	14.71	43.42
11	A6QD99	Siderophore compound ABC transporter binding protein	Siderophore (iron-chelators) uptake	1	4131-4483	4	0.68	> 2	6.68	16.06
12	A6QFV2	Dihydrolipoyl dehydrogenase	Oxidoreductase activity	1	4131-4483	7	0.53	> 2	9.2	17.95
13	A6QH25	30S ribosomal protein S1	Binds mRNA; facilitate translation initiation	1	4131-4483	4	0.51	> 2	8.18	15.86
14	A6QIM7	60 kDa chaperonin	GroEL protein	1	4131-4483	4	0.48	> 2	8	11.9
15	A6QFH3	Glucose-6-phosphate isomerase	Isomerase enzyme	1	4131-4483	12	0.45	> 2	20.88	43.12

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
16	A6QFV1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Transfer acyl group	1	4131-4483	4	0.42	> 2	9	14.42
17	A6QFV0	Pyruvate dehydrogenase E1 component, beta subunit	Acyl-transfering activity	1	4131-4483	4	0.26	> 2	8.03	24
18	A6QEIO	Glutamyl-tRNA synthetase	Catalyzes the attachment of glutamate to tRNA(Glu)	1	4065-4277	2	0.68	> 2	3.71	7.438
19	A6QG47	Thioredoxin	Protects against oxidative stress	1	4065-4277	2	0.52	> 2	1.02	27.88
20	A6QD95	Immunoglobulin G binding protein A (Protein A)	Binds Fc fragment of IgG	1	4065-4277	10	0.34	> 2	12.7	27.5
21	A6QIY3	General stress protein 20U	Oxidoreductase activity; stress response	1	4065-4277	6	0.09	> 2	8.13	44.22
22	A6QKD3	N-acetylmuramoyl-L-alanine amidase domain protein	Catalytic domain of autolysin (PGN hydrolase)	1	4338-4257	10	0.66	> 2	15.51	17.45
23	A6QFR2	Bifunctional autolysin	peptidoglycan hydrolase & amidase activity	2	4131-4483	4	0.28	< 2	8.84	4.459
24	A6QF17	Putative lipoprotein	Lipoprotein	2	4065-4277	5	0.69	> 2	6.06	15.75
25	A6QG59	Fibrinogen-binding protein	For adherence	2	4065-4277	4	0.31	< 2	7.66	24.85

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
26	A6QG56	Fibrinogen binding-related protein	For adherence	2	4065-4277	4	0.15	> 2	7.36	19.27
27	A6QF82	Phosphoglycerate kinase	Glycolytic enzyme	2	4338-4257	6	0.67	> 2	12	17.17
28	A6QKC6	Zinc metalloproteinase aureolysin	Metalloendopeptidase	2	4338-4257	2	0.66	> 2	4.05	4.72
29	A6QI23	Foldase protein prsA	Role in protein secretion by helping the post-translocational extracellular folding of secreted proteins	2	4338-4257	3	0.56	> 2	6.06	13.44
30	A6QJ77	50S ribosomal protein L6	Ribosomal protein; binds 23s rRNA	2	4338-4257	3	0.42	> 2	6	25.84
31	A6QIL6	Truncated beta-hemolysin	Extracellular region of beta hemolysin	2	4338-4257	21	0.35	< 2	28.12	49.64
32	A6QFA0	Thermonuclease	Heat-stable endonuclease	2	4338-4257	15	0.24	> 2	22.12	37.28
33	A6QG63	Alpha-hemolysin	Cytotoxicity	2	4338-4257	4	0.17	> 2	8	17.24

Table A-2. Significantly downregulated cell wall proteins in pathogenic *S. aureus* strains.

The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
1	A6QG31	Iron-regulated surface determinant protein A	Iron acquisition by degrading heme	1	4131-4483	7	0.12	> 2	10.000	20.29
				2	4131-4483	10	0.52	> 2	18.080	47.43
2	A6QGK7	Glutamine synthetase	enzyme; condenses glutamate and ammonia	1	4131-4483	24	0.18	< 2	37.510	60.31
				2	4338-4257	23	0.48	> 2	35.250	48.56
3	A6QK93	Fructose-bisphosphate aldolase class 1	Enzyme in glycolysis	1	4131-4483	17	0.63	< 2	28.580	62.84
				2	4338-4257	23	0.54	< 2	34.310	77.36
4	A6QIM8	10 kDa chaperonin	GroES protein GroES; Heat shock protein 10	1	4131-4483	3	0.69	> 2	6	27.66
5	A6QDB7	Superoxide dismutase	Stress resistance	1	4131-4483	3	0.61	> 2	6	25.63
6	A6QEG5	Cysteine synthase	Cysteine biosynthesis	1	4131-4483	3	0.58	< 2	6	24.52
7	A6QHL6	Threonyl-tRNA synthetase	Protein biosynthesis	1	4131-4483	2	0.56	> 2	4.24	4.651
8	A6QFH3	Glucose-6-phosphate isomerase	Isomerase enzyme	1	4131-4483	6	0.54	> 2	10	26.19
9	A6QFV0	Pyruvate dehydrogenase E1 component, beta subunit	Acyl-transferring activity	1	4131-4483	5	0.39	> 2	9.16	22.15
10	A6QFA1	Cold-shock protein CSD family protein	Heat shock protein	1	4131-4483	2	0.35	> 2	2.76	39.39
					4170-4396	4	0.37	< 2	4.93	68.18
11	A6QFT1	Bifunctional purine biosynthesis protein PurH	IMP synthesis	1	4131-4483	3	0.23	> 2	6.02	10.16

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
12	A6QD95	Immunoglobulin G binding protein A (Protein A)	Binds Fc fragment of IgG	1	4170-4396	11	0.68	< 2	17.08	32.5
13	A6QIV7	Serine hydroxymethyltransferase	Interconversion of serine and glycine	1	4170-4396	3	0.67	> 2	6.52	9.709
14	A6QFU9	Pyruvate dehydrogenase E1 component, alpha subunit	Catalytic site that binds magnesium ion and pyrophosphate fragment	1	4170-4396	4	0.66	> 2	5.07	11.62
15	A6QF81	Glyceraldehyde 3-phosphate dehydrogenase 1	Oxidoreductase enzyme in glycolysis	1	4170-4396	14	0.61	< 2	13.44	33.04
16	A6QGF7	Elongation factor Ts	Associates with EF-Tu	1	4170-4396	6	0.55	> 2	10	21.16
17	A6QEJ9	Elongation factor G	Catalysis ribosomal translocation step	1	4170-4396	13	0.46	< 2	20.67	22.22
18	A6QHK9	Trigger factor	Chaperone in protein export	1	4170-4396	5	0.43	> 2	4	6.928
19	A6QGV3	Major cold-shock protein CspA	Stress related	1	4170-4396	2	0.35	> 2	2.19	40.91
20	A6QE47	30S ribosomal protein S6	Translational activity	1	4170-4396	2	0.25	> 2	4	35.71
21	A6QJ86	30S ribosomal protein S3	Translational activity	1	4170-4396	2	0.25	> 2	4.13	15.67
22	A6QJ68	30S ribosomal protein S13	Translational activity	1	4170-4396	5	0.19	< 2	6.44	23.14
23	A6QJ90	50S ribosomal protein L23	Translational activity	1	4170-4396	2	0.12	> 2	2	18.68
24	A6QE61	Alkyl hydroperoxide reductase subunit F	Protects cell against DNA damage by alkyl hydroperoxides	1	4338-4257	9	0.47	> 2	14	26.04

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
25	A6QE62	Alkyl hydroperoxide reductase subunit C	Protects cell against DNA damage by alkyl hydroperoxides	1	4338-4257	9	0.25	> 2	10	42.86
26	A6QIY3	General stress protein 20U	Oxidoreductase activity; stress response	1	4338-4257	4	0.04	> 2	6	23.13
27	A6QJ75	30S ribosomal protein S5	Translational activity	2	4170-4396	4	0.68	> 2	2	14.72
28	A6QFV1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Transfer acyl group	2	4170-4396	2	0.53	> 2	3.4	8.14
29	A6QH22	DNA-binding protein HU	Histone-like DNA-binding protein	2	4170-4396	5	0.35	> 2	7.49	52.22
30	A6QFV2	Dihydrolipoyl dehydrogenase	Oxidoreductase activity	2	4170-4396	3	0.33	> 2	7.04	12.39
31	A6QGD2	Acyl carrier protein	Fatty acid synthesis	2	4338-4257	4	0.66	> 2	4	27.27
32	A6QF82	Phosphoglycerate kinase	Glycolysis	2	4338-4257	3	0.65	> 2	5.27	9.848
33	A6QK48	Copper chaperone CopZ	Chaperone for Cu ²⁺ transport	2	4338-4257	4	0.46	> 2	6	51.47
34	A6QHC3	Chaperone protein DnaK	Stress-related	2	4338-4257	10	0.37	> 2	17.67	17.87
35	A6QK89	L-lactate dehydrogenase 2	growth during nitrosative stress	2	4338-4257	5	0.10	> 2	10	29.78
36	A6QFQ5	Naphthoate synthase	menaquinone biosynthesis	2	4131-4484	3	0.51	> 2	6.38	9.158

Table A-3. Significantly downregulated cell membrane proteins in pathogenic *S. aureus* strains.

The pathogenic and commensal strains were labeled with heavy (H) and light (L) lysine, respectively.

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
1	A6QD99	Siderophore compound ABC transporter binding protein	Siderophore (iron-chelators) uptake	1	4131-4483	8	0.47	< 2	13.21	23.33
				2	4131-4483	3	0.19	> 2	7.22	12.12
2	A6QJ89	50S ribosomal protein L2	rRNA binding protein	1	4170-4396	2	0.33	< 2	4.24	11.91
				2	4170-4396	5	0.46	< 2	6.53	16.25
3	A6QEK0	Elongation factor Tu	Promote binding of aminoacyl-tRNA to the A-site of ribosomes during translation	1	4170-4396	9	0.34	< 2	13.84	22.34
				2	4065-4277	6	0.66	< 2	8.00	12.69
4	A6QH22	DNA-binding protein HU	Histone-like DNA-binding protein; wraps DNA to stabilize, prevent denaturation	1	4170-4396	3	0.48	> 2	5.7	34.44
				2	4170-4396	12	0.46	> 2	11.7	75.56
5	A6QFV1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex	Transfer acyl group	1	4131-4483	8	0.64	> 2	13.08	29.53
				2	4170-4396	2	0.20	> 2	3.51	5.581
6	A6QFV0	Pyruvate dehydrogenase E1 component, beta subunit	Acyl-transferring activity	1	4065-4277	4	0.48	> 2	7.66	14.15
					4131-4483	8	0.68	< 2	16.68	38.77
7	A6QGK7	Glutamine synthetase	Enzyme; condenses glutamate, ammonia	1	4065-4277	8	0.46	> 2	11.03	19.73
					4131-4483	34	0.19	< 2	47.61	60.09

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
8	A6QK94	Probable malate:quinone oxidoreductase 2	Redox reaction	1	4131-4483	13	0.68	< 2	19.66	26.71
9	A6QEU1	Putative uncharacterized protein	Unknown	1	4131-4483	16	0.68	< 2	22	45.63
10	A6QEF3	Ribose-phosphate pyrophosphokinase	Nucleotide biosynthesis	1	4131-4483	3	0.68	> 2	4.57	18.07
11	A6QFU9	Pyruvate dehydrogenase E1 component, alpha subunit	Catalytic site that binds magnesium ion and pyrophosphate fragment	1	4131-4483	10	0.60	< 2	14	30
12	A6QJ18	Ferrichrome ABC transporter lipoprotein	Iron transport	1	4131-4483	6	0.53	< 2	11.08	15.9
13	A6QFT1	Bifunctional purine biosynthesis protein PurH	IMP synthesis	1	4131-4483	12	0.45	> 2	16.37	28.05
14	A6QJQ7	Immunoglobulin-binding protein sbi	Bind IgG Fc fragment & complement	1	4131-4483	3	0.35	> 2	5.41	7.569
15	A6QG31	Iron-regulated surface determinant protein A	Iron acquisition by degrading heme	1	4131-4483	5	0.24	> 2	10	27.14
16	A6QG30	Iron-regulated surface determinant protein B	Iron acquisition by degrading heme	1	4131-4483	10	0.03	> 2	17.09	20.93
17	A6QK94	Probable malate:quinone oxidoreductase 2	Malate dehydrogenase	1	4170-4396	5	0.65	> 2	8.39	11.45
18	A6QIM7	60 kDa chaperonin	GroEL protein	1	4170-4396	2	0.58	> 2	4.01	8.364
19	A6QG86	Cell division protein ftsZ	Cell division	1	4170-4396	3	0.51	> 2	6	16.92

S. No	Accession ID	Protein	Remarks	SILAC expt	SILAC Pair	Peptides (95%)	H:L	EF H:L	Unused	%Cov (95)
20	A6QIU7	ATP synthase subunit beta	Catalytic site	1	4170-4396	7	0.47	< 2	13.3	25.11
21	A6QHQ3	30S ribosomal protein S4	Translational activity	1	4170-4396	5	0.35	> 2	8	19.5
22	A6QJV4	Oligopeptide permease, peptide-binding protein	Belong to ABC transporter family	2	4131-4483	2	0.13	> 2	4	5.827
23	A6QJ80	50S ribosomal protein L5	Translational protein	2	4170-4396	4	0.39	> 2	4	20.11
24	A6QJ75	30S ribosomal protein S5	Translational protein	2	4170-4396	6	0.27	> 2	8	41.1

Appendix B. Detailed protocols

Recipes – SILAC Media

Purpose: To culture and grow bacteria for SILAC experiment.

Reagents:

Custom RPMI 1640 devoid of lysine, arginine (Athena ES, Inc., MD, USA)

SILAC amino acid kit: L-lysine. HCl, $^{13}\text{C}_6\text{L}$ -lysine. HCl, L-arginine (Invitrogen-Life technologies, Carlsbad, CA, USA)

0.22 μm filter flask (Nalgene, Rochester, NY, USA)

Procedure:

1) To make light SILAC medium, add the following reagents in specified quantities

a) Custom RPMI 1640 – 1 L (Athena ES, Inc., MD, USA)

b) L-lysine. HCl – light (40 mg/L)

c) L-arginine – (200 mg/L)

Mix and filter through 0.22 μm filter; store at 4°C

2) To make heavy SILAC medium, add the following reagents in specified quantities

a) Custom RPMI 1640 – 1 L

b) $^{13}\text{C}_6\text{L}$ -lysine. HCl – heavy (40 mg/L)

c) L-arginine – (200 mg/L)

Mix and filter through 0.22 μm filter; store at 4°C

CFU determination using microtiter dilutions in 96-well plate

Purpose: To dilute bacterial cultures for determining CFU/ml.

Reagents:

Bacterial culture (grown in TSB or SILAC media)
96-well plate
Multi-channel pipettor and tips
Reagent Reservoir
PBS
TSA plate

Procedure:

1. Prepare 96-well plate by adding PBS to well in the following amounts:
 - a. Row A: 100 μ L
 - b. Row B: 200 μ L
 - c. Row C-H: 225 μ L
2. Add 100 μ L of bacterial cultures to Row A (1:2 dilution), mix
3. Transfer 50 μ L from Row A to Row B (1:10 dilution),
 - a. (make sure to clip tips before mixing and transferring 25 μ L to next well).
4. Transfer 25 μ L from Row B to Row C (1×10^2)
 - a. (make sure to clip tips before mixing and transferring 25 μ L to next well).
5. Continue transferring 25 μ L down the plate to obtain 10 fold dilutions (1×10^3 in Row D through 1×10^7 in row H)
6. Plate appropriate dilutions by dropping 3 x 25 μ L drops onto TSA plate.
 - a. e.g. If original culture has 10^9 bacteria, plate dilutions 10^7 and 10^6 (~2.5 and 25 bacteria per drop respectively)
7. Incubate blood plate overnight at 37°C.
8. Count number of colonies in 3 drops:
 - a. $(\text{colony count}/3) \times 40 \times \text{final dilution} = \text{CFU/ml}$

	1	2	3	4	5	6	7	8	9	10	11	12	Final Dilution
A	100 μ L PBS + 100 μ L bacteria culture												1:2
B	200 μ L PBS + 50 μ L bacteria from row A												1:10
C	225 μ L PBS + 25 μ L bacteria from row B												10^2
D	225 μ L PBS + 25 μ L bacteria from row C												10^3
E	225 μ L PBS + 25 μ L bacteria from row D												10^4
F	225 μ L PBS + 25 μ L bacteria from row E												10^5
G	225 μ L PBS + 25 μ L bacteria from row F												10^6
H	225 μ L PBS + 25 μ L bacteria from row G												10^7

Bacterial culture in SILAC media

Purpose: Culture *Staphylococcus aureus* in SILAC media

Reagents:

Staphylococcus aureus cultures (on 5% esculin blood agar plate)

Tryptic soy broth (TSB)

SILAC light and heavy media

Procedure:

1. Inoculate single colony of bacteria (from blood plates) in 5 mL of TSB and incubate at 37°C; 190 rpm for 5 hrs.
2. Determine the CFU count by drop plating.
3. Based on CFU count, calculate the volume of TSB culture needed to inoculate 10^6 CFU/mL into 100mL of SILAC media (do serial dilutions if necessary in PBS).
 - a. e.g.: If you have a TSB culture of the concentration of 10^9 CFU/mL, carry out the following serial dilutions: (make sure you vortex at every step)
 - i. For 10^8 : dilute 100 μ L of the TSB culture in 900 μ L of PBS
4. Inoculate 10^6 CFU/mL into 100 mL SILAC media and grow until early stationary phase at 37°C; 190 rpm.
 - a. Save 1 mL aliquots at initial and final stages of culture and take OD readings and determine the CFU count.

Isolation of cell wall and cell membrane proteins

Purpose: Extract cell wall and cell membrane proteins from *Staphylococcus aureus* grown in SILAC media for MS analysis

Reagents:

S. aureus cultured in SILAC media

Lysis Buffer

50 mM Tris-HCl

20 mM MgCl₂

2 mM EDTA

pH 7.5

Raffinose (Acros, NJ, USA)

Protease Inhibitor Cocktail (Thermo Scientific, Rockford, IL, USA)

Lysostaphin (Sigma, MO, USA)

Procedure:

1. Collect 100 mL bacterial culture grown in the SILAC media (refer to protocol “Bacterial culture in SILAC media”) till early stationary phase, in two 50 mL centrifuge tubes.
2. Centrifuge at 12,500 x g for 15 min at 4°C to pellet the bacterial cells.
3. Wash pellet with PBS twice by centrifuging at 12,500 x g for 15 min at 4°C.
4. Resuspend pellet in 1 ml lysis buffer containing
 - a. 30% raffinose
 - b. Protease Inhibitor Cocktail
 - c. Lysostaphin (25 U)
5. Incubate the cell suspension at 37 °C for 90 min (no shaking).
6. Centrifuge at 7500 x g and 4 °C for 20 min.
7. Collect supernatant - **Fraction 1**; store at -20°C (contains **cell wall protein** fraction).
8. Resuspend pellet in 500 µL lysis buffer.
9. Homogenize the cell lysate at 30s on, 30s off (repeat thrice).
10. Centrifuge at 100,000 x g for 1 hour at 4°C.
11. Collect and resuspend the pellet in 1 mL lysis buffer; store at -20°C **Fraction 2** (contains **cell membrane protein** fraction).
12. Determine the protein concentration using Bradford assay.

Protein precipitation from supernatant

Purpose: Extract secreted proteins from *Staphylococcus aureus* culture media for MS analysis

Reagents:

S. aureus cultured in SILAC media

0.45 μm filter

Acetone

Trichloroacetic acid (TCA) (Alfa Aesar, Fischer, Suwanee, GA, USA)

200 mM Tris-HCL (pH 8)

Procedure:

1. Collect 100 mL bacterial culture grown in the SILAC media (refer to protocol “Bacterial culture in SILAC media”) till early stationary phase, in two 50 mL centrifuge tubes.
2. Centrifuge at 12,500 x g for 15 min at 4°C to pellet the bacterial cells.
3. Filter supernatant through 0.45 μm filter to remove the residual bacteria, if any.
4. Add 20% w/v TCA to the filtered media and incubate at 4°C overnight.
5. Centrifuge at 7,500 x g for 90 min at 4°C.
6. Wash the pellet in ice-cold acetone twice by centrifuging at 7,500 x g for 20 min at 4°C.
7. Decant supernatant and get rid of residual acetone using SpeedVac.
8. Resuspend pellet in 250 μL of 200 mM Tris-HCl (pH 8).
9. Determine the protein concentration using Bradford assay.

Bradford assay

Purpose: To quantify the protein concentration.

Reagents:

96-well microtiter plate
Microcentrifuge tubes
Multi-channel pipettor and tips
Reagent Reservoir
Coomassie brilliant blue reagent
Bovine Serum Albumin (BSA) (Calbiochem, Gibbstown, NJ, USA)
Lysis buffer
 50 mM Tris-HCl
 20 mM MgCl₂
 2 mM EDTA
 pH 7.5
200 mM Tris Cl

Procedure:

1. Prepare 2 mg/mL stock of BSA standard in either lysis buffer or 200 mM Tris Cl.
2. In microcentrifuge tubes, prepare a set of diluted BSA standards as described below.
3. Pipette 10 μ L of each standard or unknown sample (in replicates) into a 96-well microtiter plate.
4. Add 200 μ L of the coomassie blue reagent to each well.
5. Cover the plate and incubate at 37°C for 30 min on a shaker.
6. Measure the absorbance at 562 nm on a plate reader.
7. Prepare a graph by plotting the concentrations and OD₅₆₂ readings of the BSA standards. Fit a linear line in the graph and use that equation to calculate the concentrations of the unknown samples.

Vial	Volume of diluent (μ L)	Volume and source of BSA (μ L)	Final BSA concentration (mg/mL)
A	0	300 μ L of stock	2
B	125	375 μ L of stock	1.5
C	325	325 μ L of stock	1
D	175	175 μ L of vial B dilution	0.75
E	325	325 μ L of vial C dilution	0.5
F	325	325 μ L of vial E dilution	0.25
G	325	325 μ L of vial F dilution	0.125
H	400	100 μ L of vial G dilution	0.025
I	400	0	0

In solution digestion of proteins

Purpose: Digest the proteins in solution with trypsin for MS analysis

Reagents:

Ammonium Bicarbonate (Ambic)

1M stock Ambic → 79 g in 1mL water

100 mM Ambic → 100 μ L stock in 1mL water

8M urea

480 mg urea in 1 mL of 100 mM Ambic

Reducing agent (DTT)

1M DTT → 30 mg DTT in 200 μ L of 100 mM Ambic

Alkylating Reagent (Iodoacetamide)

200 mM iodoacetamide → Dissolve 36 mg in 1 mL of 100 mM Ambic

Trypsin solution

Trypsin → 0.10 μ g/ μ L

Procedure:

1. Combine the proteins in 1:1 (w/w) ratio from heavy and light samples based on Bradford results.
2. Precipitate the proteins by adding 4X methanol and incubate at -20°C for 30 minutes.
3. Pellet the precipitated proteins by centrifuging at 13,000xg for 20 minutes at room temperature.
4. Reconstitute the pellet in approx. 20 μ L of 8.0M urea in a 0.5 mL microcentrifuge tube.
5. Add 1 μ L of reducing reagent and mix the sample by gentle vortex.
6. Reduce the mixture for 1 hour at RT or in incubator for 37° C. Don't go over 37°C as urea will react with sample and generate carbamylated artifacts.
7. Add 20 μ L of alkylating reagent and alkylate for 30 mins at RT in dark (use aluminium foil if necessary).
8. Add 4 μ L of reducing agent to consume any leftover alkylating agent (so trypsin is not alkylated).
9. Add 60 μ L of Ambic to dilute the urea before digesting it with trypsin.
10. Add trypsin in appropriate ratio (1:50) (w/v) to approximate amount of protein by weight. Digest overnight at 37° C.
11. In the morning, add 1 μ L of acetic acid to stop digestion. Vortex and centrifuge.