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Transduction as the method of horizontal gene transfer of the Staphylococcal Chromosomal Cassette mec (SCCmec)

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Transduction as the method of horizontal gene transfer of the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*)

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) gains resistance to β-lactam antibiotics through a mutated penicillin binding protein (PBP2a) encoded on the SCC*mec* element. In combination with the recombinase encoded by *ccr*, these two genes are used as markers of the mobile genetic element (SCC*mec*). Due to recent increases in community acquired MRSA infections, the mechanisms of antibiotic resistance gene transfer have gained attention. Transduction, a method of horizontal gene transfer mediated by bacteriophage, is believed to be responsible for the movement of the SCC*mec* element. Recent studies have shown the transduction of the SCC*mec* element in clinical isolates; however, this study is more concerned with transduction in the environment. The preliminary study presented here was based on two studies demonstrating the presence of the *mecA* gene in viral fractions from environmental sources by polymerase chain reaction (PCR). This study aimed to confirm the presence of the SCC*mec* element in environmental bacteriophage populations through PCR analysis and sequencing. Approximately 22% of the environmental samples collected contain *mecA* and/or *ccr*. One positive sample was sequenced, confirming the presence of the *mecA* gene and defining it as Type 1. Samples from non-fecal sources were more likely to contain one or both genes, and compost samples have the greatest percent (65%) positive. This preliminary study left many questions unanswered, spurring a second study with goals to determine the frequency of transduction and the allotype of the SCC*mec* element most frequently transduced. A number of bacterial isolates were collected and characterized. This works sets the stage for isolation of phages and transduction experiments in the future. The

results of this work will lead to a better understanding of how antibiotic resistance genes are transferred in the environment, which could lead to preventative applications.

Introduction

The increasing population of antibiotic resistant bacteria in hospital settings and in the environment is concerning, in particular, the increasing incidence of community acquired methicillin resistant *Staphylococcus aureus* (MRSA) (Colomer-Lluch *et al*., 2011). *Staphylococcus aureus* is an opportunistic pathogen that presents health concerns for both humans and animals (Larkin *et al*., 2009). In addition to methicillin resistance, many MRSA strains have gained resistance to vancomycin, linezolid (an oxazolidinone), daptomycin (a lipopetide), and tigecycline (a modified tetracycline) leaving few options for treatment (Fuda *et al*., 2006, Lemaire *et al*., 2008). These resistance genes are believed to occur naturally in bacteria that produce antibiotics and are likely transmitted to nearby bacteria experiencing the antibiotic pressures (Colomer-Lluch and Muniesa, 2011).

The genes responsible for methicillin resistance in *S. aureus* include blaZ (beta-lactamase) and *mecA* (penicillin binding protein 2a). Penicillin binding protein 2a (PBP2a) confers methicillin resistance through a low binding affinity for methicillin and a slow acylation rate (Fuda *et al.*, 2004). This gene is transferred on a mobile genetic element named the Staphylococcal Chromosomal Cassette *mec* (SCC*mec*) (Figure 1). When *S. aureus* acquires the SCC*mec* element, it is termed methicillin resistant *S. aureus* or MRSA (Colomer-Lluch and Muniesa, 2011). The *SCCmec* element has 11 allotypes in *S. aureus* and 5 allotypes in coagulase negative Staphylococcus (CoNS). The allotype is determined by the combination of the *mecA* and *ccr* genes present on the SCC*mec* element. The *ccr* gene encodes a recombinase that assists with chromosomal excision and incision of the element (Kondo *et al.,* 2007).

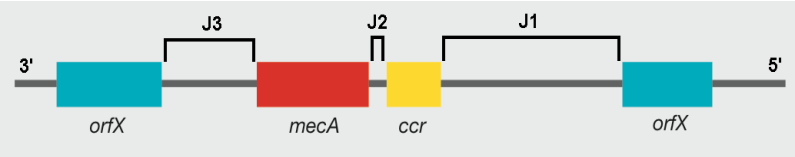


Figure 1. A generalized schematic representation of the SCC*mec* element. OrfX represents insertion sequences flanking both ends of the element which is the site the *ccr* encoded recombinase binds. The three J regions are referred to as Junkyard regions and contain various genes such as repressors and inducers. These junkyard regions are highly variable and contain a lot of non-coding DNA.

A better understanding of how the SCC*mec* element is transferred between bacteria could have potential therapeutic or prophylactic implications. The most common method of transfer of antibiotic resistance genes between bacteria is horizontal gene transfer (HGT) of mobile genetic elements (Dzidic and Bedeković, 2003). There are three known methods of horizontal gene transfer among bacteria – transformation, conjugation, and transduction. Transformation occurs when competent bacteria take up DNA from the environment; conjugation occurs when a bacterium transfers a plasmid or chromosome through a pilus to another bacterium; and transduction occurs when a bacteriophages packages bacterial DNA and transfers in to another bacterium (Jiang and Paul, 1998). Although transformation and conjugation have not been exhausted as possibilities, transduction is the hypothesized method of transfer and is explored here.

Transduction has been documented in many environmental settings including lakes, oceans, rivers, sewage treatment plants, soil, shellfish, mice, and on the surface of plants (Miller, 2001). The frequency of transduction in the Tampa Bay Estuary was estimated to be 1.3×10^{14} events per year (Davison, 1999). Due to the presence of *Staphylococcus* in marine environments (Soge *et al.*, 2009), the occurrence of

transduction in the Tampa Bay Estuary can be related to SCC*mec* transduction among Staphylococcal species.

Metagenomic studies have shown that antibiotic resistance genes are present in environmental samples, but transduction of these genes in the environment has not been shown (Balcazar, 2014). Additionally, certain bacteriophages are known to induce the excision of pathogenicity islands, which are then packaged in bacteriophages (Chen and Novick, 2009). Together, these studies suggest that it is possible for the SCC*mec* element to be transduced in the environment.

Although transduction studies have not been completed using environmental isolates, two recent studies demonstrate transduction of the SCC*mec* element in clinical isolates (Scharn *et al.*, 2013 and Maslanova *et al.*, 2013). SCC*mec* elements Type I and Type IV were transduced at a low frequency by the highly studied *Staphylococcus* phages 80α and 29 suggesting that transduction could be the method of antibiotic resistance gene transfer. A penicillinase plasmid is required for the transduction of the SCC*mec* element and an incubation time of 48-72 hours is required before *mecA* is expressed (Scharn *et al.*, 2013). Maslanova *et al.* showed that the frequency of transduction of genes located on the SCC*mec* element is approximately 10^{-4} for transducing phages and 10^{-8} for non-transducing phages (2013).

Two recent studies prompted the work presented in this study. The first study quantified the presence of the blaZ and *mecA* genes in bacteriophage populations from wastewater (sewage) and river water collected in Spain. From the samples obtained, bacteria were first quantified and characterized (Colomer-Lluch and Muniesa, 2011).

The methods used gave a good indication of the total number of bacteria easily cultured in a lab setting, but did not analyze the quantities of bacteria that are not easily cultured in the lab. The ampicillin resistant bacteria were then characterized and determined to be *S. aureus* based on their production of a yellow pigment and catalase (an enzyme which breaks down hydrogen peroxide) (Colomer-Lluch and Muniesa, 2011). These methods for assuming *S. aureus* are not sufficient since they only tested two characteristics not only shared by several species within the genus *Staphylococcus*, but also shared with member of distinct genera. After characterizing the bacteria in their sample, Colomer-Lluch and Muniesa searched for phage using transmission electron microscopy (2011). The presence of phage was confirmed and quantitative PCR of the viral fractions showed that 10^2 to 10^4 copies of blaZ were detected per milliliter of sewage, and river samples contained an order of magnitude fewer copies. The results for *mecA* were less consistent and only showed a maximum of 10^2 copies of the gene/mL (Colomer-Lluch and Muniesa, 2011). These amplicons were not confirmed by sequencing and larger portions of the SCC*mec* element were not shown to be present in the phage populations.

Colomer-Lluch *et al.* published a follow-up study focusing on the detection of blaZ and *mecA* in animal wastes. Seventy-one samples (8 mixed cow fecal slurries, 28 individual cow fecal samples, 9 mixed pig fecal slurries, 16 mixed poultry fecal slurries, and 10 waste samples from a mixed variety of animals) were collected from slaughter houses and farmland in Spain (Colomer-Lluch *et al.*, 2011). Quantitative PCR targeting a single *mecA* variant indicated that the gene was present in some of these samples. Sequence confirmation was briefly mentioned for the antibiotic resistance genes (Colomer-Lluch *et*

al., 2011). However, only 24 genes were confirmed and no data was presented (nor is it stated) that any of these sequenced genes were *mecA*.

The gaps and failed confirmations in the two Colomer-Lluch studies presented above led to the development of protocols to confirm the claims that *mecA* can be detected in wastewater, river water, and animal fecal samples. These protocols were then optimized for detection of *mecA* and altered to gather more information about the presence of the gene in the environment. A number of positive samples were found and one was confirmed by sequencing. Finally, protocols were established to determine if *mecA* is actually transmitted in the natural environment. These protocols will eventually be expanded to determine the requirements and frequency of transduction of the SCC*mec* element between environmental bacteria.

Methods

Extracting bacteriophages from agricultural samples

Samples were collected from environmental sources where *S. aureus* is known to naturally occur. These sample sources included stream water, lake water, feces, compost, sewage, and miscellaneous samples. Bacteriophages were extracted from the solid samples by doubling or tripling the volume with the addition of 1X *S. aureus* phage buffer. The samples were then incubated for at least 24 hours at room temperature.

Filtration

To separate the liquid and solid portions of the samples, an extensive process of filtration and centrifugation was used. After some optimization, an efficient way of separating solid and liquid portions was developed and that method is described here. Solid/liquid mixtures were strained through cheese cloth to initially remove large particulates. Next, the flow through was centrifuged at low speeds (speed and time varied) to separate the solid from the liquid. The liquid supernatant was decanted and passed through gravity filters of varying pore sizes to prepare them for sterile filtration. Liquid samples also went through a filtration process, but typically did not need more than gravity filtration. Once the samples were void of large particulates, the samples were sterilized through 0.4μm filters (Nalgene).

Phage Precipitation

The bacteriophages were precipitated using polyethylene glycol (PEG-8000) and sodium chloride (NaCl). For every 200 mL of liquid, 20 grams of PEG-8000 and 5.8 grams of NaCl were dissolved in the sample. Then the solution was centrifuged at 8,000

x g for 50 minutes to precipitate the phage into a pellet. The supernatant was decanted and the pellets were allowed to dry. The pellet remaining at the bottom of the tubes was resuspended in 1mL of distilled water. The same 1mL of water was used to wash out all of the tubes, if the sample had to be spilt into multiple tubes to be centrifuged.

DNA extraction

DNA was extracted from the bacteriophages by resin extraction. To every 1mL of resuspended pellet, 2mL of DNA purification resin (Promega Wizard PCR Preps) were added and mixed by pipetting. Next, 0.8mL of the mixture was added to two columns (Zymo) which were then placed into 2 mL tubes and spun in a microcentrifuge at 13,000 x g for 1 minute. The flow through was discarded and any remaining DNA-resin mixture was added to the columns and centrifugation was repeated. After discarding the flow through, the PEG and NaCl were washed off with 0.5 mL of isopropanol. The columns were spun at 13,000 x g for 1 min, rotated 180° and spun for an additional 30 seconds. The flow through was discarded. Next, DNA was eluted with 50 μL of hot water (approximately 85^{o}C). After the water was added to the column, it was allowed to sit for 1 min then was spun at 13,000 x g for 1 minute into a sterile micro tube. To ensure all DNA was eluted from the column, the elution process was repeated 3 more times, each time using a new micro tube to collect the flow through. DNA concentration was detected using a nanodrop spectrophotometer.

Multiplex PCR

Since there are many varieties of the *mecA* and *ccr* genes, multiplex PCR was used to amplify any genes present. Two multiplexes were created – one for each gene. The *mecA* multiplex contained 10 μM of mA1, mA2, *mecA*400fwd, *mecA*400rev,

*mecA*2_500fwd, and *mecA*2_500rev. The *ccr* multiplex contained 10 µM of a1, a2, a3, a4.2, B4.2, yR, and yF and 30 µM of Bc since it is a primer designed to anneal to a region conserved among three allotypes (Table 1). To each PCR tube 10µL of 2X master mix (DNA polymerase, dNTPs, and buffer), 1µL of the primer multiplex, and 9µL of DNA were added for a total volume of 20µL. The DNA added to each reaction was maximized to account for the possibility of mixed samples. Three controls were used. DNA from *S. aureus* strains N315 and COL were used as positive controls – each amplifying different versions of the mec and *ccr* genes. *S. aureus* strain RN4220 was used as a negative control. The controls were set up by adding 10µL of 2X master mix, 1µL of the primer multiplex, 1µL of DNA, and 8µL of sterile water for a total volume of 20µL. The thermocycler was set for 94°C (2 min); 45 cycles of 94°C (2 min), 57°C (1 min), and 72°C (2 min); and a final step of 72°C (2 min) (Figure 2). After PCR, 10µL of each sample was mixed with 2µL 6x loading dye. The samples were electrophoresed on a 1% agarose gel using 1X TBE buffer. Two ladders were used for size comparison – 1kb and 100bp. The gel was then imaged using the BioRad gel doc.

Table 1. Primer sequences used in multiplex PCR. Two sets were used and each primer was added in a 10µM concentration.

***mecA* multiplex primers**			
Primer name	**Sequence**	**Gene target**	**Primer pair (expected band size)**
*mecA*_400F	TGCTAGAGTAGCACTCGAATTAGGC	*mecA*	*mecA*_400R (407 bp)
*mecA*_400R	GTTCTGCAGTACCGGATTTGCC	*mecA*	*mecA*_400F (407 bp)
*mecA*2_F	GCCGTGTTTATCCATTGAACGAAGC	*mecA*2	*mecA*2_R (496 bp)
*mecA*2_R	TGGGTTGAACCTGGTGATGTAGTG	*mecA*2	*mecA*2_F (496 bp)
mA1	TGCTATCCACCCTCAAACAGG	*mecA*	mA2 (286 bp)
mA2	AACGTTGTAACCACCCCAAGA	*mecA*	mA1 (286 bp)
***ccr* multiplex primers**			
Primer name	**Sequence**	**Gene target**	**Primer pair (expected band size)**
α1	AACCTATATCATCAATCAGTACGT	*ccr*A1-*ccr*B	βc (695 bp)
α2	TAAAGGCATCAATGCACAAACACT	*ccr*A2-*ccr*B	βc (937 bp)
α3	AGCTCAAAAGCAAGCAATAGAAT	*ccr*A3-*ccr*B	βc (1791 bp)
βc	ATTGCCTTGATAATAGCCITCT	*(see α1, α2, and α3)*	
α4.2	GTATCAATGCACCAGAACTT	*ccr*A4-*ccr*B4	β4.2 (1287 bp)
β4.2	TTGCGACTCTCTTGGCGTTT	*ccr*A4-*ccr*B4	α4.2 (1287 bp)
γF	CGTCTATTACAAGATGTTAAGGATAAT	*ccr*C	γR (518 bp)
γR	CCTTTATAGACTGGATTATTCAAAATAT	*ccr*C	γF (518 bp)

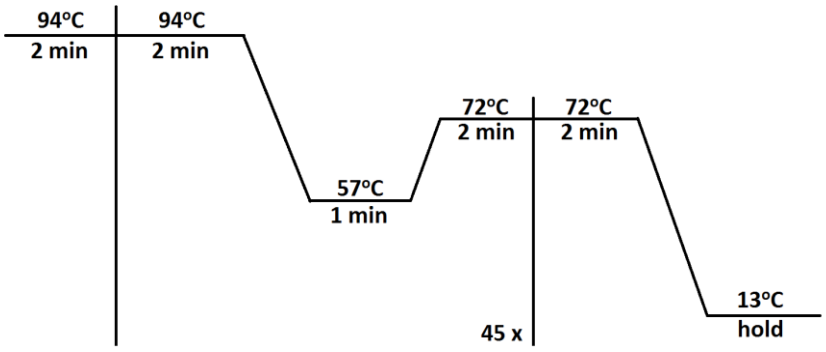


Figure 2. PCR scheme for both the *mecA* and *ccr* multiplex primer sets.

Cloning and Transformation

In order to ensure that a single PCR product was sent off for sequencing, the positive samples were cloned. If a bright, clearly defined band was obtained, 4 μL of the PCR product, 1 μL of PCR 4 TOPO TA cloning vector (Invitrogen), and 1 μL of salt solution (Invitrogen) were immediately mixed. After incubation on ice for at least 5 minutes, the vector mixture was added to 50 μL TOP10 competent *E. coli* cells (Invitrogen). A 50 μL tube of the competent cells was split in half to use for positive and negative controls. The negative control had no plasmid added to the competent cells, and the positive control had 2 μL of pUC19 added. Next, the cells were heat shocked for 30 seconds at 42°C and immediately put on ice. Next, 250 μL of room temperature SOC medium was added. The tubes were then shaken horizontally at 200 rpm at 37°C for 1 hr. Then, two volumes were plated on AMP/xgal plates because the vector and pUC19 contained ampicillin resistance. Xgal was used a marker for blue/white selection because the PCR products would interrupt the *lacZ* gene. An AMP/xgal plate was prewarmed and 50μL of the sample was spread over the plate. On another prewarmed plate, the remaining 250μL of the sample was spread. A volume of 250μL of each control was spread onto a prewarmed plate. The plates were then incubated at 37°C for 48 hours.

Colony PCR was performed on the single white colonies from the AMP/xgal plates to ensure that the colonies contained the *mecA* or *ccr* gene. A single colony was touched with a sterile loop and mixed with 20 μL of sterile water. The mixture was then boiled for 15 minutes to release the DNA. PCR was set up as previously described using the boiled cells as DNA.

Plasmid purification

If the colony PCR was positive, a miniprep procedure was performed to isolate the DNA from the colonies. In order to isolate enough DNA a liquid culture was made from the progeny of the positive colony. The liquid culture was incubated at 37°C for two days. Next, 1.5 mL of the liquid culture was transferred to a micro tube. The tube was centrifuged at max speed for 3 min and the supernatant was discarded. Then, 100 µL of lysis buffer (Qiagen) and 350 µL of cold neutralization buffer (Qiagen) were added to the sample and mixed with a pipette. The tube was then centrifuged on max speed for 3 minutes. The supernatant was transferred into a column and centrifuged for 15 seconds. The flow through was discarded. The sample was washed with 200 µL of endowash (Qiagen). The wash was applied then centrifuged on max for one minute. The flow through was discarded. Next, 400 µL of Zippy wash was applied. The sample was centrifuged on max speed for 1 min and the flow through was discarded. The column was then transferred to a clean micro tube. The DNA was eluted with 50 µL of hot water and centrifuged for 30 seconds. A second elution was performed in the same manner as the first into a new tube. PCR was performed using the previous protocol to ensure that the genes were present. If PCR confirmed the presence of the gene, the sample was sent off for sequencing.

Bacterial isolation

Bacterial were isolated from environmental samples collected from streams and common household objects. Environmental samples were enriched for *Staphylococcus* by incubating the sample with LB and 6.5% NaCl at 37◦C overnight. Volumes used for bacterial enrichments varied by sample and are detailed in Table 2. After serially diluting

the bacterial sample, 10 µL was spread onto a mannitol salt agar (MSA) plate containing 6 µg/mL of oxacillin to isolate salt resistant bacteria containing the SCC*mec* element. The same protocol was followed to isolate bacteria that did not contain the SCC*mec* element except the bacterial enrichment was plated on MSA without any oxacillin. Naïve strains were replica plated on MSA with and without oxacillin to ensure sensitivity to the antibiotic. Liquid cultures of possible naïve isolates were grown overnight and diluted. Dilutions were plated on MSA and plates with individual colonies were selected. These plates were pressed against sterile velvet, the velvet then was pressed against an oxacillin plate followed by a MSA plate without oxacillin. Growth on the plate without oxacillin and no growth on the plate containing oxacillin suggested a naïve strain was isolated.

Table 2. Details of bacterial enrichments.

Sample	LB volume	Oxacillin concentration	Sample volume
Untreated HRRSA	100 mL	6 ug/mL	100 mL
Treated HRRSA	100 mL	6 ug/mL	100 mL
Dog food bowl	5 mL	6 ug/mL	1 mL of sample in PBS
Dog water bowl	5 mL	6 ug/mL	1 mL of sample in PBS
Bathroom faucet	5 mL	6 ug/mL	1 mL of sample in PBS
Cattle poop	5 mL	0	1 mL
Goat poop	5 mL	0	1 mL
Untreated HRRSA 2	3 mL	0	1 mL
Ehall	3 mL	0	~1 mL solid
UREC	3 mL	0	~1 mL solid
Hanson	3 mL	0	~1 mL solid
Shenandoah	3 mL	0	~1 mL solid
Sponge	3 mL	0	1 mL
Shenandoah 2	3 mL	0	~1 mL solid
Sunchase	3 mL	0	~1 mL solid
Treated HRRSA 2	3 mL	0	1 mL
Richmond sewage	3 mL	0	1 mL
Sink drain	3 mL	0	1 mL
Sink handle	3 mL	0	1 mL

Bacterial characterization

In order to ensure that the methicillin resistant isolates contained the SCC*mec* element, a multiplex PCR was used to determine the presence of the *mecA* and *ccr* genes.

In order to determine the genus and possibly the species of bacteria, several tests were run. Bacterial isolates were streaked on blood agar plates to determine their ability to hemolyze red blood cells. The isolates were also placed onto a BBL dryslide to determine if the isolates produced cytochrome c oxidase; a color change indicated a positive result. A small amount of bacteria was placed onto a microscope slide and a drop of hydrogen peroxide was added. If bubbles were produced, the isolate was determined to have the enzyme catalase. Rabbit plasma was inoculated with each of the isolates to determine if the bacteria contained the enzyme coagulase. Coagulated samples are positive for the enzyme. Motility was tested by stab inoculating motility medium. Bacteria that migrate away from the initial stab are considered motile. Lastly, 16S rRNA sequencing was performed and the sequence was aligned using BLAST.

Phage hunting

Several environmental samples were collected for phage isolation. The samples were enriched for bacteriophages specific for the environmental isolates. For each isolate, an enrichment tube containing LB + 6.5% NaCl, the sample, and the bacterial isolates was incubated in a shaking incubator for 24 hours at 37°C. The volumes were adjusted to best fit the sample volume and are detailed in Table 3. The samples were then spun down at 8,000 x g for 2 min. The supernatant was decanted and filtered through a 0.22 μm syringe filter. The filtered sample (10 μL) was spotted on a lawn of each isolate as an initial test for infectivity. Samples that produced plaques were individually used to

infect the corresponding host and were added to 5 mL of 0.75% LB agar and poured over a LB plate. The plate was incubated at 37°C for 24 hours.

Table 3. Details of phage enrichments.

Sample	LB volume	Sample volume	Bacteria
Untreated HRRSA	25 mL	25 mL	3.5 mL suspension
Treated HRRSA	25 mL	25 mL	3.5 mL suspension
Dog food bowl	5 mL	200 uL	200 uL suspension
Dog water bowl	5 mL	200 uL	200 uL suspension
Bathroom faucet	5 mL	200 uL	200 uL suspension
Untreated HRRSA 2	*Bacterial cultures from table 2 centrifuged and filtered*		
Ehall	*Bacterial cultures from table 2 centrifuged and filtered*		
UREC	*Bacterial cultures from table 2 centrifuged and filtered*		
Hanson	*Bacterial cultures from table 2 centrifuged and filtered*		
Shenandoah	*Bacterial cultures from table 2 centrifuged and filtered*		
Sponge	*Bacterial cultures from table 2 centrifuged and filtered*		
Shenandoah 2	*Bacterial cultures from table 2 centrifuged and filtered*		
Sunchase	*Bacterial cultures from table 2 centrifuged and filtered*		
Treated HRRSA 2	*Bacterial cultures from table 2 centrifuged and filtered*		
Richmond sewage	*Bacterial cultures from table 2 centrifuged and filtered*		
Sink drain	*Bacterial cultures from table 2 centrifuged and filtered*		
Sink handle	*Bacterial cultures from table 2 centrifuged and filtered*		

Results and Discussion

Detection of SCC*mec* genes in viral fractions from environmental samples

Samples of stream water, lake water, feces, compost, sewage, and miscellaneous samples were collected and analyzed. Although the sample set is small, *mecA* and *ccr* were more abundant in compost samples than fecal samples and even less abundant in sewage samples (Table 4). These results do not correlate with the high abundance of *mecA* detected in fecal material (Colomer-Lluch *et al.*, 2011) and sewage (Colomer-Lluch and Muniesa, 2011). Use of multiplex primer sets targeting a wider variation of the genes should have detected an even greater number of these antibiotic resistance genes than the previous studies. However, the multiplex PCR revealed that 22% of these environmental samples contained *mecA* and/or *ccr* (Table 4, Figure3). One of these samples was cloned and sequenced to confirm the *mecA* gene. The cloned portion was determined to be 221 base pairs (bp) which is slightly shorter than the expected 286 bp fragment normally amplified by the mA1 and mA2 primer pair. This sequence was analyzed using BLASTn to determine its similarity to sequences in the database. The sequence showed similarity to those found the type I SCC*mec* element.

Table 4. Categorization of samples collected and the number of PCR confirmed positives.

	Number of samples	Number of positives	Percentage
Streams	12	2	17%
Lakes	7	2	29%
Feces	26	2	8%
Compost	6	4	67%
Sewage	3	0	0%
Miscellaneous	9	4	44%
Totals	63	14	22%

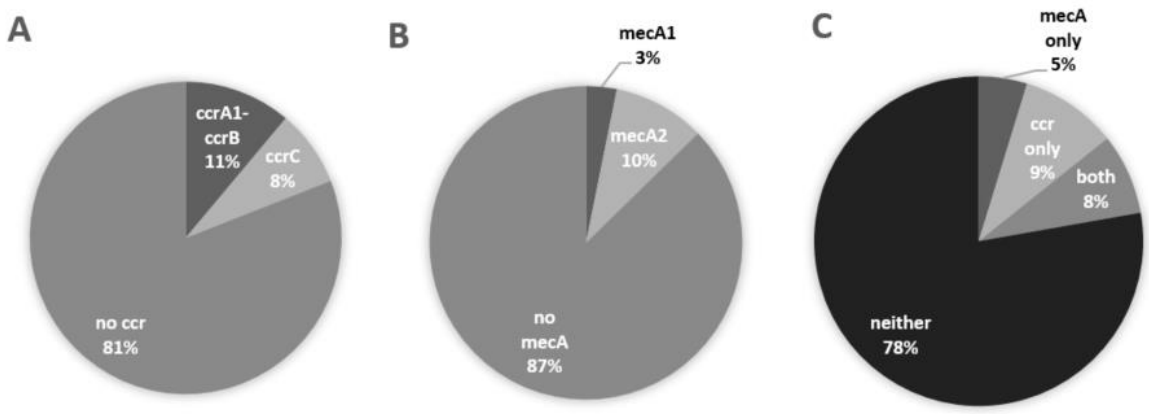


Figure 3. Pie charts showing the relative abundance of each gene in this sample set as shown by multiplex PCR analysis. (A) *ccr* gene allotype abundance (B) *mecA* gene allotype abundance (C) abundance of either or both genes.

This preliminary study suggested that pieces, if not the entirety, of the SCC*mec* element are present in the bacteriophage population. The finding of the methicillin resistance genes in 22% of samples from the environment is alarming. The presence of the *mecA* gene in environmental bacteriophage populations was confirmed, but led to many unanswered questions. To determine how much of the element is being carried and if the element is actually transduced into naïve bacteria, a new protocol was developed.

Bacterial Isolation and Characterization

Initially, twelve SCC*mec* PCR positive isolates of *S. sciuri* were obtained from Dr. James Herrick's lab (Biology Department, James Madison University). These isolates were grown on LB agar, then transferred to LB agar containing oxacillin. Two of the isolates grew and were renamed *S. sciuri* 1 and *S. sciuri* 2. Multiplex PCR was performed on these isolates, but neither showed a band for *mecA* or *ccr*. Although these isolates once contained the SCC*mec* element, the element was likely lost in the

absence of selective pressures. Therefore, these bacterial strains were abandoned and new isolates were collected.

In an attempt to isolate bacterial strains containing the SCC*mec* element, environmental samples previously and freshly collected were enriched for bacteria that tolerate high salt (6.5% NaCl). These enrichments were diluted and plated on MSA, which yielded many different colony morphologies. Isolation streaks of individual colonies were performed on MSA. Three colonies were selected for further testing because they turned the MSA media yellow (a characteristic of *Staphylococcus*) and consistently grew well. The bacteria were isolated from horse feces, goat feces, Black's Run in Purcell Park (Harrisonburg, VA), and cattle feces. Multiplex PCR revealed that the Purcell Park isolate contained both *mecA* and *ccr*. The *ccr* band was located at approximately 700 bp meaning that the band corresponds with the α1-βc primer set and the *ccr*A1-*ccr*B gene. The *mecA* band was located at approximately 250 bp corresponding with the mA1-mA2 primer set amplifying the *mecA* gene type. The multiplex primer sets did not amplify any DNA in the other three bacterial strains (Figure 4). The SCC*mec* positive strain isolated from Black's Run in Harrisonburg, Virginia (GPS coordinates 38.436716, -78.882616) was named BSR1. The isolates from horse feces, goat feces, and cattle feces were oxacillin resistant, making them poor candidates for naïve strains.

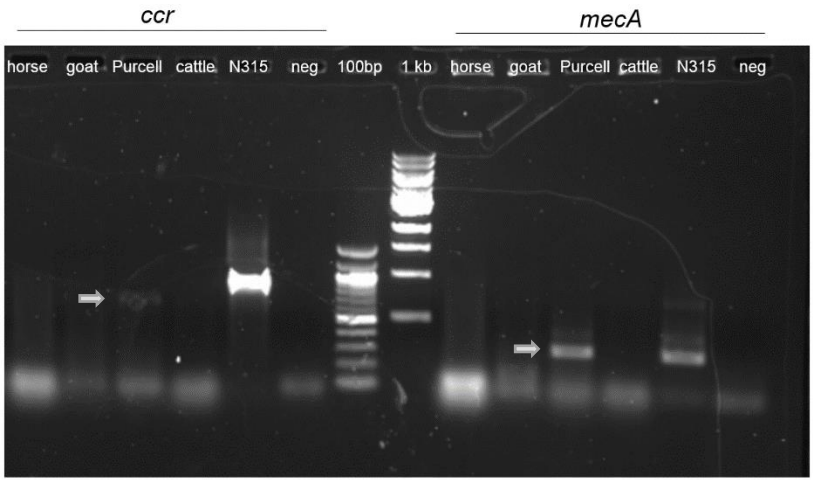
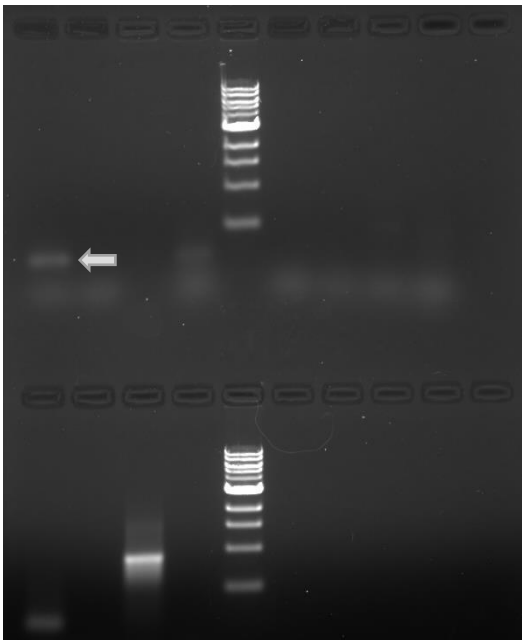


Figure 4. PCR results of the first set of samples tested for the SCC*mec* element. The left side of the gel shows results from the *ccr* multiplex and the right side of the gel shows results from the *mecA* multiplex. Every lane is labelled with the sample it contains. Arrows point to positive bands of samples.

Subsequently, more samples were collected and enriched for salt tolerant bacteria. These samples included treated and untreated sewage from Harrisonburg Rockingham Regional Sewage Authority (HRRSA), swabs from dog food and water bowls, bathroom faucets, toilet handles, sponges, and sink drains. The *mecA* multiplex PCR revealed a band of approximately 300 bp in the dog's food bowl isolate (Figure 5). This size corresponded to the mA1-mA2 primer set. Although *ccr* was not amplified in this sample, it is likely that the sample contains the SCC*mec* element. It is possible that the *ccr* allotype was not recognized by the primer multiplex used in this study. Therefore, the isolate from the dog's food bowl was labeled BSR2.



Lane	Primer multiplex	Sample
1	*mecA*	Dog food bowl
2	*mecA*	Dog water bowl
3	*mecA*	Bathroom faucet
4	*mecA*	Treated HRRSA sewage
5	---	1 kb ladder
6	*ccr*	Dog food bowl
7	*ccr*	Dog water bowl
8	*ccr*	Bathroom faucet
9	*ccr*	Treated HRRSA sewage
10	---	Empty
11	*mecA*	N315
12	*mecA*	Negative control
13	*ccr*	N315
14	*ccr*	Negative control
15	---	1 kb ladder

Figure 5. PCR results from the second sample set tested in search of SCC*mec* positive isolates. The table details the contents of each lane. Arrow points to positive *mecA* band from the dog food bowl.

Previously, 6ng/mL oxacillin was used in the media to select for oxacillin resistant strains, increasing the percentage of *SCCmec* positive samples. In order to isolate a naïve strain as a recipient for transduction, of the element, bacterial enrichments were performed with a high salt concentration, but no oxacillin. The same samples listed above in addition to soil samples collected around Harrisonburg, VA (including E-Hall (JMU), UREC (JMU), Hanson Hall (JMU), Shenandoah Hall (JMU), and Sunchase apartments) were incubated and spread on MSA. Individual colonies were selected and grown in liquid culture. The cultures were diluted and replica plated on MSA containing oxacillin. The bacteria grew on the MSA plates, but not the plates containing oxacillin. Two naïve strains were isolated using this procedure. An isolate from a sponge kept near a sink was named BSN1 and an isolate from a soil sample collected outside of

Hanson Hall (James Madison University, Harrisonburg, VA) and named BSN2. Unfortunately, BSN2 ceased to propagate after a few passages.

BSR1, BSR2, and BSN1 underwent biochemical testing to determine their genus and species. All three isolates tested negative for oxidase, positive for catalase, and did not hemolyze blood (Table 5). Additionally all three isolates turn the MSA media yellow. The BSR1 colonies grown on MSA appear as flat, yellow, regular colonies about 3 mm in diameter. The BSR2 colonies grown on MSA appear as flat, tan, regular colonies about 2.5 mm in diameter. The BSN2 colonies grown on MSA appear as flat, tan, regular colonies about 1 mm in diameter. These characteristics lead to the conclusion that these isolates are *S. epidermidis* rather than *S. aureus*.

Table 5. Bacterial characterization.

Isolate	oxidase	catalase	hemolysis
BSR1	-	+	α
BSR2	-	+	α
BSN1	-	+	α

Phage Isolation

In order to isolate transducing phage, samples were enriched to increase phage yield for the bacterial isolates. Similarly to the bacterial isolation, samples collected around Harrisonburg were used for phage enrichments. Initially, sample enrichments and plaque assays were incubated at 37ºC. When treated and untreated HRRSA sewage was enriched with BSR1 and assayed on BSR2, some plaque-like spots were seen. Unfortunately, when these plaques were suspended in phage buffer and used to perform further plaque assays, no plaques formed. Therefore, the plaques did not represent phages specific for BSR1 or BSR2. Due to a lack of results at 37ºC, sixteen

samples were enriched and plaque assays were performed at 25^{o}C. None of these samples produced plaques on BSR1, BSR2, or BSN1.

Future work

Because this project is still in its infancy, much work needs to be done to draw meaningful conclusions beyond the evidence for the *mecA* and *ccr* elements in viral fractions of environmental samples. The first step in moving forward is to collect more bacterial isolates and collect many Staphylococcal phages. Although collecting more samples and screening them could unveil a phage to use in transduction experiments, it is worth the time and effort to vary the protocols. There is a possibility that the bacteria do not express appropriate receptors for the phage to bind to under certain conditions. This is one of the reasons that a second phage isolation experiment was done at room temperature; however, temperature is not the only factor to alter. Additionally, the richness of the growth media could result in different protein expression on the surface of the bacteria. In order to combat this potential problem, large water samples from Black's Run (the isolation site of BSR1) could be sterilized and used as media. This would allow the bacteria to grow in a more natural environment with much fewer nutrients, possibly requiring different proteins to acquire the necessary nutrients. It is also possibly that the high salt concentration (6.5%) causes the bacteria to alter the expression of proteins on their surface. These experiments could be repeated exactly as before, but removing the high salt concentrations used for selectivity. Altering these three variables and testing more samples should unveil phages to complete future studies.

After bacteria and phage are isolated, transduction experiments will be completed. SCC*mec* positive isolates will be infected with phage samples. Phage buffer with be placed on the plates, allowed to set overnight, and collected. The lysate will be

centrifuged, filter sterilized, and DNase treated to isolate DNA protected by the capsid of a phage. After contaminating DNA is destroyed, a portion of the lysate will be used to isolate DNA for PCR testing. In parallel, another portion of the lysate will be used to infect naïve bacteria. These bacteria will be incubated in liquid culture for 48-72 hours to allow expression of the PBP2a. The liquid culture will be plated on oxacillin containing MSA to screen for newly resistant bacteria. Colonies will be propagated and used in a multiplex PCR. If the PCR is positive, transduction of the SCC*mec* element occurred.

If initial transduction experiments are successful, transduction frequencies and phage preferences will be analyzed. Analysis will include whether one SCC*mec* allotype is preferentially present in environmental bacteria and if an allotype is more likely to be transduced than the others. Additionally, naïve bacteria should be screened for the presence of the penicillinase plasmid to see if environmental transduction can be compared to clinical transduction described by Scharn *et al.* (2013). Analysis could also give insight into whether a specific group of phages is more apt to transducing the element or if highly varied phages are able to transduce the element. Lastly, experiments and bioinformatic analysis could provide data to support generalized or specialized transduction. After collecting more bacterial isolates and bacteriophages, we can begin to answer these questions.

Conclusions

Preliminary studies confirmed the presence of *mecA* and *ccr* in environmental bacteriophage populations through the use of a multiplex PCR. Because DNA yield was low, only one sample was cloned and sequenced to confirm its identity. These genes were not detected in the quantities described by Colomer-Lluch *et al.*; however, approximately 22% of the 63 samples analyzed contained *mecA* and/or *ccr* (2011). The relatively small sample size prevents definitive conclusions, but a higher proportion of compost samples contained the genes than other sample types. Unexpectedly, fecal material and sewage samples (known for being rich in phage populations) showed lower proportions containing *mecA* and/or *ccr* (table 4). From this study, it was concluded that at least pieces of the SCC*mec* element are present in bacteriophage populations. However, there was still a lack of information as to whether the bacteriophages were able to transduce the element and to what degree they are able to package the element. In order to answer these questions a secondary study was designed. This study involved collecting environmental bacterial isolates both containing the SCC*mec* element and naïve strains. Two strains of salt-tolerant oxacillin resistant and one strain of salt-tolerant oxacillin sensitive bacteria were collected, tested further, and presumed to be *Staphylococcus.* The isolation of phage populations for the transduction experiments was less fruitful. Many factors, such as temperature, salt concentration, and nutrient concentration, may have affected detection of phage. Future experiments will be conducted to gather more bacterial isolates and many phage isolates. These isolates can then be used in transduction studies to first determine if transduction is a valid hypothesis for transfer of the SCC*mec* element in the

environment. Furthermore, data can be collected to determine the degree of transduction and any preferences for specific allotypes to be transduced.

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