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Hospital and meat associated *Staphylococcus aureus* and Their Biofilm Characteristics

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Master of Science

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ABSTRACT

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and Their Biofilm Characteristics

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Biofilm phenotypes were studied in 32 *Staphylococcus aureus* strains isolated from store-bought meats and 22 from diseased patients in hospitals. Of the meat-associated strains, 21 were methicillin-resistant *Staphylococcus aureus* (MRSA) and 11 were methicillin-susceptible *Staphylococcus aureus* (MSSA). The hospital-associated strains included 15 MRSAs and 7 MSSAs. We studied the robustness and composition of the biofilms produced by these strains. We found that on average hospital-associated strains form more robust biofilms than meat associated strains. The model often used to describe *S. aureus* biofilm composition includes two biofilm types defined by the presence or absence of polysaccharide intercellular adhesin (PIA), PIA-dependent and PIA-independent respectively. In this model, PIA-independent biofilms are structurally reliant on proteins and extracellular DNA (eDNA) and PIA-dependent are structurally reliant on polysaccharides. Enzymatic degradation of the extracellular matrix can reveal which compounds are essential for the structural integrity of the biofilm, and by this model PIA-independent biofilms should be susceptible to both DNase and proteinase K. We found that hospital-associated strains are, on average, more susceptible to degradation by proteinase K. Interestingly, hospital-associated strains are less susceptible to degradation by DNase than meat-associated strains. Finding that proteinase K and DNase susceptibility for these strains are not linked gives evidence to support the idea that *S. aureus* biofilm composition can vary greatly from strain to strain and that the PIA-dependent and PIA-independent dichotomy of the standard model may be insufficient to describe the variety of *S. aureus* biofilm composition and may only apply to the extremes of the spectrum. Additionally, we saw no relationship between MRSA or MSSA strains and biofilm robustness, proteinase K degradation, or DNase degradation. Differences in biofilm characteristics between hospital-associated and meat-associated strains reinforce previous findings that these populations are genetically distinct.

Keywords: staphylococcus aureus, s. aureus, biofilm, methicillin resistant styphylococcus aureus, hospital, meat, food safety

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Introduction

Staphylococcus aureus is a common commensal bacterium and opportunistic pathogen. It establishes long-term colonization in 20-30% of the human population, and transient colonization in 60% [1-3]. Though carriage is usually asymptomatic, infection can cause a wide range of diseases including skin and soft tissue infection, bacteremia, pneumonia, and endocarditis [2, 4-6]. *S. aureus* is possibly the most common cause of food poisoning and the leading cause of death of any infectious agent in the United States and is a leading cause of hospital-associated infection throughout the developed world, being second only to *Clostridium difficile* in the United States [7-9]. Additionally, *S. aureus* infection rates have increased in recent decades, including both hospital and community acquired infections [10]. For soft-tissue infections alone there were an estimated 48.1 cases per 1,000 population in 2005 [10]. Infections associated with *S. aureus* can have mortality rates as high as 25% [11].

S. aureus can carry a variety of virulence factors including leukocidins, hemolysins, enterotoxins, the super antigen TSST-1, protein A, and biofilm genes [6, 12-15]. Antibiotic resistant strains of *S. aureus* have been known since shortly after the introduction of penicillin [2]. These first resistant strains produced a penicillinase and were still susceptible to the second-generation penicillins, such as methicillin, that were introduced in the early 1960s. However, resistance to these new drugs was reported within one year [2, 8]. These strains of methicillin-resistant *Staphylococcus aureus*, or MRSA, had acquired the gene *mecA*, an antibiotic-resistant transpeptidase which allows cell wall synthesis to carry on in the presence of beta-lactam antibiotics [8, 16, 17]. Though methicillin is no longer used clinically the term MRSA is still used to describe any *S. aureus* strain which carries *mecA*. MRSA, while originally only found in

hospitals, was found to be common in the community outside of hospitals in the 1980s, leading to the terms hospital acquired MRSA (HA-MRSA) and community acquired MRSA (CA-MRSA) to differentiate these genetically distinct families of MRSA [4, 7]. The antibiotic vancomycin is used to treat MRSA infections as a last resort, though vancomycin-resistant SA (VRSA) strains have been reported in recent years [18, 19]. Most clinical *S. aureus* infections are transmitted from person-to-person contact, in both hospital-acquired and community-acquired transmissions [4, 20, 21]. However, *S. aureus* can also be transmitted to humans from direct contact with living livestock or through exposure to contaminated meats [22-25]. In the United States, SA can be isolated from retail meat products at rates between 20% and 50% depending on the study location, and type of meat tested [26, 27].

Genetics of *S. aureus* antibiotic resistance

The antibiotic resistance gene of MRSA, *mecA*, was acquired as part of a mobile genetic element called the staphylococcal cassette chromosome *mec* (SCCmec). SCCmec is thought to have originated from a non-staphylococcal source, and twelve SCCmec variations have been described [28-30]. In addition to *mecA* the SCCmec contains a number of genes of unknown function and what is known as the *ccr* gene complex, *ccrAB* and/or *ccrC*, all of which have roles in promoting site-specific recombination [28]. The gene *mecA* encodes the membrane-bound transpeptidase penicillin-binding protein 2A (PBP2a) which catalyzes peptidoglycan crosslinking during cell-wall synthesis [16]. This class of enzymes, which were named for their affinity for penicillin, are inhibited when bound by beta-lactams. PBP2a, however, has a uniquely lower affinity for beta-lactams, allowing PBP2a to carry on cell wall synthesis when the activity of the four native SA penicillin-binding proteins is blocked [29, 31].

Some SCCmec types have a regulatory system in place consisting of a transcriptional repressor, a sensor-inducer, and an anti-repressor to control the expression of *mecA*, though most clinical MRSA strains appear to have a non-functional regulatory system [32]. High constitutive expression appears to be necessary to give the beta-lactam resistant phenotype associated with MRSA. Because of the slow response time of a functional regulatory system, strains without constitutive expression often appear susceptible to oxacillin in testing, even though they have a functional *mecA* gene [32, 33]. The varied levels of beta-lactam resistance among MRSA strains is due to varied levels of *mecA* expression [5]. MRSAs can be classified into two categories based on resistance levels – heterogeneously resistant (HeR) and homogeneously resistant (HoR). HeR strains are those able to grow in oxacillin concentrations between 2 and 100 μg/ml while HoR strains can grow in oxacillin concentrations in excess of 100 μg/ml [5].

Biofilm formation

One of the key virulence factors of *S. aureus* is its ability to form biofilms. *S. aureus* biofilms are sticky conglomerations of cells surrounded by an extracellular matrix which provide protection from mechanical removal of cells, host immune responses (both innate and adaptive), and antibiotics, giving as much as a 6-log increase in cell viability over planktonic cells following antibiotic challenge [34-36]. Additionally, *S. aureus* biofilm formation greatly increases the occurrence of horizontal gene transfer, contributing to the spread of antibiotic resistance [37]. *S. aureus* biofilms are a major concern in hospitals, not just for the danger of infection of damaged host tissue, but also because of the ability of *S. aureus* to form biofilms on implanted medical devices such as catheters, pacemakers, artificial heart valves, intravascular

lines, and joint replacements [38]. Colonization of such devices can lead serious, chronic infections that are difficult to treat [11, 34].

S. aureus biofilm formation is a highly organized process allowing for the formation of complex three-dimensional structures with channels that allow for the flow of nutrients to cells located deeper within the matrix [34, 39]. The biofilms have tightly regulated growth patterns that regulate attachment to a surface, the growth and expansion of the biofilm, and detachment and spread [34, 36]. These processes are regulated through quorum sensing, allowing the optimal cell density to be maintained by regulating the dispersal of cells for spread to new areas. During the growth phase some cells will even undergo an apparently altruistic autolysis to provide neighboring cells with the materials necessary to construct the extracellular matrix (such as DNA, for example) [39, 40].

Though there are some general characteristics that connect most *S. aureus* biofilms, the composition of the extracellular matrix from strain to strain can be drastically different [38, 41, 42]. In general, these varied extracellular matrix compositions are categorized into two classes based upon the presence of polysaccharide intercellular adhesin (PIA), PIA-dependent and PIA-independent [5, 38]. The biofilm class of any particular strain can be determined by a simple test. Biofilms are grown in 96 well plates and then treated with proteinase K which degrades proteins or sodium meta-periodate which oxidizes polysaccharide linkages. PIA-dependent biofilms are unaffected by proteinase K treatment and dispersed by sodium meta-periodate treatment, while PIA-independent biofilms are dispersed by proteinase K treatment and unaffected by sodium meta-periodate [41, 42]. While there have been thorough studies of the composition and genes associated with each class of biofilm [43-45] only an overview of the major components will be given here.

PIA-dependent

PIA-dependent biofilms are the "classic" biofilm type, they were the first studied and are what are usually described when talking about the average *S. aureus* strain [11, 44, 46]. Their extracellular matrix consists primarily of PIA, built from the polysaccharide poly-β(1-6)-N-acetylglucosamine (PNAG), but also contains a variety of proteins, extracellular DNA (Edna), and amyloid fibrils (see fig 1) [44]. Many cytoplasmic proteins and genomic DNA become associated with the extracellular matrix as cells undergo autolysis. This altruistic act of some cells, which is triggered through quorum sensing, provides the raw materials necessary to form the biofilm [34, 39, 40]. The eDNA, while not necessary for the structural integrity of PIA-dependent biofilms, is important for the formation of amyloid fibrils from phenol-soluble modulins, which contribute to biofilm stability [42, 47]. The primary component of PIA-dependent biofilms is, of course, PIA. This polysaccharide is produced and assembled into the extracellular matrix by the products of the *icaADBC* operon (see figure 3 B). *icaA* is an N-acetylglucosaminyltransferase that synthesizes PIA; *icaD* produces a product that, while not fully understood, is known to increases the efficiency of *icaA*; *icaB* produces an N-deacetylase which partially deacetylates PIA; and *icaC* is involved in the exportation of PIA to the cell surface [44, 48]. While several genes are known to influence the production of PIA the best characterized is *icaR*, a divergently transcribed repressor of the *ica* operon located just upstream of the *icaA* gene [48-50].

PIA-independent

PIA-independent biofilms are most notably characterized by the lack of PIA, instead, these biofilms rely solely on extracellular proteins and eDNA for their structural integrity, a difference which can even be seen by electron microscopy (see Fig. 1) [38, 41]. The reliance of

eDNA in PIA-independent biofilms makes the *agr* quorum-sensing system, which triggers autolysis, vital in biofilm formation [40, 51]. Once DNA is released it is thought to interact with cell surface proteins to bind cells one to another [38]. The primary proteins involved in the PIA-independent biofilm type are the membrane-bound fibronectin-binding proteins FnBPA and FnBPB [40]. The function of these proteins in biofilm formation appears to be redundant as either may be knocked out and biofilms will continue to form normally [52]. Other membrane-bound proteins, such as protein A and SasG have been shown to be involved in PIA-independent biofilm formation, though their specific functions and possible interactions with eDNA have yet to be studied [38, 43]. Extracellular proteases are of crucial importance for PIA-independent biofilms. Protease production is limited during biofilm maturation and increased, allowing for degradation of the extracellular matrix, for biofilm dispersal [35, 51, 53]

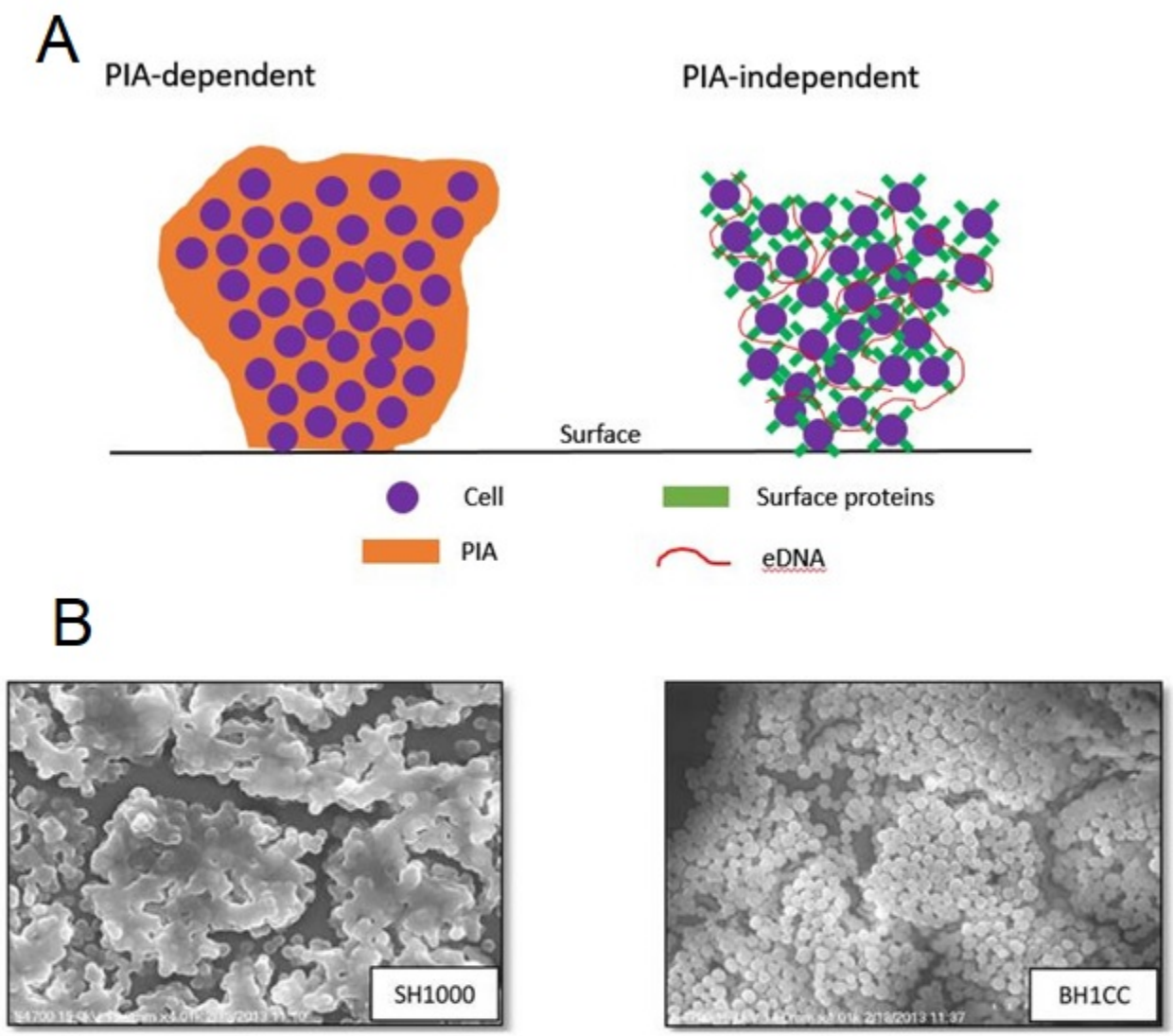

Figure 1 – *S. aureus* biofilm phenotypes

A: A simplified representation of PIA-dependent and PIA-independent biofilm structures are shown. The primary structural element of PIA-dependent biofilms is PIA whereas PIA-independent biofilms are structurally dependent upon the interaction of surface proteins and extracellular DNA (eDNA). B: Shown are scanning electron micrographs of PIA-dependent (strain SH1000) and PIA-independent biofilms (strain BH1CC). The obvious difference is the presence of PIA which largely obscures the cells of the PIA-dependent strain. Electron micrographs by McCarthy et al. [38].

The link between antibiotic-resistance and biofilm type

It has been observed that MRSA strains tend to produce a PIA-independent biofilm type while methicillin-susceptible SA (MSSA) tend to produce PIA-dependent biofilms [5, 38, 41, 43, 54]. This trend is typically described in the context of HoR MRSA strains [5, 38]. In one study by Pozzi et al, MSSA cells producing a PIA-dependent biofilm were transformed with a plasmid containing *mecA.* These cells were then put through a selection to isolate a HoR strain. A complete shift in biofilm type from PIA-dependent to PIA-independent was observed and icaA expression was drastically reduced. The plasmid was then cured (turning the strain back into MSSA) and the strain returned to a PIA-dependent biofilm type and *icaA* expression returned to normal. How this shift in biofilm type is accomplished is unknown. PBP2a is membrane-bound and is not known to have any direct effect on transcription, yet mutation of its active site abolishes the effects on *ica* transcription. Furthermore, the repression of the *ica* operon was found to be *icaR* independent (see figure 2). It has been suggested that a change in cell wall architecture through the action of PBP2a may be responsible for this drastic shift in biofilm composition, yet this is still uncertain and more research into these mechanisms has yet to be done [5, 38].

Project 1 – Mutagenesis to find genetic influencers of *ica* expression in response to PBP2a production

Description

This first project was aimed at better understanding the relationship between *mecA* and *ica* operon expression. Due to technical difficulties in cloning a reporter plasmid, this project was

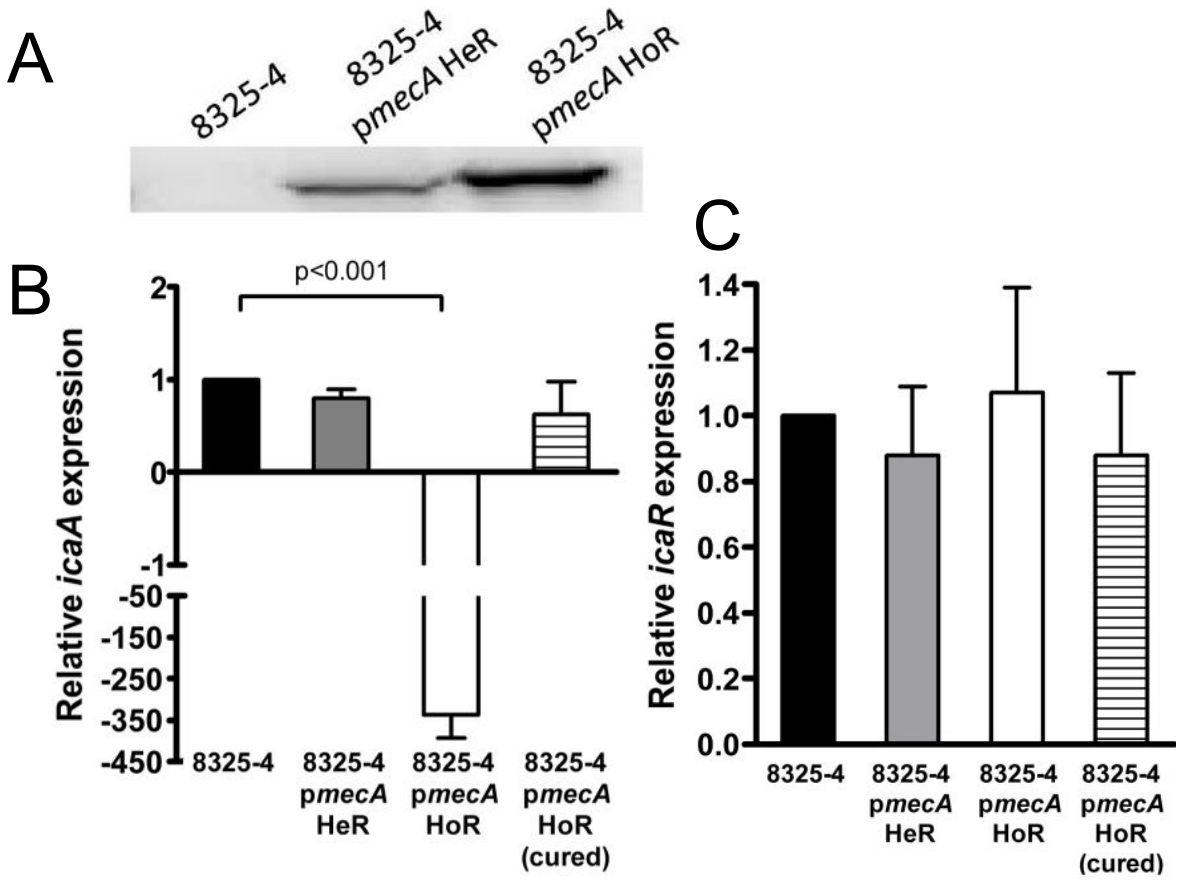

Figure 2 – Expression of biofilm genes

Figures and data by Pozzi et al. demonstrating the relationships between the expression of *mecA*, *icaA*, and *icaR* [4] A: Western blot analysis of PBP2a levels, the product of *mecA*. 8325-4 is a MSSA strain with no copy of *mecA*. When it is added by the plasmid *pmecA* it becomes an HeR MRSA. When selected for a highly resistant HoR mutant, mecA expression dramatically increased. B: Analysis of *icaA* expression by qPCR demonstrating the effect of increased *mecA* expression on *icaA* expression. 8325-4 (MSSA), 8325-4 p*mecA* HeR (HeR MRSA), 8325-4 p*mecA* HoR (HoR MRSA), and the HoR variant cured of the plasmid. From this we can see that *icaA* expression drops in response to increased *mecA* expression and returns to normal when *mecA* expression is abolished by curing the plasmid. C: Measurement of *icaR* expression of the same strains, leading us to believe that *ica* operon modulation in response to *mecA* is *icaR* independent.

put on hold while we investigated the observations described in project 2. Though it is yet to be completed, the experimental design along with all of this projects findings are described here.

As can be seen in Figure 2, high *mecA* expression, associated with homogeneously resistant MRSA strains, is accompanied by a drop in *ica* operon expression. This relationship appears to be due to some unknown repressor, as the normal repressor for this system, *icaR,* had no significant change in expression in response to *mecA* expression [5]. Furthermore, the activity of PBP2a was found to be necessary to cause a change in *ica* expression and a shift in biofilm type. Mutation of the PBP2a active site abolished any effect on biofilm phenotype or gene expression [5].

The goal of this project was to find any other genes besides *mecA* that were responsible for the observed drop in *ica* expression. These genes might include a repressor directly responsible for the repression of the polysaccharide producing *ica* genes and any genes involved in signal transduction. The hypothesis of this project was that an unknown repressor, or repressor not known to act upon the *ica* operon, was responsible for repressing *ica* expression in response to *mecA* expression and causing the PIA-independent biofilm phenotype. Alternatively, the same phenotype could be caused by the repression of an activator of the *ica* operon. The relationship between these genes could be indirect. Changes in cell wall structure resulting from differences in the enzymatic action of PBP2a from the four penicillin-binding proteins native to *S. aureus* might be the root cause of the observed phenotype. Alterations in cell wall structure could cause changes in cellular processes and alter gene expression.

Experimental approach

With nothing known about our target, and so many possibilities as to its function and identity, we elected to employ a forward genetic screen. We would use a chemical mutagen to disrupt genes at random and screen for mutants with altered *ica* expression. To be able to easily observe changes in *ica* expression we would create a reporter plasmid with *gfp* under control of the *ica* promoter. MRSA mutants with restored *ica* expression would appear green. These would be collected after observing colonies on plates, or possibly by FACS, and sequenced. Point mutations would be plotted to find mutation "hot spots" with higher mutation rates than average, indicating genes which, when mutated, lead to an increase in *ica* expression. Each of these genes would be investigated as possibly playing a role in the *mecA*-dependent repression of the *ica* operon.

Strains

The wild type strain used in this study is JE2. JE2 is a MRSA strain derived from the commonly used, highly-characterized USA300 LAC. JE2 has been cured of two plasmids found in USA300 LAC, one encoding macrolide resistance and a second cryptic plasmid. JE2 carries the *mecA* gene for beta-lactam resistance, is *ica* positive, and produces a PIA-independent biofilm type [55]. We would also be using the strain JE2 *mecA*::mar which is a transposon mutant of JE2 with a single insertion in the *mecA* gene [56]. We confirmed experimentally that this transposon insertion abolishes beta-lactam resistance and results in the reversion to the PIA-dependent biofilm type. These strains were chosen because they are genetically identical except for the transposon insertion.

We created JE2 HoR, a highly-resistant variant of JE2 created through selection on agar with high concentrations of oxacillin. In HoR strains, spontaneous mutations lead to increased *mecA* expression and higher resistance [5, 57, 58]. JE2 HoR can grow at concentrations of 100 μg/ml oxacillin, is slower growing than JE2, and exhibits a PIA-independent biofilm phenotype.

Assessment of biofilm phenotype

Biofilm phenotype was assessed by staining biofilms grown in 96-well plates. Biofilms were grown in the wells for 24 hours with no shaking, washed, and treated with proteinase K, Dnase, or sodium meta-periodate and stained with crystal violet. The crystal violet was then eluted and the absorbance at 595 nm was taken as a quantitative measure of the biofilm mass accumulated within the well. Crystal violet, which is positively charged, will stain negatively charged molecules within the cells and extracellular matrix such as proteins and DNA. Biofilm dispersal by treatment with proteinase K or Dnase indicates a PIA-independent phenotype and dispersal by sodium meta-periodate indicates a PIA-dependent phenotype.

The theory behind this assay is that without PIA production the biofilm is structurally dependent on extracellular DNA and proteins for its structural integrity and is able to be dispersed by proteinase K or Dnase treatment. If PIA is produced, proteinase K and Dnase will not be able to disperse the biofilm, but it will instead be susceptible to sodium meta-periodate treatment which oxidizes polysaccharides.

Creation of *ica* reporter plasmid

At this point in the project we encountered difficulties which led to this project being put on hold in favor of pursuing other, more fruitful experiments. What follows is the planned approach for the rest of this project. An *ica* reporter plasmid is to be used to easily assess *ica* expression levels. Determination of biofilm type by the plate based method is long and labor-intensive. A reporter plasmid allows visual assessment *ica* expression levels, and thereby biofilm phenotype, of a large number of mutant cells. This reporter is to be composed of a fusion of the *ica* promoter region and *gfp* created by overlap-extension PCR. This included 884 bp upstream of the *icaA* start codon, a region containing the entire *ica* promoter region as well as the entire *icaR* gene. We want to be sure and include any elements that may have an effect on *ica* transcription. The first 5 codons of *icaA* will be included, but will be truncated by an in-frame stop codon. A Shine-Dalgarno sequence and spacer sequence taken from the EF-TU gene of *S. aureus*, selected for its high expression, was used to ensure efficient translation. The ATG start codon of EF-TU is the precise location where we would fuse *gfp* to the *ica* promotor region (see figure 3 C). This fusion was designed with EcoRI and KpnI restriction sites on the ends for the insertion into the MCS of the plasmid pJB38 (see figure 3 A and C). This plasmid confers ampicillin resistance in *E. coli*, chloramphenicol resistance in *S. aureus*, and is temperature sensitive in *S. aureus* (requiring any *S. aureus* strains carrying the plasmid to be grown at 30°C) [59].

This plasmid is to be assembled *in vitro* from purified plasmid and the overlap-extension PCR product and transformed into *E. coli* for replication. Because DNA from *E. coli* will be restricted when introduced into *S. aureus* the plasmid would need to be passed through the intermediate strain, RN4220, a restriction-negative *S. aureus* to acquire proper DNA methylation before

transformation into the final recipient by electroporation [60]. All three of our experimental strains (JE2, JE2 *mecA*::mar, and JE2 HoR) will be transformed to serve as baselines of *ica* expression. JE2 *mecA*::mar would indicate normal *ica* expression, and JE2 and JE2 HoR would indicate reduced *ica* expression. This would also aid us in deciding whether JE2 or JE2 HoR should be used for the mutagenesis part of this study. If *ica* expression in JE2 is drastically different from JE2 *mecA*::mar we would use JE2, if not, we would use JE2 HoR. These baseline levels of fluorescence would be measured and established by flow cytometry.

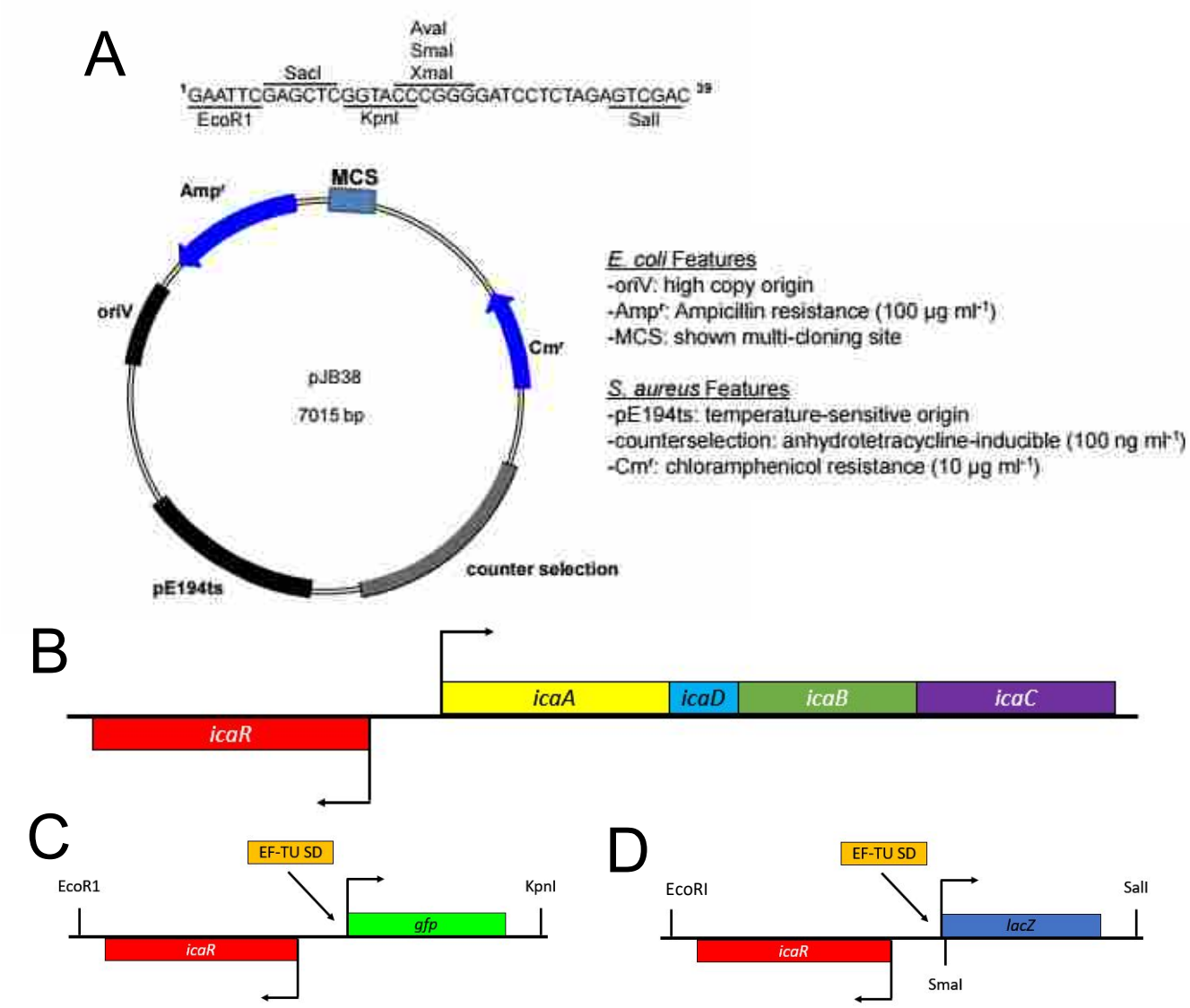

Figure 3 – Genetic maps of the cloning portion of project 1

A: Map and details of the plasmid pJB38, the backbone for *ica* reporter constructs. B: Map of the *ica* operon in its native state including the repressor of this system, *icaR*, as well as the genes responsible for PIA production, *icaA, icaD, icaB,* and *icaC*. C: Map of the insert created by overlap extension PCR for the *ica-gfp* construct. D: Map of the two inserts (shown together at the SmaI restriction site) for the *ica-lacZ* construct.

Chemical Mutagenesis

For mutagenesis we will use the mutagen ethyl methanesulfonate (EMS). EMS is a mutagenic compound which introduces random mutations through guanine alkylation, forming O-6 ethylguanine. During DNA replication, the damage becomes a permanent mutation when thymine is inserted across from the O-6 ethylguanine instead of cytosine [61]. EMS was chosen because it causes only random point mutations and it has low toxicity and high mutation rates can be achieved with minimal cell death.

To perform the mutagenesis experiment, JE2 or JE2 HoR cells containing the indicator plasmid will be grown from a freezer stock. The purity of these cells will be ensured by streaking cells to single colonies on LB agar with chloramphenicol and grown at 30°C. An isolated colony would then be picked and grown in LB until mid-log phase (approximately $1x10^8$ CFUs ml^{-1}). These cells will then be grown in media containing EMS for 1 hour at 30°C. Cells will then be pelleted and washed in a solution containing sodium thiosulphate to inactivate the EMS. A grow-out step will then be performed to allow the DNA damage to become mutation, cells will be grown in fresh LB for 3 to 3.5 hours at 30° C. Mutagenesis will be performed on 4 separate 25 ml cultures in parallel. The mutation of billions of cells in each culture, plus separate parallel cultures ensures independence of mutants recovered during the screen. Aliquots of these cultures would then be frozen in 10% glycerol at -80° C for use in the screen.

Prior to performing the mutagenesis some initial testing was needed to determine mutation rate and toxicity. A concentration resulting in approximately 90% lethality would be ideal for the high mutation rate in the surviving cells. Mutation rates as high as $5x10^{-2}$ per gene have been reported.

Screening of mutants

Mutants will be screened by fluorescence-activated cell sorting (FACS). Because the mutagenized cells would express PIA-independent biofilms, we will be screening for cells with increased *ica* expression, indicated by increased *gfp* expression. The threshold for sorting will be determined by comparison of JE2/JE2 HoR and JE2 *mecA*::mar *gfp* expression. The pooled mutant cells, both *gfp*+ and *gfp*-, will be conserved and their genomic DNA will be extracted by a phenol:chloroform extraction method or a bacterial DNA extraction kit. We plan to collect approximately 400 mutants.

Analysis of data

The pooled DNA of the collected mutants will be sequenced by next-gen Illumina sequencing. We will want at least 10X coverage of each mutant (both the *gfp*+ and much smaller *gfp*- pools). The *gfp*+ pool would have mutations in genes involved in *ica* repression, sequencing of the *gfp*- mutants would control for ancestral mutations and mutation hot spots. The raw data output from the Illumina sequencing is FASTQ format. This format gives the data for each read, including the sequence and a rating of confidence in each base. First, the reads will be mapped to a reference sequence. This reference sequence would be the sequence of JE2 in FASTA format. The program would perform an alignment and map each read to a specific location of the genome. Next, because we are only interested in mutations that occurred within an open reading frame a GTF file will be used to filter out all other mutations.

These data will then be tabulated for ease of analysis. The table is planned to contain 4 columns – gene number, number of mutations in *gfp*+, number of mutations in *gfp*-, and gene size in bp. There would be a total of 2604 rows – one for each gene. Synonymous mutations,

those which do not affect the protein product, would be removed from the mutation count. Next, the computer will analyze the number of mutations in each gene for the *gfp*+ and *gfp*- groups. Any gene with a statistically significant difference will be tagged for additional analysis. For closer analysis of each of these genes a separate file would be prepared for each. These files will give more details on the types of mutations that occurred. This file will also be in the form of a table and will list each mutation, its counts (number of reads) in each pool, nucleotide position, and amino acid change. We will then consult published literature for what is known about these genes and see if any known function gives insight into how they might function in the regulatory mechanisms of *S. aureus* biofilm production. If no known function has been assigned to these genes we would then search the sequence in BLAST to look for similar genes in other organisms that would give us insights into its functions. We would expect to see many mutations within the *mecA* gene. This will serve as a positive control for the experiment.

Site-specific knockouts of genes of interest

To confirm the role of any gene of interest found in the analysis of the mutant pool site specific mutagenesis will be done to knock out the gene. There are two strategies we could employ to do this, the choice of which would depend on whether we wanted to create the knockout in JE2 or JE2 HoR. For creation of knockouts in JE2 the process is likely already complete. The Nebraska transposon mutant library, available through BEI, contains transposon insertions in nearly all non-essential genes in JE2. We would simply need to order the mutant we want and assess its biofilm type.

If the gene of interest were unavailable in the transposon mutant library, or if we would like to create a knockout in JE2 HoR, we would need to cause a knockout through homologous

recombination. For this task we would be able to use the same plasmid, pJB38, that we used for the reporter plasmid. Instead of inserting the *ica*/*gfp* fusion we would instead clone a segment of the gene of interest. This region of homology will occasionally cause spontaneous recombination. These cells would be isolated by growing the transformed cells at 37°C. At this temperature the plasmid will be unable to replicate unless it has been integrated, through recombination, into the genome. The biofilm type would then be determined to assess the gene's impact on biofilm regulation.

Project 1 Results

Characterization of Strains

The wild-type strain used in this study was JE2, the plasmid-free variant of USA 300 LAC. The MRSA characteristics of this strain were verified through PCR amplification of the *mecA* gene and growth on LB agar containing 4 μg/ml oxacillin [62]. This strain was the wild-type HeR strain used in this study. A JE2 mutant with a transposon insertion in the *mecA* gene was used in this study as the representative MSSA strain. It was obtained from the Nebraska Transposon Mutant Library (NTML) [59]. This strain, JE2 *mecA*::mar, was verified to have a disruption in the *mecA* gene by its inability to grow on LB agar containing 4 μg/ml oxacillin [62]. For the purposes of this study a HoR strain was also needed. We created the strain JE2 HoR through spontaneous mutation by selecting for mutants able to grow on LB agar containing 100 μg/ml oxacillin as described by Pozzi et al. (see Figure 4 A) [5]. JE2 HoR was slower growing than JE2, even when both were grown on LB agar without antibiotic. JE2 HoR required two days of growth for colonies to be similar in size to JE2 colonies after a single day.

Also used extensively in this study were two strains important in the transformation process. *Escherichia coli* DH5α and *S. aureus* RN4220. The uses of these strains will be described in the section on cloning.

Assessment of the biofilm phenotypes of test strains

It was necessary to verify that the test strains used in this study exhibited the typical biofilm characteristics which had been observed by other researchers. That is, a PIA-dependent phenotype in the MSSA strain (JE2 *mecA*::mar), a PIA-dependent to intermediate phenotype in the heterogeneously resistant strain (JE2 HeR), and a PIA-independent phenotype in the homogenously resistant strain (JE2 HoR) [5, 38, 41, 43, 52, 54]. This was done through a biofilm staining assay in which biofilms were grown in 96-well plates, treated with a substance that would preferentially disrupt one biofilm type over another, and then stained do determine the degree to which biofilms were affected by the treatments (see figure 8) [5, 38, 47, 63, 64]. In this study, both proteinase K and DNase were used to indicate a PIA-independent biofilm through the disruption of extracellular DNA or proteins. Sodium meta-periodate, which disrupts polysaccharides through oxidation, is used for the identification of a PIA-dependent biofilm.

JE2, JE2 HoR, and JE2 *mecA*::mar were all tested for biofilm composition. Once stained the biofilms in the wells were inspected visually, and by optical density using a plate reader, for significant reduction in biofilm mass. JE2 saw moderate reduction in biofilm mass after treatment by both proteinase K and DNase, though the reduction by proteinase K was greater, consistent with the intermediate biofilm phenotype that it might be expected to express. JE2 HoR was highly affected by both proteinase K and DNase, demonstrating the expected PIA-

independent phenotype. JE2 *mecA*::mar was affected little by both proteinase K and DNase, consistent with the expected PIA-dependent phenotype.

We had planned to use the sodium meta-periodate assay as well as the enzymatic tests to determine the biofilm phenotypes of the test strains, but we were never able to fully develop this assay before this project was put on hold. We were never able to successfully break down PIA-dependent biofilms with sodium meta-periodate treatment. What we often saw, instead, was an increase in staining by crystal violet over the control. We suspect that there may be a chemical enhancement of the binding of crystal violet to the biofilm caused by the sodium meta-periodate.

Due to the labor-intensive nature of the 96-well plate method for biofilm analysis we wanted to develop a faster and easier assay to determine biofilm phenotype. We used Congo red agar, which has been shown to stain the amyloid fibrils of the extracellular matrix [65, 66]. After various modifications of the media formulation we were able to see slight differences in color between biofilm phenotypes (see Figure 4B). Due to the subtlety of this difference we weren't able to use this assay to reliably determine the phenotype.

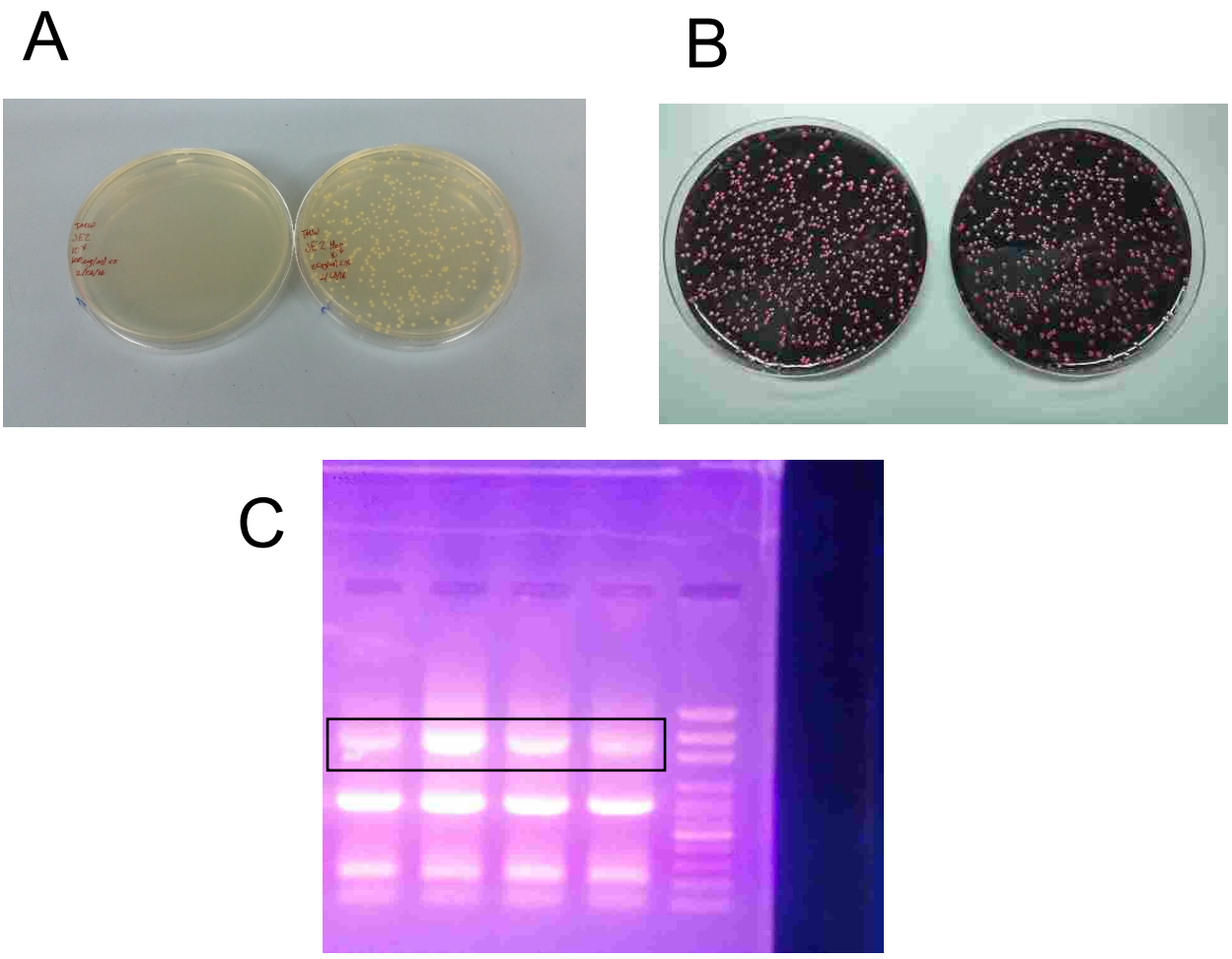

Figure 4 – Results from project 1

A: No growth of JE2 (left) vs normal growth of JE2 HoR (right) on LB agar with 100 µg/ml of oxacillin. B: Biofilm phenotype demonstrated on Congo red agar. Left is JE2 *mecA*::mar displaying slightly lighter colored colonies compared to JE2 HoR on the right. Difference was too subtle to use this method for a screen. C: An agarose gel showing the various products produced by overlap extension PCR. The boxed band is the desired 1866 bp product.

Mutagen titration

The mutagen ethyl methanesulfonate (EMS) was titrated to determine appropriate concentrations for the mutagenesis experiments. We expected a concentration of EMS with a lethality of approximately 90% would be appropriate for our mutagenesis experiments. We estimated the appropriate concentration of EMS to be between 0.25% and 1%. EMS was added to log-phase cells at the appropriate concentrations and incubated for 1 hour. Cells were centrifuged and the pellet was washed and resuspended. A serial dilution was then performed and cells were plated to compare to an un-treated control. 0.5% EMS was found to give approximately 90% lethality.

Transformation of S. aureus

S. aureus is known for being difficult to transform. Our lab had no experience transforming *S. aureus*, so we needed to develop a reliable transformation protocol. *S. aureus* transformation is a two-step transformation. After a construct is created in *E. coli* the *S. aureus* strain RN4220 is transformed by electroporation. RN4220 lacks the mechanisms that would normally destroy incorrectly methylated DNA (e.g. DNA originating in *E. coli*). The incoming plasmid will receive proper methylation in RN4220 from which it can be transformed into the final recipient strain.

Our final protocol was developed from a general protocol for *S. aureus* electroporation and modified through trial and error until it gave reliable results. We were able to take plasmids from *E. coli*, through RN4220, and into JE2, the final recipient. These transformants were confirmed by antibiotic selection and plasmid extraction.

Cloning of the reporter plasmid

The cloning of a reporter plasmid to indicate the expression of the *ica* operon began with the synthesis of the upstream promoter region of the operon linked with *gfp* through overlap-extension PCR. This was a three-step process with the first two steps being the cloning of the two genetic elements to be fused with the addition of restriction sites and overlap region and the third step being the fusion through overlap extension. The *ica* promoter region and *icaR* gene was cloned from JE2 genomic DNA with an EcoRI restriction site added at the 5' end and the EF-TU Shine-Dalgarno sequence (which doubled as the overlap between the two PCR products) added at the 3' end. The second product was the *gfp* gene, cloned from the plasmid pGFP-F acquired from the Nebraska Transposon Mutant Library, a superfolder GFP proven to be functional in *S. aureus* [59]. Primers were used to add the EF-TU Shine-Dalgarno sequence to the 5' end of this product and a KpnI restriction site to the 3' end. Both components were cloned without issue, but we encountered difficulty performing the final fusion step. When the PCR product was imaged by electrophoresis the correct molecular weight band of the final product was visible. Lower and higher molecular weight bands were visible as well (see Figure 4 C). After excision and cleanup and restriction digest we attempted to ligate the product into pJB38, but this yielded no transformants. We also attempted the process without first excising the band of interest to simplify the process, but once again were unable to recover any transformants.

Due to the lack of progress in this cloning project, we decided to start over with a new strategy. We would use *lacZ* instead of *gfp* to simplify the screen and we would clone the *ica* promoter region and *lacZ* separately. These would be ligated into pJB38 individually, removing the overlap extension step. The *ica* promoter region would be cloned to include an EcoRI restriction site at the 5' end and the EF-TU shine-dalgarno sequence and a KpnI restriction site at

the 3' end with a total product size of 1134 bp. This was cloned without issue and successfully ligated into pJB38. We designated this new plasmid pICA Next, *lacZ* was cloned from pUC19 with an existing SmaI restriction site used at the 5' end and the addition of a SalI restriction site at the 3' end giving a product of 400 bp. The 3' restriction site of the *ica* product and the 5' restriction site of the *lacZ* product did not match to keep *lacZ* in frame with the start codon and Shine-Dalgarno of EF-TU from the *ica* product. This product was successfully cloned from pUC19, but we were unable to ligate it into pICA. We were never able to recover any transformants. Many different methods of preparation, including CIP treatment of the insert, as well as various incubation times and temperatures were tried, but we were never able to successfully insert *lacZ* into pICA. During these set-backs we observed other interesting characteristics of *S. aureus* biofilms while testing our collections of hospital-associated and meat-associated strains. We decided to put this project on hold while we pursued a second project.

Project 1 Discussion

Though the goals of this project have not yet been met, most of the tools necessary to complete this project have been developed and it may yet be completed by future students. This project has also provided, for our lab, foundational tools which will likely be used in future *S. aureus* research projects. This includes our familiarization with the genetic tools available for *S. aureus*, the development of a reliable transformation protocol for *S. aureus*, and refinement of biofilm dispersal and staining assays. Additionally, the plasmid pICA may be a powerful tool for future biofilm regulation studies. It is perfectly situated for the addition of any number of genes for control by the *ica* operon, reporter or otherwise.

The final difficulty that forced us to pause work on this project was the inability to insert the *lacZ* fragment into pICA. We initially suspected that the ligation issues could be due to the use of SmaI, which produces blunt ends, causing the insert fragments to fuse together. We added an alkaline phosphatase (CIP) treatment to our procedures to prevent insert to insert fusions, but there was no change in outcome. Control plasmids that had been digested only did not yield transformants, while those that were digested and re-ligated did. One explanation for our inability to insert *lacZ* would be a restriction site disrupting mutation in pICA, pUC19, or the cloned *lacZ* fragment. In future work, the cloning process could be started over to solve a mutation issue. Alternatively, the entire cloning process could be redesigned to remove any unknown issues in the cloning design.

Project 2 – *Staphylococcus aureus* isolated from retail meats forms biofilms of composition and mass distinct from strains associated with disease in humans

Background

Due to difficulties in cloning the reporter plasmid as had originally been planned, a new project was begun to analyze some of the biofilm characteristics we had observed during the first project. We would use some of the same techniques that we had already been using to analyze the biofilm characteristics of our collections of hospital-associated and meat-associated strains which we had collected in the course of other related projects.

Meat-associated *S. aureus* collection

Our collection of meat-associated *S. aureus* isolates are all environmental isolates collected by our lab and include 32 strains, of which 11 are MSSA and 21 are MRSA. They were collected from samples of retail meat products purchased from 11 different retailers in the state of Utah and includes samples from a variety of meat types including beef, pork, chicken, and turkey. The breakdown of these strains, their origins, and resistance-profile is shown in Table 1. These isolates were confirmed as *S. aureus* through rigorous identity testing that included growth and fermentation on mannitol salt agar (MSA), gram staining, and catalase and coagulase testing [67, 68]. Isolates that had passed these tests were confirmed *S. aureus* through PCR amplification of the *S. aureus* 16S rRNA and *nuncA* genes [69]. Additionally, these strains were identified as MRSA or MSSA through their ability to grow in media containing 2 μg/ml of oxacillin and through PCR amplification of the *mecA* gene [69].

Table 1 – List of S. aureus strains used in project 2

Meat-associated strains are shown on the left, hospital-associated strains are shown on the right. A total of 32 meat-associated strains were studied (21 MRSA, 11 MSSA) and a total of 22 hospital-associated strains were studied (15 MRSA, 7 MSSA). Strain names: C strains are chicken origin, P are pork, T are turkey, and B are beef.

Strain name	*Type*	*Source*
C1	*MRSA*	*Raw chicken*
C2	*MRSA*	*Raw chicken*
C3	*MRSA*	*Raw chicken*
C7	*MRSA*	*Raw chicken*
C11	*MRSA*	*Raw chicken*
C15	*MRSA*	*Raw chicken*
C20	*MRSA*	*Raw chicken*
C4	*MSSA*	*Raw chicken*
C6	*MSSA*	*Raw chicken*
C9	*MSSA*	*Raw chicken*
C10	*MSSA*	*Raw chicken*
P3	*MRSA*	*Raw pork*
P4	*MRSA*	*Raw pork*
P5	*MRSA*	*Raw pork*
P6	*MRSA*	*Raw pork*
P11	*MRSA*	*Raw pork*
P14	*MRSA*	*Raw pork*
P7	*MSSA*	*Raw pork*
P9	*MSSA*	*Raw pork*
P10	*MSSA*	*Raw pork*
P12	*MSSA*	*Raw pork*
P13	*MSSA*	*Raw pork*
T2	*MRSA*	*Raw turkey*
T4	*MRSA*	*Raw turkey*
T5	*MRSA*	*Raw turkey*
T6	*MRSA*	*Raw turkey*
T7	*MRSA*	*Raw turkey*
T10	*MRSA*	*Raw turkey*
T14	*MRSA*	*Raw turkey*
T12	*MSSA*	*Raw turkey*
T13	*MSSA*	*Raw turkey*
B8	*MRSA*	*Raw Beef*

Strain name	Type	Source
HA 1	MRSA	Hospital pathology lab
HA 2	MRSA	Hospital pathology lab
HA 3	MRSA	Hospital pathology lab
HA 4	MRSA	Hospital pathology lab
HA 5	MRSA	Hospital pathology lab
FR1913	MRSA	Culture collection
USA 300 LAC	MRSA	Culture collection
HFH 30364	MRSA	Culture collection
NY336	MRSA	Culture collection
CO34	MRSA	Culture collection
GA92	MRSA	Culture collection
TN112	MRSA	Culture collection
CA127	MRSA	Culture collection
USA 400	MRSA	Culture collection
USA 300-0114	MRSA	Culture collection
SH 1000	MSSA	Culture collection
SA 6538	MSSA	Culture collection
SA 29213	MSSA	Culture collection
SA 25923	MSSA	Culture collection
SA 43300	MSSA	Culture collection
SA 4651	MSSA	Culture collection
SA 12600	MSSA	Culture collection

Hospital-associated *S. aureus* collection

Our collection of hospital-associated *S. aureus* consists of 22 strains acquired through culture collections and verified MRSA strains acquired through a contact in a hospital pathology department. This collection of hospital-associated strains consists of 15 MRSAs and 7 MSSAs; see Table 1.

Hypothesis

We hypothesized that the biofilm characteristics of these two *S. aureus* populations would be different, that is, that we would see distinct trends in overall biofilm mass and composition for both hospital-associated and meat-associated strains. While the origin of the strains that contaminate retail meat-products is not well understood, there is some evidence to suggest that they are of livestock origin [70-73]. Livestock-associated strains are, in general, genetically distinct from their human-derived counterparts, though there is occasional transmission from one population to the other [74-76]. Demonstrating general phenotypic differences between the biofilms formed by meat-associated strains and hospital associated strains would provide additional evidence that meat-associated strains do not generally originate from a human source. These findings would also have implications for our understanding of the virulence potential of these strains.

We also hypothesized that we would see differences in the biofilm characteristics of MRSA strains and MSSA. Research has shown a correlation between oxacillin susceptibility and biofilm composition. Highly-resistant MRSA strains tend to have proteinaceous biofilms while less-resistant MRSAs and MSSAs tend to have polysaccharide-based biofilms [5, 38, 40]. We

expected to see these differences in our collection of MRSA strains and MSSA strains from hospital and meat sources.

Experimental design

Evaluation of biofilm characteristics was performed in a 96-well plate based assay wherein biofilms were allowed to grow and adhere to the bottom of each well. Biofilms were then stained by filling each well with 0.1% crystal violet, washing, and then eluting stain from the biofilm as a measure of total organic mass accumulated over the growth period. This was performed in quadruplicate for each strain. Composition was measured through treatment with biofilm-disrupting substances before staining. Dispersal by proteinase K was used to indicate a reliance on extracellular proteins for biofilm structural integrity; DNase demonstrated eDNA as an essential structural component; and sodium meta-periodate, which degrades polysaccharides demonstrated structural dependence on PIA. Together, degradation by proteinase K and DNase indicate a PIA-independent (protein) biofilm type, whereas degradation by sodium meta-periodate would indicate a PIA-dependent (polysaccharide) biofilm type. Because SA biofilms exist in a spectrum of compositions and PIA-dependent and PIA-independent are only general categories, quantitative measurements of enzymatic or chemical impact on biofilm mass was taken by measuring the absorbance of eluted crystal violet at 595 nm. This information was used to show general trends for each SA category as well as show quantitative data for each individual strain.

Project 2 Results

The first characteristic that we analyzed was over-all biofilm mass formed by hospital-associated and meat-associated strains. Biofilm mass, as measured by crystal violet staining of biofilms formed over 24 hours of growth, includes both the cells of the biofilm as well as the extracellular matrix. The positively charged crystal violet binds to the negative charges of DNA and proteins. After cells had been grown without shaking for 24 hours in the wells of 96-well plates, the biofilms were fixed, stained, and the stain was eluted using organic solvents. The absorbance was measured at 595 nm to give a quantitative measure of biofilm mass.

Biofilm mass was found to vary considerably from strain to strain. Some strains, such as P13 or C15 (see Figure 5A) had very little biofilm formation, while others like HA1 or SH 1000, for example, had considerable biofilm accumulation (see Figure 5B). Both categories, meat-associated strains and hospital-associated strains, had strong biofilm formers and weak biofilm formers. The averages between these categories, however, were different. The average OD 595 reading for meat strains was found to be 0.887 while the average for hospital strains was 1.286 with a p value for these averages of 7.09×10^{-3} (two-sample T-test assuming equal variances) (see figure 5C).

Next, we examined the susceptibility of the biofilms formed by each strain to degradation by proteinase K. A biofilm broken down by proteinase K depends on proteins, either attached to the cell surface or in the extracellular matrix, for structural integrity of the biofilm. After biofilms had been grown for 24 hours in a 96-well plate, planktonic cells were gently washed from the wells and a solution containing proteinase K, or a mock treatment, was added to each well. The mock treatment consisted of the appropriate buffer without the enzyme. After incubation at 37° C for two hours the wells were gently washed and the biofilms were fixed and

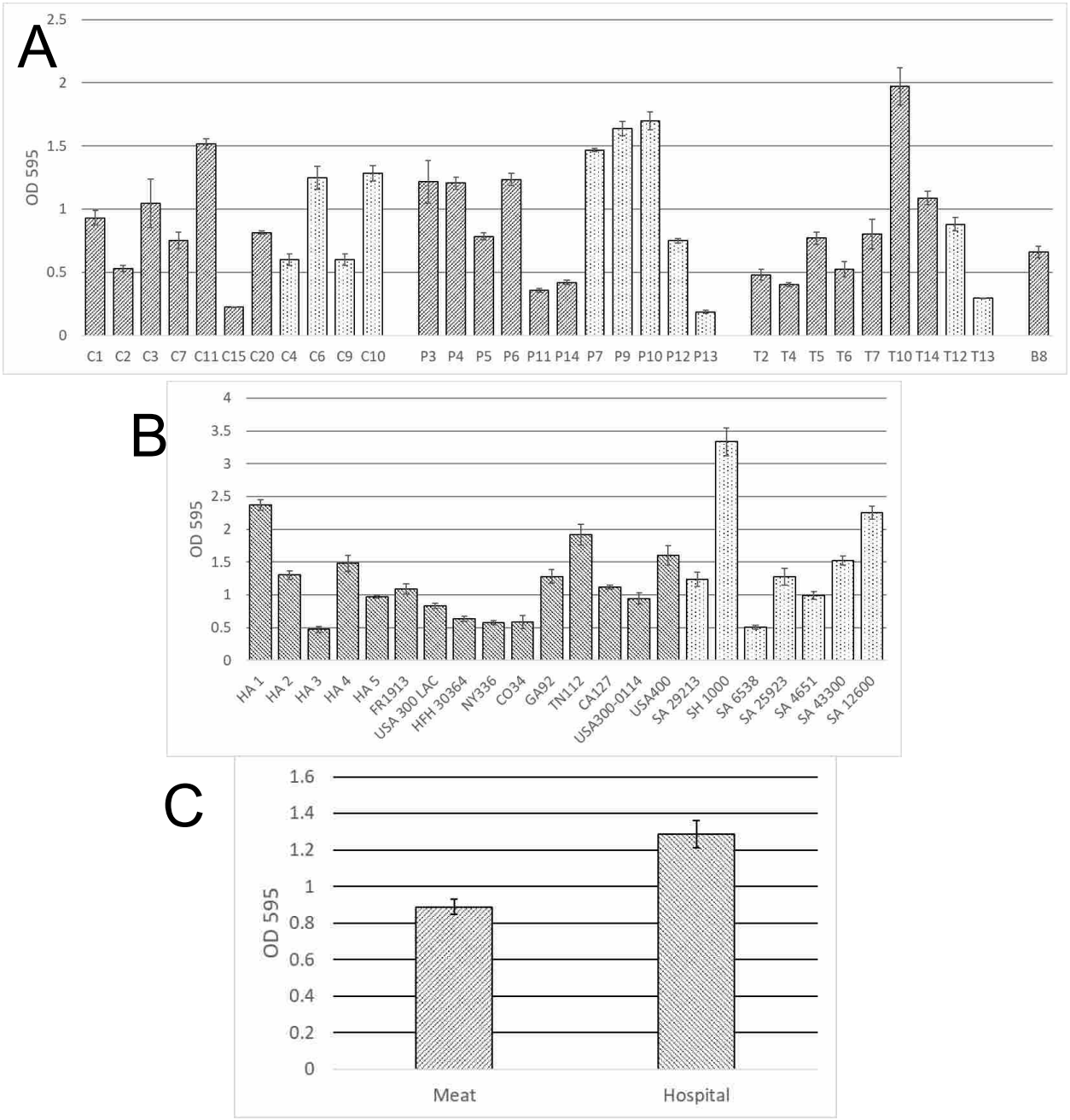

Figure 5 – Biofilm mass measured by OD

Shown is the OD 595 of crystal violet eluted from biofilms formed within the wells of a 96 well plate. Darker colored bars represent MRSA strains, light colored bars represent MSSA strains. Error bars show the standard error of the 4 replicates for each strain. A: Biofilm mass of meat-associated strains. B: Biofilm mass of hospital associated strains. C: Average biofilm mass of each category, error bars show standard error, $p = 7.0 \times 10^{-3}$.

stained. The stain was then eluted and OD was measured at 595 nm. To make valid comparisons between strains with high biofilm-mass and those with lower biofilm-mass the effect of the enzyme was measured as a percent reduction of biofilm mass calculated as the percent difference between mock treated and treated.

Both hospital-associated and meat-associated strains had some strains which were highly-affected by proteinase K (such as P5 or HA2) and some strains which were unaffected (such as C5 or SA 25923) (see figure 6 A and B). We did not observe a correlation between biofilm mass and proteinase K susceptibility. We did, however, observe a significant difference in proteinase K susceptibility between hospital-associated and meat-associated strains. The average reduction of meat strains was 33.24% while the average reduction in hospital strains was 52.01% with a p value for these averages of 8.09×10^{-4} (two-sample T-test assuming equal variances)(see figure 6 C).

Next, we measured biofilm susceptibility to degradation by DNase. As before, each strain was grown in the wells of a 96-well plate and a biofilm was allowed to form for 24 hours. Planktonic cells were gently washed away and the biofilm was treated with a solution containing DNase (or a mock treatment) for 2 hours before a final wash, fixing of the biofilm, and staining. Degradation by DNase shows reliance on extracellular DNA for the structural integrity of the biofilm. Biofilm stain was eluted and OD 595 measured. The OD reading of the treated biofilm was subtracted from that of the mock treated and the difference was given as a percentage to normalize the data so as to compare robust biofilm forming strains with weak biofilm forming strains.

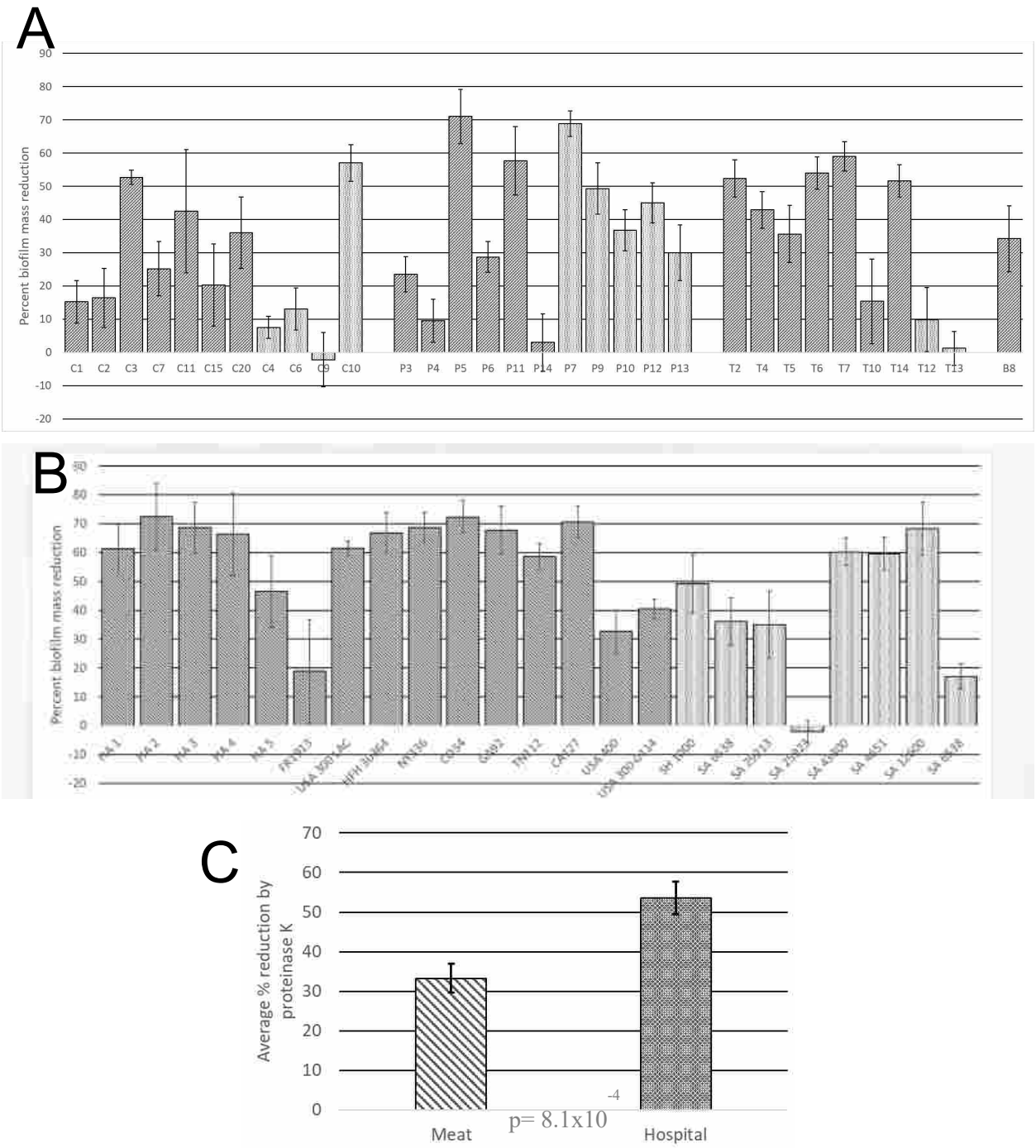

Figure 6 – Percent biofilm reduction by proteinase K

Percent reduction measured by percent change in OD 595 from mock treatment. Error bars show standard error for difference. A: Percent reduction by proteinase K for meat-associated strains. Dark bars are MRSA strains, lighter bars are MSSA strains. B: Percent reduction by proteinase K for hospital-associated strains. Dark bars are MRSA strains, lighter bars are MSSA strains. C: Average percent reduction for each category, $p = 8.1 \times 10^{-4}$

We observed that, like proteinase K susceptibility, meat-associated and hospital-associated strains had a mix of highly-affected strains (C20 and SA 43300) and unaffected strains (P10 and HA1) (see figure 7 A and B). We observed no correlation between biofilm mass and DNase susceptibility. However, there was a significant difference between the DNase susceptibility of hospital-associated and meat-associated strains. The average percent reduction in biofilm mass DNase for meat-associated strains was 47.90% and the average for hospital-associated strains was 35.93% with a p value of 3.38×10^{-3} (two-sample T-test assuming equal variances) (see figure 7 C).

We also compared each of these characteristics among MRSA strains and MSSA strains for each category (biofilm mass, proteinase K susceptibility, and DNase susceptibility) and found no significant differences. Differences were only observed when comparing strains from different sources. These characteristics had no observable relation to oxacillin resistance.

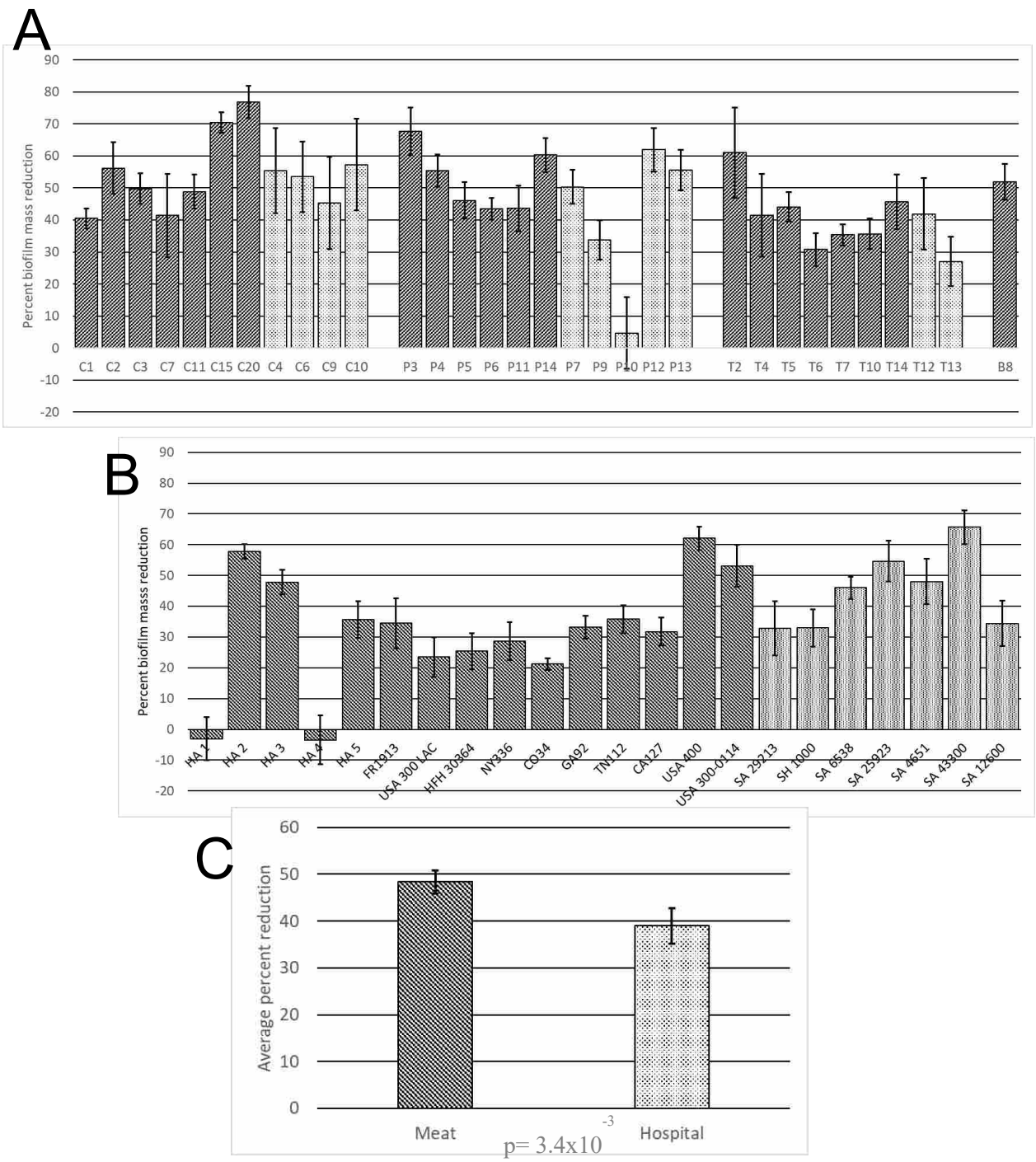

Figure 7 – Percent biofilm reduction by DNase

Percent reduction measured by percent change in OD 595 from mock treatment. Error bars show standard error for difference. A: Percent reduction by DNase for meat-associated strains. Dark bars are MRSA strains, lighter bars are MSSA strains. B: Percent reduction by DNase for hospital-associated strains. Dark bars are MRSA strains, lighter bars are MSSA strains. C: Average percent reduction for each category, $p = 3.4 \times 10^{-3}$

Project 2 Discussion

As we had hypothesized, we saw many differences between hospital-associated and meat-associated strains. This reinforces previous research showing that meat-associated *S. aureus* typically originates from an animal source since animal strains and human strains are genetically distinct populations. Hospital strains, on average, formed more robust biofilms with an average OD reading of 1.286 while meat strains had an average OD reading of 0.887 ($p=7.09x10^{-3}$). These differences are likely the result of selective pressures found in the environments to which they have adapted. Since all our hospital strains were isolated from active infections, this relationship may be influenced by the fact that biofilms are such an important virulence factor and any strain able to invade a host and establish an infection is more likely to have a strong biofilm-forming capacity. There may be other selective pressures in the hospital environment that cause hospital-associated strains to have more robust biofilms such as adaption to hard plastic surfaces, a defense against cleaning agents, or to provide some resistance to antibiotics as has been shown in prior research [34]. On the other hand, if meat-associated strains come from an animal source as research has suggested, they will be adapted to an entirely different environment [70-73]. We can assume that this environment would be much dirtier, that these strains will be subjected to different drug-challenges, and that they may be commensal.

The differences in biofilm composition between hospital-associated and meat-associated were the most surprising results. While we had expected to see compositional differences between the two strain categories, we hadn't expected proteinase K susceptibility and DNase susceptibility to be independent of one another. The average reduction of meat strain biofilms by proteinase K was 33.24% while that of hospital strains was 52.01% ($p=8.09x10^{-4}$) and the

average reduction of meat-associated strain biofilms by DNase was 47.9% while that of hospital strains was 35.93% ($p=3.38x10^{-3}$).

Because proteinase K affected hospital strains greater than meat strains and DNase affected meat strains greater than hospital strains we can see that these characteristics are not necessarily connected, as we had hypothesized. While the protein and polysaccharide biofilm dichotomy may be a useful model when considering the extremes of *S. aureus* biofilms, it appears that there is a spectrum of *S. aureus* biofilm characteristics in between with a mixture of dependence on protein, eDNA, and polysaccharides for structural integrity of the biofilm. When PIA-independent (protein) biofilms are described they are typically HoR MRSA strains [5, 38, 41, 43]. If we assembled a collection of HoR strains the relationship between high *mecA* expression and decreased *ica* expression would likely hold true and we would see high susceptibility to both proteinase K and DNase. When separated into MSSA and MRSA categories we didn't see any significant differences in the biofilm mass or composition of our strains.

Some of the interesting strains that illustrate these findings are highlighted in figures 6 and 7. For example P14 is unaffected by proteinase K, but highly affected by DNase. P10 is the opposite, it is affected by proteinase K, but DNase has no affect. Other strains, such as SA4651 were reduced by both enzymatic treatments. Interestingly, we didn't have any strains that were unaffected by both treatments. This could imply that strains may depend on proteins or eDNA, or both for structural integrity, but we have not characterized any strains that depend on neither for biofilm integrity.

In summation, we have found that on average *S. aureus* hospital-associated strains form more-robust biofilms than meat-associated strains, hospital-associated strains were more

susceptible to proteinase K degradation than meat-associated strains, and meat-associated strains were more susceptible to DNase degradation than hospital-associated strains. This implies the effect of environmental selective pressures on biofilm formation and composition.

Due to the dangerous nature of *S. aureus* as a pathogen, continued study of its biofilms is needed to improve patient outlook and public health. Biofilm mass and composition surely have great implications for virulence and may help us to understand the danger of common exposure sources such as raw meats.

Methods

Isolation and identification of *S. aureus*/MRSA from meat samples

Strains isolated from retail meats were identified as *S. aureus* through a variety of tests. First, meat samples were swabbed with sterile swabs or pipetting 10 µl of meat juices and streaked directly onto Mannitol Salt Agar (MSA) plates (Thermo Scientific, Waltham, MA) and grown overnight at 37° C. Isolates were assessed for their ability to grow on this media as well as for their ability to ferment mannitol (indicated by the yellowing of the media around colonies). Possible MRSA strains were identified by their ability to grow on MSA plates containing 2 µg/ml oxacillin, the standard substitute for methicillin which is no longer used clinically. Gram stains were performed to confirm that these isolates were gram positive cocci, followed by catalase and coagulase tests, for which all of our SA/MRSA strains were positive. As a final confirmation of the identities of these environmental isolates, genotypes were assessed by a triplex PCR assay to detect Staphylococcus-specific 16S rDNA sequences, the *nucA* gene which is specific to *S. aureus*, and the *mecA* gene which is specific to MRSA as described by Maes et al. [69]. See primers in table 2.

Selection of homogeneously-resistant (HoR) strain

A homogeneously-resistant (HoR) mutant of JE2 was selected through random mutation selected for on media with high oxacillin concentration. JE2 was grown in LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) containing 2 µg/ml oxacillin overnight. 1 ml of liquid culture was pelleted and resuspended in 100 µl of LB broth and spread on LB agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, 12 g/l agar) containing 100 µg/ml oxacillin and incubated overnight at 37° C. JE2 HoR was selected from these colonies and was streaked for purity before archiving at -80° C in 20% glycerol and used in future experiments.

Mutagen titration

The mutagen ethyl methanesulfonate (EMS) was titrated to determine appropriate concentrations for SA mutagenesis experiments. We expected a concentration of EMS with a lethality of approximately 90% would be appropriate for our mutagenesis experiments. We estimated the appropriate concentration of EMS to be between 0.25% and 1%. Cells were grown to mid-log phase in LB broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) at 37° C and 200 rpm shaking, approximately 5 hours and aliquoted into 2.5 ml volumes. EMS was added at concentrations of 0.25%, 0.5%, 0.75% and 1% and the cells were incubated 1 hour at 30° C and 200 rpm shaking. A lower temperature of 30° C was used to mirror the lower temperature required when using a temperature-sensitive plasmid in later experiments. Cells were then pelleted, washed, diluted in increments of factors of 10, and plated. The number of resulting colonies was counted and compared to an untreated control to determine EMS lethality.

Biofilm assay

Biofilm mass was measured through an assay in which biofilms were grown and stained in 96-well plates (see figure 8). Strains to be tested were grown overnight at 37° C in tryptic soy broth (TSB) (Sigma-Aldrich, St. Louis, MO). Cultures were then diluted 1:200 in 66% TSB with 0.5% glucose added. 200 µl of culture dilution were added to the wells of a 96-well, flat-bottomed, tissue-culture plate (Corning Incorporated, Corning, NY) in quadruplicate, covered, and incubated for 24 hours at 37° C. The liquid and non-adhered cells were then removed from the wells by gently overturning the plate onto paper towels. Each well was then gently washed with phosphate-buffered saline (PBS) and allowed to dry. Once dry, 205 µl of 100% ethanol was added to each well to fix the biofilms, incubated for 15 minutes at room temperature, and then emptied onto a paper towel. Once the ethanol had dried 205 µl of 0.1% crystal violet dye was added to each well and incubated for 15 minutes at room temperature. The dye was then emptied as before, by overturning the plate onto paper towels, and three washes with 210 µl of ddH_2O were performed, dumping the water between each wash. Once dry, stain was eluted from the biofilms with 205 µl of a mixture of 1/3 volume of EtOH with 40 mM HCl and 2/3 volume of acetone added to each well. The wells were sealed and incubated with this solution for 15 minutes at 37° C with 100 rpm shaking. 80 µl of eluted stain was removed from each well and transferred to a new plate for reading and the absorbance at 595 nm was measured for each well.

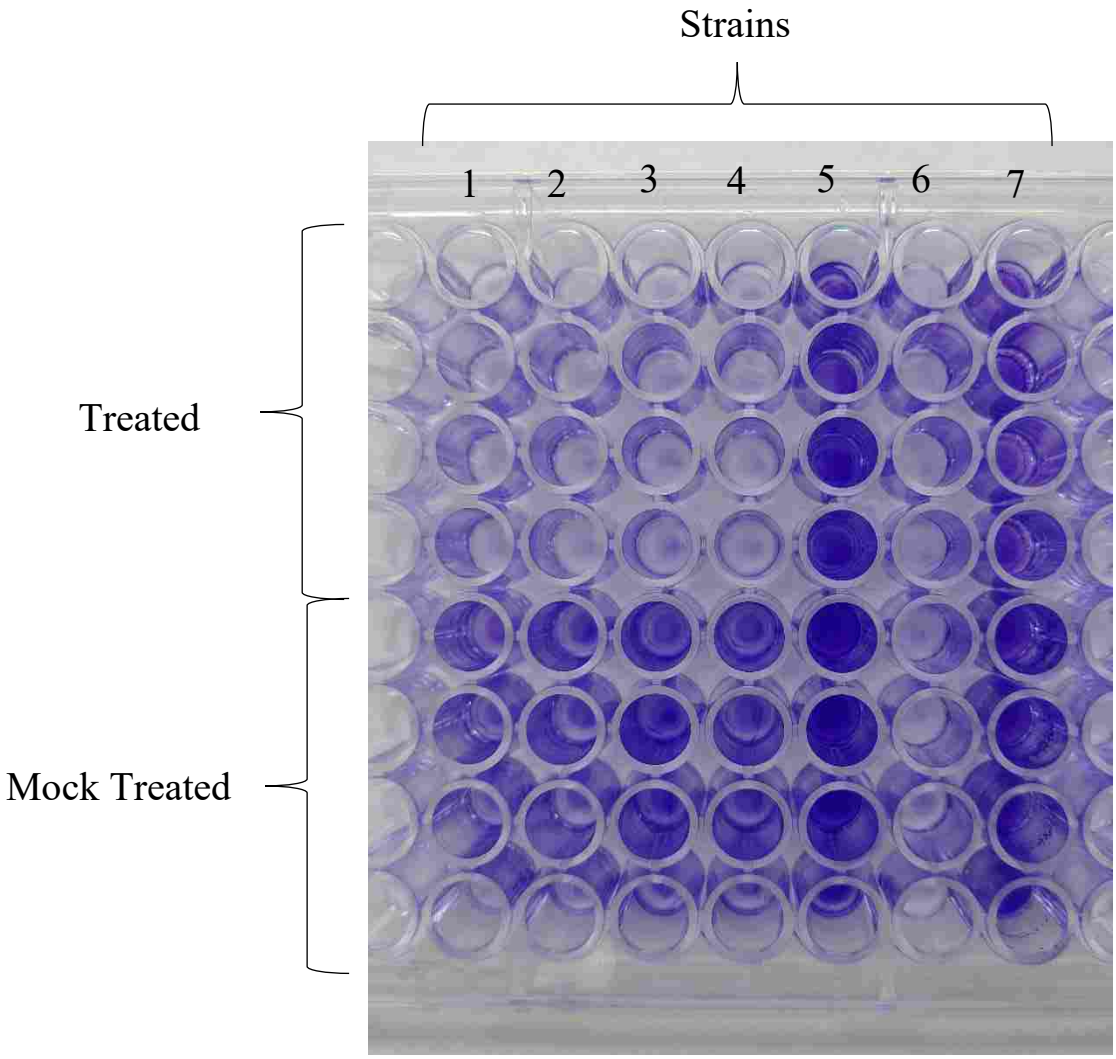

Figure 8 – 96-well plate biofilm assay

Shown is an example of a biofilm reduction assay as would be performed with proteinase K or DNase. Strains being tested are organized in columns with four treated wells and four mock treated wells. Various biofilm characteristics can be observed in this picture. Column 5, for example forms very robust biofilms that are affected very little by enzyme treatment. Column 4 forms moderately robust biofilms but are heavily affected by the enzyme treatment. Column 6 forms weak biofilms, but is less affected by the enzyme treatment.

Biofilm dispersal assay

Biofilm dispersal assays were performed using a similar method to that described for the general biofilm assay with an added enzymatic treatment step after the biofilms are first drained and washed with PBS. 8 wells of each strain are grown, 4 control wells and 4 test wells (see figure 8). A solution containing the desired enzyme, DNase or proteinase K, is added to each well and incubated for 2 hours at 37° C. The wells are then drained, washed with PBS, and allowed to dry before the assay proceeds with ethanol fixation as described for the general biofilm assay. The DNase solution consists of 140 U/ml DNase in TSB. The proteinase K solution consists of 100 µg/ml proteinase K in 10 mM Tris-HCl, pH 7.5. The controls for each treatment were mock treated with the respective solution without the enzyme. Percent difference in absorbance at 595 nm was used as a measurement of biofilm dispersal by these enzymes.

Cloning of lacZ and the ica promoter region

Both *lacZ* and the *ica* promoter region were cloned by PCR using primers to add restriction sites and the EF-TU Shine-Dalgarno sequence. For *lacZ*, pUC19 plasmid DNA was used as the source of the gene. A PCR program of 94° C for 5 minutes, 40 cycles of 94° C for 30 seconds, 59° C for one minute, and 72° C for one minute, followed by a final extension of 72° C for 10 minutes was used with the primers for *lacZ* (see table 2), yielding a product of 400 bp. The *ica* promoter region was amplified from JE2 genomic DNA using a PCR program consisting of 94° C for 7 minutes with 40 cycles of 94° C for 30 seconds, 55° C for 30 seconds, and 72° C for one minute, followed by a final extension at 72° C for 10 minutes with the primers for *ica-lacZ* ica (see table 2), yielding a product of 1134 bp.

Table 2 – Primers used in project 1 and project 2

lacZ* for *ica-lacZ	
Forward	5’-GCGGTCGACGACGGTGAAAACCTCTGACACA-3’
Reverse	5’-TGTGTGGAATTGTGAGCGGAT-3’
ica* for *ica-lacZ	
Forward	5’-CGCGAATTCCAGGGGAACATTACACTTTTATAA-3’
Reverse	5’-GCGGGTACCCATTATAAAATCTCTCCTACAGGCTTCTTGTTCAATGAA-3’
***ica* for *ica-gfp* (overlap extension)**	
Forward (out)	5’-CGCGAATTCCAGGGGAACATTACACTTTTATAA-3’
Reverse (in)	5’-CATTATAAAATCTCTCCTACAGGCTTCTTGTTCAATGAA-3’
***gfp* for *ica-gfp* (overlap extension)**	
Forward (in)	5’-AACTAGGAGAGATTTTATAATGCCCGGGAGCAAAGG-3’
Reverse (out)	5’-GCGGGTACCTTATTTGTAGAGCTCATCCATG-3’
mecA	
Forward	5’-AAAATCGATGGTAAAGGTTGGC-3’
Reverse	5’-AGTTCTGCAGTACCGGATTTGC-3’
16S rDNA	
Forward	5’-GCGGATCCTGACTGCAGTGCCAGCAGCCGCGGTAA-3’
Reverse	5’-GCGGATCCGCGGCCGCGGACTACCAGGGTATCTAAT-3’
nucA	
Forward	5’-GCGATTGATGGTGATACGGTT-3’
Reverse	5’-AGCCAAGCCTTGACGAACTAAAGC-3’

Overlap extension PCR

An overlap-extension PCR technique was used to first amplify and then splice the *ica* promoter region and *gfp*. The *ica* promoter region was amplified from JE2 genomic DNA with the primers for ica (forward out and reverse in) (see table 2) and a program consisting of 94° C for 5 minutes followed by 20 cycles of 94° C for 30 seconds, 38.9° C for 30 seconds, and 72° C for one minute, followed by a final extension of 72° C for 10 minutes. *gfp* was amplified from the plasmid pGFP-F with the primers for *gfp* (forward in and reverse out) (see table 2) and a program consisting of 94° C for 5 minutes followed by 20 cycles of 94° C for 30 seconds, 46.9° C for 30 seconds, and 72° C for one minute, followed by a final extension of 72 ° C for 10 minutes. 5 µl of each of these reactions was added to a new PCR reaction tube and run at 94° C for 5 minutes followed by 15 cycles of 94° C for 30 seconds, 38.9° C for 30 seconds, and 72° C for one minute and once finished 2 µl of both the primers For_out and Rev_out were added to the reaction which was then run 30 cycles consisting of 94° C for 5 minutes followed by 30 cycles of 94° C for 30 seconds, 46.9° C for 30 seconds, and 72° C for one minute, followed by a final extension at 72° C for 10 minutes. The product of 1137 bp was visualized on and excised from a 1% agarose gel and cleaned up using the Zymoclean™ Gel DNA Recovery Kit by Zymo Research.

Recovery of DNA from agarose gels

We used the Zymoclean™ Gel DNA Recovery Kit from Zymo Research for purification of DNA from agarose gels and followed the protocols provided by the manufacturer.

Plasmid Mini-prep

Plasmid mini-preps were performed using the E.Z.N.A® Plasmid DNA Mini Kit I from Omega Bio-Tek. The protocols provided for this kit by the manufacturer were followed.

Genomic DNA extraction

Genomic DNA extraction was performed using the E.Z.N.A.® Bacterial DNA Kit from Omega Bio-Tek. We followed the protocols provided by the manufacturer for this kit.

Restriction digest and CIP treatment

The restriction enzymes used were purchased from New England Biolabs. The restriction enzymes EcoRI-HF, KpnI-HF, SmaI, and SalI-HF were used. We followed the manufacturer's instructions and used the provided buffer for digestion. All digestions were performed at 37° C with the exception of SmaI which is active at 25° C.

We acquired our Calf Intestinal alkaline phosphatase (CIP) from New England Biolabs. When CIP treatment was included, the manufacturer's instructions were followed. The phosphatase was added directly to the digestion reaction and incubated for 30 minutes at 37° C, the DNA was then purified by spin column prior to ligation.

DNA cleanup and purification

Prior to ligation, DNA was purified using the E.Z.N.A Cycle Pure Kit by Omega Bio-Tek. The protocols provided by the manufacturer for this kit were followed.

Ligation

T4 DNA ligase from Thermo scientific was used for ligations. 20 µl reactions were performed which included 2 µl of 10X DNA ligase buffer, 1 µl of DNA ligase, and the remaining volume consisting of ddH_2O, vector DNA, and insert DNA. Several different concentrations of vector DNA and insert DNA were used in the troubleshooting of this step. The reaction mixture was incubated for 2 hrs at room temperature or overnight at 16° C.

Preparation of chemically competent *E. coli* cells

An overnight culture of *E. coli* DH5α is used to inoculate a flask with 50 ml of LB broth and grown to logarithmic phase. The culture is pelleted at 8000 rpm for 5 min and resuspended in ice-cold 30 mM $CaCl_2$. Cells are distributed by 0.5 ml into microcentrifuge tubes (all kept on ice) and pelleted at 30 seconds at 10,000 g. Each pellet is resuspended in 0.5 ml ice-cold $CaCl_2$ and flicked to mix. 50 µl volumes were then distributed into pre-chilled microcentrifuge tubes and immediately frozen at -80° C.

Transformation of *E. coli*

E. coli DH5α was used as the recipient cells for our transformations of *E. coli*. Cells are thawed on ice for 20 minutes, 1-5 µl of DNA is then added to the bottom of a 15 ml conical centrifuge tube and the thawed *E. coli* cells are added to the tube and incubated on ice for 20 minutes. Tubes are then transferred directly from the ice to a water bath at 42° C and incubated for 90 seconds and then placed back on ice for two minutes. 250 µl of LB broth is then added to

the tube and incubated at 37° C in the shaking incubator for 45 minutes. Cells were then plated on selective media and allowed to grow overnight at 37° C.

Preparation of electrocompetent *S. aureus* cells

10 ml of overnight culture is added to 90 ml of LB media and grown to OD578 = 0.6. Two 50 ml centrifuge tubes are filled with bacterial culture and placed on ice for 15 minutes. They are then centrifuged at 4000 rpm for 7 minutes at 4° C. Each is then washed with 50 ml ice-cold sterile water and again centrifuged as before and washed one more time with 50 ml of ice-cold sterile water and centrifuged as before. The cells are then washed with 16 ml of ice-cold sterile water and pelleted at 4000 rpm for 5 minutes at 4° C. Cells are then washed with 2 ml ice-cold sterile 10 % glycerol and centrifuged at 4000 rpm for 3 minutes at 4° C. Cells are then washed with 1 ml of ice-cold 10% glycerol and pelleted at 4000 rpm for 3 minutes at 4° C. The cells are then resuspended in 700 µl of ice-cold 10% glycerol and immediately frozen in 70µl aliquots at -80° C.

S. aureus transformation by electroporation

Electrocompetent *S. aureus* cells are thawed on ice for approximately 5 minutes and then incubated at room temperature for 10 minutes. Cells are pelleted at 10,000 rpm for 5 minutes and resuspended in 500 µl of EC buffer (0.5 M sucrose and 10% glycerol). Cells are pelleted at 10,000 rpm for 5 minutes and resuspended in 85 µl of EC buffer. 5-15 µl of DNA is then added and gently mixed. Mixture is transferred to a 1 mm gap electroporation cuvette and pulsed twice manually, with approximately 1 second between pulses, at 2.4 kv using a Bio-Rad MicroPulser

electroporator. Immediately add 900 µl of LB media and transfer to a culture tube. Incubate at 30° C (for temperature sensitive plasmid) for 2 hours and then spread on selective media and grow overnight at 30° C.

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