8-2010

Methicillin Resistance in Staphylococcus pseudintermedius

Chad Christopher Black
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To the Graduate Council:

I am submitting herewith a dissertation written by Chad Christopher Black entitled "Methicillin Resistance in Staphylococcus pseudintermedius." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

Stephen A. Kania, Major Professor

We have read this dissertation and recommend its acceptance:

David A. Bemis, Robert N. Moore, John C. New

Accepted for the Council:

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Methicillin Resistance in *Staphylococcus pseudintermedius*

A Dissertation Presented for the

Doctor of Philosophy Degree

The University of Tennessee, Knoxville

Chad Christopher Black

August 2010

Dedication

For Belinda and in spite of the cat.

"An ocean in a drop of water – and unknown, uncharted, unexplored by man! By man, who gives all his time to the Africas and the poles, with this unsearched marvelous world right at his elbow." – *Mark Twain, "The Great Dark"*[1]

Acknowledgments

Thanks to Robert E. Black and Robert D. Black for their example; to Belinda Black and Susan Black for their patience and understanding; to Stephen Kania and David Bemis for their sound guidance throughout this process; to the members of my graduate committee and the many fine people at the at the University of Tennessee College of Veterinary Medicine who have created a rich and supportive learning environment.

Abstract

Staphylococcus pseudintermedius affecting dogs is analogous to *S. aureus* on humans, acting as both normal flora and opportunistic pathogen. Methicillin resistance in *S. pseudintermedius* is recent, with the first documented occurrence of an isolate bearing the methicillin resistance gene, *mecA,* in 1999. This gene encodes penicillin binding protein 2a, which renders all beta-lactam drugs ineffective and functions as a "gateway" antibiotic resistance determinant. In the presence of ineffective antibiotics, opportunities for mutational events and acquisition of mobile genetic elements increase as microbial densities increase, often leading to multi-drug resistance. Methicillin-resistant *S. pseudintermedius* (MRSP) infections have become increasingly common. For example, approximately 30% of the *S. pseudintermedius* isolates tested by the University of Tennessee College of Veterinary Medicine Diagnostic Bacteriology service are resistant to methicillin. An increasing number of MRSP isolates are also resistant to most clinically useful antibiotics available to veterinarians except for chloramphenicol, and resistance to this antibiotic is common among European MRSP isolates. Chloramphenicol resistance has begun to appear in the US and if this trend continues there may soon be few viable antibiotic treatment options.

Compared with the arrival of methicillin-resistant *S. aureus* in the 1960s, the opportunity currently exists to apply advanced molecular methods early in this recognized emergence of MRSP. To that end I have pursued projects utilizing multilocus sequence typing, pulsed-field electrophoresis, and SCC*mec* characterization of both susceptible and resistant *S. pseudintermedius*. The initial result was the detection of a clonal population of MRSP in the southeastern United States. Further characterization of this and other clonal lineages using genomic sequencing and real-time RT-PCR expression analysis of antibiotic resistance and quorum sensing genes revealed a marked difference in the regulation of antibiotic resistance between regional clones. These discoveries have interesting epidemiological implications and provide a foundation for the development of novel therapeutics to circumvent the expanding antibiotic resistance repertoire of MRSP. Potential targets identified by this work include membrane-bound beta-lactamase receptors responsible for the regulation of *mecA*, non-cognate auto-inducing peptides, and synthetic antisense oligonucleotides.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

agrD – accessory gene regulator, locus D, encodes AIP and serves as one of the genes for Bannoehr et al. MLST

AIP – auto-inducing peptide

ATCC – American Type Culture Collection

blaZ – gene encoding β-lactamase

CA-MRSA – community-acquired methicillin-resistant *Staphylococcus aureus*

CoNS – coagulase-negative staphylococci

cpn60 – chaperonin 60, gene for Bannoehr et al. MLST

HA-MRSA – hospital-acquired methicillin-resistant *Staphylococcus aureus*

HGT – horizontal gene transfer

mecA – gene encoding penicillin-binding protein 2α

MGE – mobile genetic element

MLST – multilocus sequence type*(ing)*

MLVA – multiple-locus variable-number tandem-repeat analysis

MRS(P)I – methicillin-resistant *Staphylococcus pseudintermedius* originally reported as *intermedius* but isolated from dogs

MRSA – methicillin-resistant *Staphylococcus aureus*

MRS – methicillin-resistant staphylococci

MRSP – methicillin-resistant *Staphylococcus pseudintermedius*

MSSA – methicillin-susceptible *Staphylococcus aureus*

MSSP – methicillin-susceptible *Staphylococcus pseudintermedius*

PBP2a – penicillin-binding protein 2a

PFGE – pulsed-field gel electrophoresis

pta – phosphate acetyltransferase gene, gene for Bannoehr et al. MLST

RT-PCR – reverse transcriptase polymerase chain reaction

SIG – *S. intermedius* group (*S. delphini*, *S. intermedius*, and *S. pseudintermedius*)

ST – sequence type, refers to MLST

sodA – superoxide dismutase

spa – staphylococcal protein a

tuf – elongation factor tu, gene for Bannoehr et al. MLST

UTCVM – University of Tennessee College of Veterinary Medicine

Chapter 1: Literature Review

Overview

A synthetic form of penicillin, methicillin, was introduced in 1959 to treat *Staphylococcus aureus* resistant to beta-lactam antibiotics. Within a year the first methicillin-resistant *S. aureus* (MRSA) isolates began to appear in hospital settings.[2] Methicillin-resistance is a "gateway" antibiotic resistance determinant. It causes penicillins and cephalosporins to become ineffective, establishing a MRSA foothold in the hospital environment and on the patient and hospital staff repeatedly exposed to first-line antibiotics. From that foothold, the organism has the opportunity to acquire mutations and further transmissible elements that increase its multi-drug resistance repertoire. Methicillin-resistance often leads to multi-drug resistance. In the fifty years since methicillin resistance appeared in *S. aureus*, MRSA infection has become the sixth-leading cause of death in hospitalized human patients in the United States, with costs estimated to be between 4 and 6 billion dollars annually.[2,3]

Staphylococcus pseudintermedius on dogs is analogous to *S. aureus* on humans, acting as both normal flora and opportunistic pathogen. It is the primary cause of pyoderma, the most common dermatologic disease seen in dogs, and is also frequently associated with urinary tract infections and wound and surgical site infections.[4] The first documented isolate of *S. pseudintermedius* bearing the methicillin resistance gene *mecA* occurred in 1999.[5] Since that discovery, methicillin-resistant *S. pseudintermedius* (MRSP) infections have become increasingly common. Approximately 30% of the staphylococcal isolates tested by the University of Tennessee College of Veterinary Medicine (UTCVM) Bacteriology Service are resistant to methicillin.[6-8] These bacteria also carry resistance to almost all clinically useful antibiotics except for chloramphenicol and the ones reserved for use in humans. Resistance to chloramphenicol is now beginning to appear, leaving no viable therapeutic options.

Methicillin resistance in *S. aureus* has been studied in far greater detail than in *S. pseudintermedius*; despite significant dissimilarities in genomic sequence the two species share a natural history, as well as many of the same regulatory, antimicrobial, and virulence determinants. Biochemical and molecular techniques used in the study of *S. aureus* can and have often been adapted to *S. pseudintermedius*. Therefore, much of the literature regarding *S. aureus* is applicable to the study of staphylococci as a whole.

The following is a review of the nomenclature, phenotype, genetic characterization of methicillin resistance, and typing methodologies for *S. pseudintermedius*.

The Taxonomic History of *Staphylococcus pseudintermedius*

The defining characteristics of bacterial species have changed with advances in technology. With the proliferation of molecular characterization techniques and the increased accessibility of genomic sequencing platforms, the inevitable taxonomic trend is toward diversification within the bacterial genus. The 19th century Scottish physician Alex Ogston was an early proponent of the role of microscopic spherical organisms ("micrococci") in acute inflammation and suppuration associated with abscess formation in skin. He was also one of the first to make the leap connecting microbial abscessation with the circulatory phenomena of septicemia and pyemia.[9,10] These foundational observations hold true in the clinical presentation of staphylococcal infection today. In 1882 he coined the term *Staphylococcus* – from the Greek *staphulê* for *bunch of grapes* – to differentiate cluster-forming micrococcus from the chain-forming variety then known as Billroth's streptococci.[11] Rosenbach further subdivided staphylococci in 1884 on the basis of color on culture media – *S. aureus* and *S. albus* for gold and white colony formation respectively.[12] For the next ninety years *Staphylococcus* was widely held to have only three distinct species. During this period gram-positive, coagulase-positive, hemolytic isolates with variable colony coloration and associated with disease in both humans and animals were presumed to be *S. aureus*; whereas non-pathogenic, coagulase-negative staphylococci (CoNS) with white colony coloration were classified as either *S. epidermidis* (formerly *albus*) or *S. saprophyticus* and considered to be environmental contaminates.[12-14]

Review of veterinary microbiology textbooks from the 1930s through the late 1960s supports the bias towards *S. aureus* as the catchall designation for pathogenic gram-positive and coagulase-positive clustering cocci. Variations in *S. aureus* microscopic size, hemolytic ability, and colony coloration were often noted, prompting calls for greater research into the validity of species designation.[15-18] From Merchants 1968 edition of Veterinary Bacteriology and Virology the follow observation is made concerning the use of pigmentation as the sole criteria for staphylococcal differentiation,

(*S. epidermidis*) has been considered by many to be only a non-pigmented variant of the aureus strains. It is readily apparent to those who study the staphylococci that numerous non-pigmented strains can be isolated. In fact, unless isolation is made upon special media, such as no. 110, colonies of staphylococci are sometimes white.

The designation of strains of any organism, one variety or another, on the basis of colony color without specification of growth is most confusing, not only to the student but to anyone interested in taxonomy. . . .

It is quite apparent that additional research must be done on the genus *Micrococcus* (*Staphylococcus*) in order to clarify many of the interrelationships of the members of this genus.[15]

This admonition in a standard veterinary text of the time suggests that as late as the early 1970s some veterinary practitioners were dismissing the as yet uncharacterized *S. intermedius* as nonpathogenic based primarily on colony color.

In the latter half of the 20th century, spurred by the emergence of new hospital-acquired and antibiotic-resistant infections, both human and veterinary medical microbiologists began to actively pursue a better understanding of which organisms were associated with specific types of infections and hosts. The improvements made in both phenotypic biochemical testing and genotypic molecular tests resulted in the diversification of both coagulase-positive and negative staphylococcal species.[13,16,19] At the time of writing there are 41 species and 24 subspecies cited in the genus *Staphylococcus*.[20]

The earliest call for a more precise designation for coagulase-positive staphylococci of canines came in 1947, with Smith's observation that not all suspected pathogenic strains from canines reacted uniformly in tube, slide and plate agglutination tests; and that canine isolates were discernable from those of other animals by cultural characters and toxin formation.[21] Based upon confirmation of Smith's work, a 1967 report proposed the name *S. aureus var. canis* and described biochemical and staining differences between isolates from different host species. The study showed that canine isolates consistently failed to coagulate human serum, pigmentation lacked the characteristic golden hue, and crystal-violet staining, fibrinolysin production, hemolysis type and phage type did not consistently match between human and canine isolates.[22] A series of cross-reactivity

experiments by I. Live of Pennsylvania and V. Hajek of Czechoslovakia further explored the coagulase and fibrinolysis disconnect observed when isolates of one background were exposed to blood components of the other. Not only did these findings have bearing on speciation, they were an early suggestion that staphylococci of canine origin may be more host-adapted than the human equivalent, *S. aureus*.[23-25] In 1976 Hajek reported the results of a battery of additional biochemical tests that when applied to coagulase-positive staphylococci of animal origin (pigeon, canine, mink, and horse) justified a new species name, *S. intermedius*. The name was chosen to indicate the group's intermediate phenotypic position between the coagulase-positive *S. aureus* and the white colony formation of *S. epidermidis*. Despite evidence of biochemical differences between isolates from the different animals within the study, a pigeon isolate became the type strain for *S. intermedius*.[16] With the biochemical profile established for species identification, reports from the 1980s through 2007 consistently demonstrated *S. intermedius* to be the primary staphylococcal normal flora and opportunistic pathogen of canines.[26-30]

The improvement of molecular typing methods in the 1980s and 90s provided additional tools for the description of bacterial species. In 2005 Devriese et al. described a novel staphylococci based on 16S rRNA gene sequence analysis and confirmed by DNA-DNA hybridization its dissimilarity with *S. intermedius*, *S. delphini* and *S. schleiferi* subsp. *schleiferi* type strains. The name *S. pseudintermedius* was chosen because the new species was difficult to distinguish from *S. intermedius* with standard phenotypic tests.[31] Because 16S rRNA gene sequence differed so little (>99% homology) among the *S. intermedius* group (SIG includes *S. intermedius*, *S. delphini,* and *S. pseudintermedius*), subsequent research expanded phylogenetic analysis with *sodA* and *hsp60* gene sequences. Whereas the Devriese group based its findings on four isolates, one each from a cat, dog, horse and parrot, Sasaki et al. analyzed 117 isolates with the *S. intermedius* phenotype and primarily of canine and pigeon origin. They found that all canine isolates (n=78) were *S. pseudintermedius* and that all pigeon isolates were not. The ATCC *S. intermedius* type strain (acquired from the Hajek 1976 study) clustered only with isolates from wild pigeons. This finding was the first strong indication that the newly identified *S. pseudintermedius* was inherently linked to the canine host.[32]

Further evidence for replacing *S. intermedius* with *S. pseudintermedius* as the opportunistic staphylococcal pathogen of canines was given by Bannoehr et al. in 2007. The group created a four-component multilocus sequence typing (MLST) analysis using chaperonin 60 (*cpn60*), phosphate acetyltransferase (*pta*), elongation factor tu (*tuf*), and accessory gene regulator locus D (*agrD*) in addition to 16S rRNA. They tested 105 SIG

isolates from various hosts and from ten countries in North America, Europe, and Asia. Their findings mirrored those of Sasaki et al. in that all *S. pseudintermedius* isolates identified with this method were found on dogs.[33] Based upon these studies the current consensus for SIG nomenclature is to categorize as *S. pseudintermedius* all canine isolates that have the *S. intermedius* phenotype. Therefore, the following terms and abbreviations are a convenient method of classification[31,34,35]:

1. *S. (pseud)intermedius* when isolates previously identified as *S. intermedius* are likely *S. pseudintermedius* (obtained from a canine host)
2. MRS(P)I for methicillin-resistant *S. (pseud)intermedius* (formerly classified as MRSI but obtained from a canine host)
3. MRSP for isolates identified as *S. pseudintermedius* via methods that have appeared in more recent literature. [34,35]

The History of Methicillin Resistance in *S. pseudintermedius*

In studies from the 1980s, all veterinary staphylococcal isolates tested were susceptible to methicillin.[36,37] The first report of methicillin resistance in *S. (pseud)intermedius* confirmed by PCR detection of *mecA* occurred in 1999 from a single isolate in a survey of 25 staphylococcal isolates of canine origin. In that study, both coagulase-positive and negative isolates were screened; but interestingly, all other coagulase-positive methicillin-resistant isolates were found to be *S. aureus*. This occurred at the University of Illinois, and the sample set had been collected from 1995 forward. Several possible explanations exist for the detection and/or emergence of a single and novel MRS(P)I at this time. The first is that a confluence of events provided the necessary elements for acquisition of the *mecA* gene by *S. (pseud)intermedius*:

1. A high prevalence of MRSA among staphylococci responsible for hospital-acquired infections (>50%) meant that the raw numbers of mobile genetic elements (MGE) bearing *mecA* were high relative to the past.[35]
2. The emergence of CA-MRSA infections not linked to HA-MRSA indicated that MGEs had made the intra-species transition from one set of lineages to another – *mecA* and SCC*mec* was on the move.[38]
3. The use of beta-lactam antibiotics and cephalosporins in human and animal medicine, as well as in food animal production may have reached

a critical mass, facilitating not only the acquisition, but also the maintenance of the SCC*mec* bearing *mecA* in *S. (pseud)intermedius*.[39,40]

While all these elements were certainly in place, another reason for the later arrival of methicillin resistance in the *S. intermedius* literature relative to its arrival in *S. aureus* may simply be due to the sensitivity of speciation and screening methodologies. The Spanish group Piriz et al. reported an apparently novel and large proportion of methicillin-resistant isolates in a sampling of *S. (pseud)intermedius* collected between 1988 and 1992; however, they did not verify the presence of *mecA* and found widely varying levels of methicillin resistance when adjusting the culture alkalinity for canine physiology.[41] It is important to consider that the speciation of coagulase-positive staphylococci was nebulous prior to the mid-1970s, and that appropriate standards for speciation and methicillin resistance determination in staphylococci of animal origin continue to evolve through the present.[6] When molecularly screening UTCVM isolates and samples submitted by collaborators for species identity, the Bacteriology and Immunology Services often find *S. aureus*, *S. pseudintermedius*, *S. intermedius*, *S. delphini* and *S. schleiferi coagulans* phenotypically misidentified. Perhaps *S. intermedius* lineages bearing the *mecA* gene existed for some time prior to the mid-1990s and were misclassified as MRSA or expressed *mecA* at less detectable levels when tested using *S. aureus* standards. The exact point in time where *S. pseudintermedius* acquired *mecA* is not vital to present treatment and control of MRSP expansion. However, historical review of the literature provides a foundation and informs upon how *mecA* might move from one organism to another, and from what ecological repositories resistance genes like *mecA* arise. Understanding the frequency and mode of SCC*mec* horizontal transfer, through in-depth molecular population analysis, enables the identification of clinically relevant MRSP lineages and sheds light on the frequency at which novel lineages appear.

The Utility of Genotypic Methods for Staphylococcal Population Analysis

Horizontal gene transfer (HGT) incidence is an important part of determining zoonotic potential of MRSP. As yet there is no evidence that SCC*mec* from canine normal flora *S. pseudintermedius* can transfer to human *S. aureus* or vice versa. Interspecies SCC*mec* transfer is likely a rare event in that no research group has yet demonstrated such movement experimentally. However, there is compelling evidence from a 2001 Dutch

case report concerning a premature infant treated with potentiated amoxicillin while in a neonatal intensive care unit. Prior to antibiotic therapy a *mecA* negative MSSA was cultured. Following antibiotic treatment failure ten days later, a MRSA was isolated with the same genetic background as the previously identified MSSA, as well as CoNS with an identical SCC*mec* to that of the MRSA.[42] The neonatal intensive care unit had strict infection control procedures in place, and cultured at all stages of treatment. One plausible explanation for the appearance of this novel MRSA was that the SCC*mec* transferred from CoNS to MSSA and the antibiotic given insured selection of the newly formed MRSA.

Investigations of MRSA genotype in large populations are essentially attempting to replicate the findings on the Dutch baby at the regional or national level. Understanding the movement of SCC*mec* requires that researchers adopt a consistent typing scheme. Prior to the molecular age, tools for bacterial characterization included phenotypic speciation, antibiogram profile, and phage typing. Over the past two decades methodologies have been developed that allow investigators to apply numerical values to genetic relatedness within a species. Molecular techniques have also been developed that describe the relatedness of resistance cassettes SCC*mec*. The following is a summary of genotypic methods applied to staphylococci. These are some of the approaches that can demonstrate associations between bacterial lineages and resistance determinanants.

Staphylococcal Cassette Chromosome (SCC*mec*) Typing

Known SCC*mec*s are relatively large for MGEs, ranging in size from 30 to 55KB. The essential components used to characterize SCC*mec* are the genetic sequences of the *mec* and *ccr* gene complexes. The *mec* operon is composed of *mecA*, a promoter region, and some or all of the regulatory genes *mecI* (promoter inhibitor) and *mecR1* (membrane-bound β-lactam sensor). The *ccr* gene complex encodes the recombinases responsible for cassette excision from and integration into the bacterial chromosome.[43] To date sequence analysis has identified five classes of *mec* complex (A, B, C1, C2, and E based upon the completeness of the operon) and five *ccr* allotypes (*ccrAB1* to *ccrAB4* and *ccrC*) in major MRSA lineages. SCC*mec* type is designated by a roman numeral (I-VIII) and represents a combination of *mec* class and *ccr* allotype.[44] Because the cassettes are so large, extensive sequencing would be required to fully characterize each one. To

bypass this laborious process, numerous multiplex PCR schemes have been described, amplifying sequences specific to each *mec* and *ccr* region by SCC*mec* type. The inherent drawback to these PCR-based techniques is that any novel cassette type is either misclassified because the small regions amplified by the assay are by chance similar to the described cassette type, or it is untypeable and must be fully sequenced and a new multiplex approach developed. Low frequency untypeable cassettes are often reported in large surveys of methicillin-resistant staphylococci. The greatest utility of the cassette-typing multiplex PCR approaches are their ability to identify the most common SCC*mec*s in the predominant MRSA clonal lineages. As will be discussed in proceeding studies, the multiplex PCR developed by Zhang et al. for MRSA is sufficient to classify MRSP SCCmec types II-III and V_T in predominant clonal lineages ST 71 and ST 68 respectively.[45,46]

The MRSA SCC*mec* typing nomenclature has been used for characterization of SCC*mec* in other staphylococci. Cassettes are likely similar across species; SCC*mec* is a MGE believed to be restricted to staphylococci, and similar cassette types would presumably be interchangeable between *S. aureus*, *S. pseudintermedius* and the CoNS. Three cassettes associated with *S. pseudintermedius* have been fully characterized.[47,45] Descloux et al. described two cassettes. SCC*mec* type II-III was identified in 14 of 15 MRSP isolates screened, and SCC*mec* type VII in a single isolate. SCC*mec* II-III was found to be a hybrid of *S. aureus* SCC*mec* III and *S. epidermidis* SCCmec II, and associated with the most common European MRSP sequence type, ST 71. MRSP SCC*mec* VII had novel *ccr* regions, some similarity to MRSA SCC*mec* III, but an incomplete *mec* operon. The "take-home" message from the Descloux study is that even though the emergence of methicillin-resistance in *S. pseudintermedius* is recent, the movement of cassettes between staphylococci is not a simple transaction whereby the recipient receives an exact copy from the donor. No MRSP has yet been shown to carry an *exact* copy of a MRSA SCC*mec*. Something about the as yet unidentified mechanism of cassette transfer between staphylococci causes some level of recombination or deletion. An alternative explanation could be that direct MRSA to MRSP cassette transfer does not occur, and the genetic shuffling of the cassettes happens in a reservoir species, such as a commensal nonpathogenic CoNS.

The third known MRSP SCC*mec* type (type V_T associated with ST 68) was sequenced and described by Eberlein and Kania at UTCVM, and will be part of the study described in chapter two.[45]

Methods for Sub-Typing *S. aureus* and *S. pseudintermedius*

Because SCC*mec* is mobile, cassette typing alone is insufficient to characterize MRS and incapable of describing susceptible lineages. Therefore, typing methods have been developed that focus on areas of genetic variability shared by all isolates. Depending upon the desired resolution of the bacterial population picture, the following strategies have been used to type staphylococcal isolates (listed in order from highest to lowest specificity): pulsed-field gel electrophoresis (PFGE), multiple-locus variable-number tandem-repeat analysis (MLVA), *spa* typing, and multilocus sequence typing (MLST).[48]

Pulsed-Field Gel Electrophoresis

PFGE is among the most discriminative of the typing methods, and is the foundation of the Center for Disease Control (CDC) Pulsenet initiative for standardized molecular subtyping of foodborne disease-causing bacteria such *Escherichia coli* O157:H7, *Salmonella*, *Shigella*, *Listeria*, or *Campylobacter*.[49] No PCR amplification is required. Chromosomal DNA is isolated and then digested with a restriction enzyme; for staphylococci the enzyme most often used is SmaI. Depending upon the number and location of restriction sites within the genome, a characteristic number of fragments are produced. Those fragments range from >100KB to <5KB and are separated on agarose gel by alternating voltage across an electric field. The gel banding patterns are then assessed by statistical software and assigned a percent similarity relative to one another.[50] Because the frequency of point mutations that can add or delete restriction sites is high, pulsed-field patterns change relatively quickly through successive bacterial generations. PFGE is most useful when comparing isolates within a close population, such as within a single hospital or between a series of patients. The primary impediment to wide scale use of PFGE is difficulty with reproducibility between laboratories. It is a labor-intensive process, and the banding patterns vary greatly depending upon the agarose gel, power supply settings, and analysis software used. Attempts to standardize PFGE for *S. aureus* have occurred at the federal level in the United States and Europe, with its addition to Pulsenet by the CDC and the European Union Harmony protocol respectively.[51,52] No such attempt has been made to standardize *S. pseudintermedius* PFGE. Researchers of animal staphylococci typically use PFGE to add validity to the results of less discriminating typing methodologies (MLST

or *spa* typing)[45,46,53], or to track closely related isolates in carriage studies between animals or on different locations of a single animal.[54,55]

Multilocus Variable Number Tandem Repeat Analysis (MLVA) and spa Typing

MLVA and *spa* typing fall between PFGE and MLST in their discriminatory ability, and are therefore used where appropriate for study focus, sample size and cost-effectiveness. Like MLST, both are PCR-based techniques that have the advantage of being highly reproducible between facilities. MLVA is a multiplex PCR analyzing the variation in number of repeats in seven individual *S. aureus* genes (*sspA*, *spa*, *sdrC*, *sdrD*, *sdrE*, *clfA*, and *clfB*).[56] In several MRSA studies MLVA was found to be comparable in discriminatory power with PFGE.[48,57] Due to reduced need for expensive single-purpose equipment and specialized training, MLVA may eventually replace PFGE as the method of choice for CDC Pulsnet.[58-60] Unlike PFGE however, MLVA requires an extensive genus and species-specific survey and validation of candidate genes with tandem repeat regions exhibiting appropriate levels of variability. To date MLVA has not been developed for sub-typing of *S. pseudintermedius*.

spa typing is an older single locus technique and subset of MLVA developed by Frenay et al.; the sequence variation in a highly polymorphic region of the *S. aureus* protein A is measured and typed, and statistical software determines relatedness between isolates.[61] Its primary advantage is that only one PCR and sequencing reaction are required to characterize each isolate. The polymorphic region is classified by number of 24bp repeats and point mutations. Some hospitals use *spa* typing as their high throughput baseline MRSA typing modality, monitoring for unusual homogeneous spikes in *spa* types that might require PFGE or MLVA for finer characterization. Among its disadvantages include this need for verification by other methods, and the potential for mischaracterization of an isolate due to a recombination event that includes *spa*.[62] Within the last two years the *spa* locus has been characterized for *S. pseudintermedius* and used in conjunction with PFGE[53] and MLST[46] to illustrate clonality in large regional samples of MRSP. This technique appears promising as a tool to gather information on sample sizes in the hundreds or thousands, and could be invaluable for an as yet unperformed comprehensive survey of MSSP and MSRP on a national scale.

Multilocus Sequence Typing

The resolution provided by PFGE is more in step with the parsing of small differences between isolates. On the other end of the sub-typing spectrum, for measurement of bacterial genomic variation through time and over great distances, MLST is the method of choice.[63,64] MLST for *S. aureus* requires seven individual PCRs (400-500bp) and sequence analyses in the housekeeping genes: carbamate kinase (*arcC*), shikimate dehydrogenase (*aroE*), glycerol kinase (*glp*), guanylate kinase (*gmk*), phosphate acetyltransferase (*pta*), triosephosphate isomerase (*tpi*), and acetyl coenzyme A acetyltransferase (*yqiL*).[65] *S. aureus* allelic profiles and STs are assigned by comparing sequence data for each locus to the MLST repository at http://saureus.mlst.net/. Variations between loci in different isolates are designated as numbered alleles. A distinct sequence type is made up of a unique combination of arbitrarily numbered alleles, e.g. MRSA ST5 has the assigned allelic profile 1-4-1-4-12-1-10.

Essentialness of gene function is the primary selection criteria for MLST loci. All isolates of a species must have all loci. The loci should be areas of genes that are not prone to recombination, and should exhibit an average rate of point mutation through time. The Feil et al. phylogenetic analysis of MLST showed that *S. aureus* alleles are at least 15-fold more likely to change by point mutation than by recombination; indicating recombination at these sites is rare, that they are stable enough to maintain function, but not too conserved either.[63] Sequence differences between isolates at just one locus indicate a closer phylogenetic relationship than isolates sharing three or fewer alleles. eBURST analysis is a graphical representation alleles shared between sequence types.

The four loci MLST developed for SIG by Bannoehr et. al has been described previously in this review. Of the seven gene loci used in the standard *S. aureus* MLST, only the *pta* locus is shared between the two methods.[33] *S. pseudintermedius* MLST has proven valuable in the examination of isolates collected over large geographic areas and through many bacterial generations, enabling broader associations than can be made with other methods. In the proceeding studies MLST will be the primary tool used for the characterization of the emergence of MRSP in the southeastern United States.

Quorum-sensing in S. pseudintermedius

As one of the gene loci examined for sequence typing, the accessory gene regulator (*agr*) quorum-sensing locus D serves a dual purpose as both a molecular target and a phenotypic driver of species selection in a mixed species environment. The *agrD* locus encodes auto-inducing peptides (AIP) capable of modulating virulence under stress and in the presence of competing bacteria. These peptides are seven to nine amino acids in length, and are constitutively expressed and deposited into the surrounding environment. Once a threshold density of AIP is reached in the immediate bacterial environment (wound, biofilm, lysosome, etc.) the expression of RNA III is triggered, and a cascade of virulence-enhancing genes is activated. AIPs of one *agr* type have been shown to inhibit the RNA III expression and virulence of staphylococci with a different *agr* type. The agr system is thought to have evolved along lines of speciation within the genus; the assumption being that dominant AIPs have the ability to inhibit the SOS response in other bacteria within the species, genus, and potentially across genus boundries.[66,67] However, in the case of *S. pseudintermedius* the four elucidated AIP variants are shared with *S. delphini*, and *S. intermedius*. This suggests that the SIG shares common quorum-sensing capacity that has been conserved in spite of species differentiation with tropism towards fairly distinct ecological niches.

SIG isolates encode AIPs structurally different than those of *S. aureus*. While a majority of staphylococcal quorum sensing research has focused on interaction among different types of *S. aureus* AIPs, some evidence exists that *S. aureus* AIP may also dampen *S. pseudintermedius* virulence expression.[66] Little is known about AIP interaction among different *S. pseudintermedius agr* types. Recent evidence suggests that expression of methicillin resistance may be triggered by the RNA III-mediated SOS response.[68] These findings suggest that staphylococcal quorum-sensing cross-reactivity may inhibit the signally cascade responsible for the expression of virulence and methicillin resistance.

Summary Statement

At its inception the goal of this doctoral project was to clarify the population genetics of MRSP in the United States. This is largely unexplored territory. Prior to this work MLST had been performed on only five methicillin resistant isolates from the United States as

part of the broader SIG multi-country analysis[33], but no in-depth analysis of a specific referral center population had yet been undertaken. To that end a molecular characterization scheme focusing on both the whole genome and the *mecA* cassette was implemented as the foundational study for this project. Methicillin-resistant and susceptible isolates from the UTCVM collection were analyzed using *mecA* cassette sequencing and typing, PFGE, and MLST. Our key findings (Chapter 2) are that the spread of methicillin-resistance within *S. pseudintermedius* is clonal (vertical), and that the southeastern US clone bears a cassette type similar to that of a Taiwanese MRSA lineage and wholly different than that of the European clonal MRSP.

The next step was to capitalize on this population genetic information. By segregating isolates according to clonal lineage, applying an external stimulus, and analyzing *mecA* expression as a response variable, the effect of genotype was correlated with antibiotic resistance behavior. Our external stimuli (Chapter 3) include oxacillin over different lengths of time and different concentrations, as well as quorum-sensing AIPs.

The problem as well as the study of methicillin-resistance in *S. pseudintermedius* is relatively new. Defining the movement and activity of resistance genes and the isolates that carry them has both intrinsic and clinical value. We hypothesize that the expansion of MRSP is proceeding in a clonal manner, analogous to the early expansion of MRSA. During this nascent phase of expansion there may be as few as two predominant regional clones. The over-arching theme for this work is that once characterized, the clonality of methicillin-resistant staphylococcal lineages can be exploited by linking genotype with expression characteristics. Clonality allows the molecular researcher to inform the clinician of conserved characteristics. These discovered characteristics will in turn inform decisions on antibiotic therapy choice and infection control strategies.

Chapter 2: Identification of a predominant multilocus sequence type, pulsed-field gel electrophoresis cluster, and novel staphylococcal chromosomal cassette in clinical isolates of *mecA*-containing, methicillin-resistant *Staphylococcus pseudintermedius*

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This chapter is a revised paper co-authored by S.M. Solyman and published in the journal Veterinary Microbiology, appearing in November 2009, volume 139.

My primary contributions to this paper include: (i) assisting with experiment design and isolate selection, (ii) development of the pulsed-field gel electrophoresis protocol and processing of *S. pseudintermedius* samples, (iii) most of the gathering and reviewing of literature, (iv) analysis of data and formulation of discussion topics, (v) pulling various contributions into a single paper, (vi) most of the writing.

Abstract

Methicillin resistance encoded by the *mecA* gene is increasingly observed in *Staphylococcus pseudintermedius*. Little is known about the population genetics of veterinary staphylococci bearing methicillin resistance. The aim of this study was to determine the relatedness of resistant bacteria and to compare them with methicillin-susceptible isolates. Multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) fragment profiling were performed on methicillin-resistant *S. pseudintermedius* (MRSP) and methicillin-susceptible *S. pseudintermedius* (MSSP) isolates obtained from canine samples submitted to the veterinary teaching hospital bacteriology service between 2006 and 2008. Multilocus sequence typing detected 20

different sequence types, 16 of which were not previously described. Methicillin-resistant isolates were predominantly ST68, possessed the *Staphylococcus aureus*-associated staphylococcal chromosomal cassette *mec* (SCC*mec*) type VT and fell within the largest PFGE cluster; whereas methicillin-susceptible strains were more genetically diverse. This suggests that most methicillin resistance within the population of isolates tested originated from a single source, which has persisted and expanded for several years.

Introduction

Coagulase-positive hemolytic *Staphylococcus* species, particularly *S. pseudintermedius*, are associated with clinically important infections in dogs. The prevalence of MRSP in dogs is rising. Prior to 1999 reported frequencies of MRSP isolates from dogs were low (<5%); in several surveys from the 1980s, all veterinary staphylococcal isolates tested were susceptible to methicillin.[5,36,37] An increasing number of multidrug-resistant isolates from dogs have been associated with MRSP.[8,35]

Little is known about the population genetics of MRSP. Epidemic MRSA lineages have emerged through horizontal transfer of SCCmec into successful methicillin-susceptible lineages. Once established, MRSA clonal complexes typically dominate specific geographic regions.[69] It is likely that this has occurred with MRSP, and that *S. pseudintermedius* gained SCC*mec* through horizontal transmission within recent history.[33]

The purpose of this study was to use standard MLST, PFGE, and SCC*mec* typing techniques to explore relationships among a collection of geographically linked, MRSP and MSSP isolates.

Materials and Methods

Bacterial strain selection

S. pseudintermedius isolates were obtained from clinical samples submitted to the University of Tennessee College of Veterinary Medicine Clinical Bacteriology Laboratory. A total of 60 non-duplicate isolates from dogs were arbitrarily selected from nine groups, and then were sorted by year of isolation and by presumptive categories of relative *in vitro* susceptibility to oxacillin. Samples included 20 from 2006, 21 from 2007, and 19 from 2008. Isolates with oxacillin zone diameters equal to or less than 10 mm were categorized as high-level-resistant (n=19), isolates with zone diameters from 11 to 17 mm were categorized as low-level-resistant (n=21) and isolates with zone diameters equal to or greater than 18mm were categorized susceptible (n=20).[70] Each group contained six to eight isolates. Bacterial isolation and identification procedures were those routinely used in the laboratory as previously described.[8] Isolates phenotypically identified as "*S. intermedius"* were presumed to be *S. pseudintermedius*. Species identity of *S. pseudintermedius* was confirmed by partial 16S rRNA and *pta* gene sequencing associated with the MLST process (Bannoehr et al., 2007). The type strain of *S. intermedius* isolated from a pigeon (ATCC 29663) and *S. pseudintermedius* (ATCC 51874, isolated from a dog and originally designated *S. intermedius*) served as reference strains for this study.

DNA isolation

Isolates were grown on blood agar plates overnight at 37°C and bacteria derived from a single colony were suspended in 0.5 ml of TE buffer mixed with an equal volume of glass beads. DNA was extracted by cell disruption following pulsed vortexing.

SCCmec typing and characterization

Multiplex PCR designed for characterization of SCC*mec* types I-V in *S. aureus* was used to characterize all MRSP cassette types.[43] A representative ST68 was chosen for full cassette sequencing using a primer walking strategy. Sequences were obtained from PCR products at the University of Tennessee Molecular Biology Resource Facility. Overlapping contiguous sequences were aligned using DNA analysis software (Lasergene Sequence Manager). PCR primers for the detection of the *ccrC2* sequence in SCC*mec*

type V_T were used to confirm the similarity of the type V cassettes associated with ST68 isolates.[71]

Multilocus Sequence Typing

Oligonucleotide primers specific for *tuf, mecA, 16s rRNA, cpn60, pta* and *agrD* genes were designed in previous studies.[33,72] PCR was performed as previously described.[33] PCR products of expected sizes were treated to destroy single-stranded DNA (ExoSap-IT, USB Corp., Cleveland, OH) and submitted to the University of Tennessee Molecular Biology Resource Facility for DNA sequencing. PCR primers were used for direct DNA sequencing of PCR amplification products. STs were assigned using the method established by Bannoehr et al. Briefly, this involved matching *pta*, *cpn60*, *tuf*, and *agrD* sequences determined from study isolates with corresponding reference sequences available in GenBank. Designations for each allele correspond to a code used to assign ST. For alleles that were not previously reported, new STs were assigned beginning sequentially and following the previous designations.

Pulsed-field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed using the protocol described by McDougal et al. with minor modifications.[52] Briefly, a single colony from each catalogued isolate was grown aerobically on a blood agar plate for 24 hours at 37°C. From the plate a cell suspension in saline was made to a reading of 0.50 using the MicroScan turbidity meter, (Dade Behring Inc., Deerfield, IL). Plugs were formed in disposable ~ 100 µl molds by mixing equal amounts of cell suspension with 1.2% SeaKem Gold agarose (FMC, Rockland, Maine). Formed plugs where incubated in a solution of TE buffer (2.8 ml, 10 mM Tris HCl, 1 mM EDTA [pH 8]), Lysozyme (200 µl, 10 mg/ml) and Lysostaphin (20 µl, 10 mg/ml) for two hours at 37°C. The previous solution was poured off. The plugs were then incubated in 5 ml of cell lysis buffer (1M Tris HCL, 0.5M EDTA, 10% Sarcosyl solution, Sterile Type 1 water) and 10U proteinase K at 54°C for two hours in a shaking water bath. Plugs were washed twice with Type 1 water for 5 minutes and four times with TE buffer for 15 minutes each. All washes took place in the 54°C shaking water bath. Plugs were cut in half and digested using 2 µl BSA, 20 µl of

buffer 4 New England BioLabs (NEB, Ipswich, MA), 40U SmaI (NEB) and 176 µl of Type 1 water per section. Digestion was achieved over a minimum of two hours in a 25°C water bath. Restriction fragments were separated by PFGE using a CHEF Mapper (Bio-Rad Laboratories, Hercules, CA). Running conditions consisted of 6.0 V/cm, optimized for separation of 30 kb low molecular weight to 600 kb high molecular weight fragments, and 40 second initial switch time and 45 second final switch time for 18 hours. Isolates unable to elicit a distinct band pattern with *SmaI* were restricted with *ApaI* using the same protocol.

Gels were stained with ethidium bromide, destained in deionized water and the images were digitally captured using a GelDoc 2000 UV transilluminator and Quantity One software (Bio-Rad Laboratories, Hercules, CA). The *Salmonella* Braenderup H9812 global standard was used for gel normalization using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium) and banding patterns were compared in the normalized view using PulseNet *E. coli* scripts. PFGE DNA fingerprint types were assigned using the Tenover criteria.[73]

Results

SCCmec typing and characterization

Identical *mecA* gene sequences were obtained from all methicillin-resistant strains, matching *S. pseudintermedius* (accession no. AM 904732) and *S. aureus* (accession no. EU 790490). Thirty-seven of 38 MRSP SCC*mec* types characterized via multiplex PCR[43] corresponded with *S. aureus* type V. Thirty-seven of 38 MRSP SCC*mec* types characterized via *ccrC2* PCR[71] corresponded with *S. aureus* SCC*mec* type V_T. A single MRSP isolate was untypeable by either method.

Sequence of a representative type V *S. pseudintermedius* cassette (GenBank accession no. FJ544922.1) was homologous, with the exception of one deleted section of a gene, to SCC*mec* type V_T (accession no. AB462393) first described in a community-associated methicillin-resistant *S. aureus* (CA-MRSA) from Taiwan.[74]

Multilocus Sequence Typing

A total of 600 fragment sequences including forward and reverse reactions, representing five genes (16S rRNA*, pta, tuf, cpn60* and *agrD*) from 60 strains, were analyzed. 16S rRNA gene sequences were identical among all isolates in this study and matched those reported for *S. pseudintermedius* (accession no. EU157264).

Thirty-seven of 38 methicillin-resistant isolates belonged to ST68, while a single methicillin-resistant isolate, from 2007 and phenotypically classified as low-level resistant, had a novel ST pattern (TABLE 2). No apparent association was found between oxacillin disk zone diameter for methicillin-resistant isolates and ST. Fifteen of the novel sequence types were detected among 19 of the 22 methicillin-susceptible strains examined.

The *pta* sequences examined had five different alleles (TABLE 1). The sequences of four alleles were identical to *S. pseudintermedius pta* sequences previously reported.[33] A novel *pta* sequence, representing a fifth allele, with a single nucleotide difference, was identified (accession no. FJ170820).

Eight variable sites were detected in *cpn60* sequences and were identical to those reported previously.[33] A novel *cpn60* sequence was found in the same isolate that contained a novel *pta* sequence (accession no. FJ170819).

One variable site occurred in *tuf* sequences with two alleles. These sequences were identical to *S. pseudintermedius* sequences reported previously.[33]

agrD sequencing revealed the presence of the four previously described alleles and known *agrD* (auto-inducing peptide) types.[33] MRSP ST68 was type IV; as were eight of the MSSP isolates. Type III was found in six of 20 MSSP isolates, as well as in the novel MRSP ST105. Type II was found in seven of 20 MSSP isolates. Type I was found in only one novel susceptible strain.

Pulsed-field Gel Electrophoresis

A dendrogram of percent similarity, calculated with Dice coefficients revealed a single major cluster of methicillin-resistant isolates containing all 37 that were ST 68 (FIGURE

1). Within the pulsed-field cluster that contained ST 68, 22 subtypes were identified with 79.7% similarity. Three ST68 isolates, representing two subtypes, were positioned outside the 80% cutoff for close relatedness by the Tenover criteria.[73]

The single MRSP isolate with the novel sequence type ST105, and containing an SCC*mec* type other than MRSA SCC*mec* type V_T, exhibited a pulsed-field pattern unrelated to any other isolates tested.

All methicillin-susceptible isolates exhibited unique pulsed-field patterns. Three susceptible groups were closely related – one group with three isolates, two groups with two isolates each. All isolates within >80% similarity groupings, other than the group containing all ST68 isolates, had distinct sequence types.

Discussion

MLST and PFGE are both highly discriminatory methods for characterizing bacterial isolates.[52,65] In contrast to single locus classification systems, whole genome or multilocus identification methods provide more clarity concerning bacterial relatedness and can be applied to both susceptible and resistant strains. MLST was used in a multi-country survey to determine the population genetic structure of *S. pseudintermedius*; however, in that study only five methicillin-resistant samples from the United States were included.[33] Other recent surveys using PFGE have demonstrated relatedness amongst MRSP isolates in small sample sizes from specific geographic regions.[53,75]

Results from our study add strength to the horizontal transmission and geographic clonal dissemination model of methicillin-resistant staphylococci.[69] Our most notable finding is that 37 of 38 methicillin-resistant isolates in this regional sampling were of a single sequence type, ST68, and demonstrated relatedness in pulsed-field type. This finding parallels the Bannoehr European sample wherein seven methicillin-resistant isolates from dogs with clinical infections in Germany and Sweden were also from a single clone, ST71, while a single canine commensal isolate from Sweden was of a different clonal group, ST69.[33]

The 37 ST68 isolates appeared to carry a type V cassette when tested with the *S. aureus* multiplex PCR typing described by Zhang et al., but DNA sequencing of the cassette

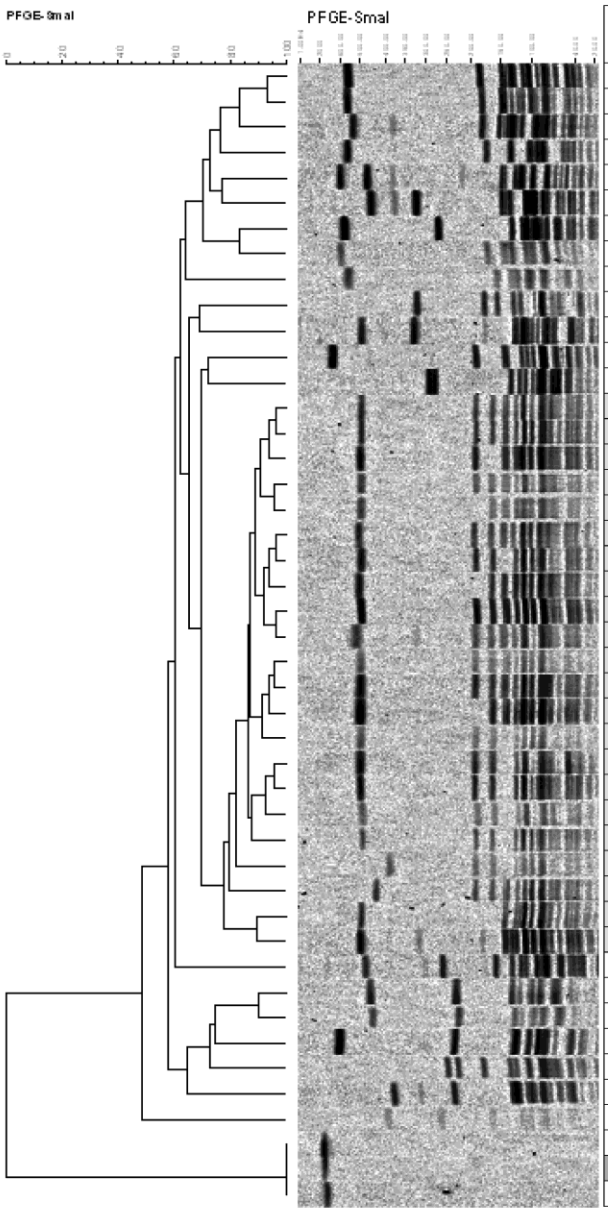
Sequence type	*tuf*	*cpn60*	*pta*	*agrD*	*agrD* (AIP) type	Methicillin resistance profile
68	**1**	**10**	**4**	**3**	**IV**	**Resistant**
92	1	8	1	2	II	Susceptible
96	1	28	19	3	IV	Susceptible
97	1	8	1	1	III	Susceptible
98	1	27	1	3	IV	Susceptible
99	1	27	8	2	II	Susceptible
100	2	7	1	3	IV	Susceptible
101	2	8	1	1	III	Susceptible
102	2	8	2	2	II	Susceptible
103	2	9	1	3	IV	Susceptible
104	2	9	2	3	IV	Susceptible
105	2	10	1	1	III	Resistant
106	2	13	1	2	II	Susceptible
107	2	13	1	3	IV	Susceptible
108	2	13	1	4	I	Susceptible
109	2	27	1	1	III	Susceptible
110	2	27	1	2	II	Susceptible

The sequences used as a reference to identify each gene and allele are: *tuf* 1, EU157712; *tuf* 2, EU157714; *cpn60* 7, EU157497; *cpn60* 8, EU157461; *cpn60* 9, EU157503; *cpn60* 10, EU157505; *cpn60* 13, EU157985; *cpn60* 27, EU157494; *cpn60* 28, FJ170819; *pta* 1, EU157607; *pta* 2, EU157608; *pta* 8, EU157598; *pta* 19, FJ170820; *agrD* 1, EU157400; *agrD* 2, EU157391; *agrD* 3, EU157402; *agrD* 4, EU157356. Predominant MRSP ST 68 in bold print.

TABLE 2.1. Allelic profiles and *agrD* types of novel *S. pseudintermedius* sequence types (ST) isolated from dogs compared to the predominant MRSP ST68.

Gene	PCR product (bp)	Variable sites	Alleles
16s rRNA	217	0	1
pta	426	4	5
cpn60	307	8	9
tuf	353	1	2
agrD	109	3	4
mecA	140	0	1

TABLE 2.2. Nucleotide sequence variations and alleles in five housekeeping genes and mecA of *S. pseudintermedius.*



PFGE Type/ MLST ST	Isolate No.
1/96	9
2/17	13
3/108	25
4/106	17
5/100	33
6/92	60
7/109	18
8/19	56
9/103	12
10/103	23
11/99	57
12/107	10
13/61	45
14/68	30, 31
15/68	47
16/68	48
17/68	43, 53
18/68	49
19/68	37, 44
20/68	42, 50
21/68	20, 41
22/68	1, 2, 4, 28, 35
23/68	29
24/68	7
25/68	3, 5, 8, 32,34
26/68	52
27/68	6
28/68	15
29/68	50
30/68	14, 51
31/68	19, 39
32/68	11
33/68	46
34/68	38, 40
35/68	54
36/15	59
37/98	26
38/97	27
39/104	21
40/110	24
41/102	55
42/101	16
43/101	22
44/105	36
45/103	58

FIGURE 2.1. Dendrogram based on pulsed-field gel electrophoresis analysis of 60 *S. pseudintermedius*. Shaded rows represent isolate groupings that are methicillin-resistant and contain the mecA gene.

revealed that it was a truncated version of SCC*mec* type V_T.[74] This cassette was first reported in a successful multidrug-resistant CA-MRSA lineage from Taiwan, and has recently been reclassified as SCC*mec* type VII.[71] SCC*mec* types II, III and V were previously described in *S. pseudintermedius* using multiplex PCR procedures used in the present study, and the complete cassette sequence from two isolates that were characterized as having type III cassettes by the multiplex PCR method revealed a new hybrid sequence that has been called type II-III.[47] The SCC*mec* cassette associated with the ST105 isolate has not yet been characterized, as this cassette is untypeable using the methods employed for this study. However, the presence of *mecA* in ST105 has been confirmed using PCR, and the ST105-associated cassette has been differentiated from the ST 68-associated cassette by applying primers used for type V sequencing. The circumstances and mechanism by which the ST68 *S. pseudintermedius* lineage in the present study acquired a cassette associated with a disease-causing human *S. aureus* lineage are unknown. The finding implies, however, that inter-species transfer of antibiotic resistance between human and canine pathogens may have occurred.

A single methicillin-resistant isolate with a different and novel ST and pulsed-field type was found – ST105. Only five MRSP sequence types have been described – STs 29, 68, 69, 70 and 71.[33] ST 105 differed from ST 68 at three alleles, indicating a distant phylogenetic relationship. This genetic dissimilarity supports the independent acquisition of *mecA* by each lineage.[33] Sequence type 105 may represent an emerging MRSP lineage, or it may be a transient cassette carrier that will fail to successfully compete with the predominant ST68. Collectively these data indicate that clonal expansion, regional selection and maintenance of the more successful clone have occurred.

Only three previously reported MSSP sequence types, STs 15, 17 and 19, were identified from one isolate each. Sixteen novel sequence types were identified among clinical isolates of *S. pseudintermedius* from dogs; all but one were from methicillin-susceptible isolates. Among methicillin-susceptible strains in our study 19 of 22 (86%) belonged to novel STs and all had distinct pulsed-field patterns. This broad range of clonal diversity amongst MSSP contrasts with the limited number of MRSP lineages seen in the United States and Europe, where ST68 and ST71 predominate respectively.[33]

It is not known if the apparent tendency of antibiotic resistance cassettes to remain in so few lineages is due to the rarity and relative difficulty of cassette transfer, or is a function of the ability of an MSSP lineage to successfully incorporate the mobile genetic

element into its genome. Based upon the variety of MSSP isolates observed, this question warrants further investigation.

In this study, we report an association between ST 68, PFGE type and SCC*mec* type V_T in contemporary clinical isolates of *S. pseudintermedius* from dogs in the southeastern United States. The known genetic diversity among canine isolates of *S. pseudintermedius* was increased by the discovery of 16 novel sequence types and a strain that contained novel sequences for both *pta* and *cpn*60 genes. Regional, national and global sharing of molecular surveillance data generated for MRSP strains through the use of techniques such as single and multi-locus gene sequencing and PFGE will help determine the epidemiological significance and patterns of spread for these newly emerging strains.

Chapter 3: Variation in *mecA* expression among regional clones of methicillin-resistant *Staphylococcus pseudintermedius* and characterization of *mecA* and *blaZ* regulatory elements.

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This chapter is a manuscript that will be submitted to an appropriate journal in summer 2010.

My contributions to this paper include: (i) experiment design and isolate selection, (ii) preliminary PCR and RT-PCR expression data collection, (iii) the gathering and reviewing of literature, (iv) sample processing, (v) analysis of data and formulation of discussion topics, (v) all of the writing except statistical analysis in the Materials and Methods section.

Abstract

Regional clones of methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) affecting veterinary clinical practice have recently been identified in Europe and North America – multilocus sequence types (MLST) 71 and 68 respectively. One notable difference between the two clones is a deletion in the *mecI/mecR1* regulatory apparatus of the staphylococcal chromosome cassette (SCC*mec*) carried by ST68. This deletion in analogous methicillin-resistant *Staphylococcus aureus* (MRSA) results in more responsive and greater expression of the *mecA* encoded penicillin-binding protein 2a, a characteristic associated with community-acquired MRSA lineages. The aim of this study was to characterize *mec* and *bla* regulatory apparatuses in MRSP. Seventeen wild-type *S. pseudintermedius* isolates representing nine methicillin-resistant lineages were screened via PCR for the presence of the repressors *blaI* and *mecI* and sensors *blaR1* and *mecR1*. The *bla* and *mec* operons for each isolate were sequenced and compared for homology between the repressor open-reading frames (ORF), sensor ORFs, and *mecA*

promoter regions. A real-time reverse transcriptase PCR expression assay was developed, validated and applied to nine isolates determining the effect of low-level oxacillin induction and quorum-sensing reporter RNAIII on *mecA* transcript production. Comparisons of the induction of *mecA* expression were made between isolates with a full regulatory complement (*mecI*/*mecR1* and *blaI*/*blaR1*) and those with truncated and/or absent regulatory elements.

Introduction

Staphylococcus pseudintermedius on dogs is analogous to *S. aureus* on humans, acting as both normal flora and opportunistic pathogen. It is the primary cause of pyoderma, the most common dermatologic disease seen in dogs, and is also frequently associated with wound, urinary tract and surgical site infections.[4] The first finding of *S. pseudintermedius* bearing the *mecA* methicillin resistance gene occurred in 1999.[5] In the decade since its discovery, methicillin-resistant *S. pseudintermedius* (MRSP) has become increasingly common, reaching prevalence as high as 30% in studies of referral center isolates.[6,35,76]

Recent multilocus sequence typing (MLST) studies have established geographic clonal emergence of two MRSP lineages.[33,45,77,46] Sequence type 71 predominates in Europe, bears the hybrid resistance cassette SCCmec II-III, and has acquired resistance to all clinically useful veterinary antimicrobial drugs expect lincosamides.[77,47] Whereas ST68 is the most common MRSP sequence type in the United States and Canada, bears SCCmec V_T, and has acquired resistance to all clinically useful veterinary antimicrobial drugs except chloramphenicol.[45,77]

As is the case with MRSA, methicillin resistance in *S. pseudintermedius* results most commonly from the acquisition of the *mecA* gene encoding penicillin-binding protein 2a (PBP2a). PBP2a contains an altered beta-lactam binding site, preventing antibiotics of that class from disrupting gram-positive cell wall metabolism. In addition to synthetic beta-lactam resistance mediated by *mecA*, all isolates characterized as ST68 and ST71 have thus far also demonstrated penicillin resistance through *blaZ*-encoded beta-lactamase.[33,45,77,46]

The controlling mechanisms for *bla* and *mec* systems are different from other bacterial signal transduction pathways in that they lack a kinase-based system for signal

transduction.[78] Instead they are modulated by two-component systems consisting of the analogous repressors *mecI* and *blaI* and sensors *mecR1* and *blaR1* respectively. BlaR1 and MecR1 represent transmembrane spanning and signal transducing proteins. Acylation of BlaR1/MecR1 subsequent to external interaction with beta-lactam antibiotics is followed by their autoproteolytic cleavage on the cytoplasmic side of the cell membrane. This separated intracellular portion of BlaR1/MecR1 travels to the bacterial chromosome and removes its cognate repressor BlaI/MecI via proteolysis. Once the repressor dissociates from its promoter-region binding site, transcription of the *blaZ/blaR1/blaI* and *mecA/mecR1/mecI* genes begins. The structure and function of MecI and BlaI are similar but not identical; they share approximately 60 percent sequence homology.[79,80] The repressors can act interchangeably, binding to the promoter-operator region of either the *blaZ* or *mecA* operon. However, *mecA* induction occurs only through the cognate sensor; the intracellular peptidase portion of transmembrane BlaR1 only has activity on BlaI, and MecR1 only cleaves MecI.[81]

The *mec* promoter region consists of ~100 nucleotides situated between the *mecA* and *mecR1* open reading frames. The *mec* operator is a subset of the promoter region and is a 30 bp palindrome covering *mecA* -10 and *mecR1* -35 promoter sequences. Within the palindrome lies two 4 bp inverted repeats (TACA/TGTA) that serve as binding motifs for the repressors MecI and BlaI.[79,82] Others have shown that mutations within this operator region can affect the ability of the repressors to bind appropriately, and thereby account for differences in *mecA* expression.[83]

In MRSA it has been demonstrated that *mecI*/*mecR1* and *blaI*/*blarR1* configuration has a significant impact on the speed and character of *mecA* induction. While a majority of epidemic strains are *blaZ* positive with functional *blaI*/*blaR1* elements, many have deletions of *mecI* and varying levels of truncation in *mecR1*; the completeness of the *mecA* operon has become a defining characteristic of MRSA SCC*mec* type. Clinical MRSA isolates as well as transformed laboratory strains with only *mecI*/*mecR1* induced *mecA* expression more slowly than those with complete *blaI*/*blaR1* and without functional *mecI*/*mecR1*. Those isolates with both regulatory apparatuses, with both MecI and BlaI available for promoter binding, appear to be even more tightly controlled.[84,85]

Methicillin resistant staphylococcal isolates cured of their SCC*mec* have improved growth rates relative to their *mecA* containing counterparts.[86] This discovery and the presence of some level of *bla* and *mec* regulatory apparatus in all successful and widely-disseminated methicillin-resistant lineages suggests a metabolic cost is associated with unregulated expression of *mecA*. The exact benefit derived from differing levels of

mecA control is unclear. However, a recent historical examination of antibiotic resistance in *S. aureus* by Chambers and DeLeo demonstrates four waves in MRSA evolution beginning with *blaZ* in response to penicillin therapy, followed by three *mecA* acquisitions with differing levels of *mecA* control. The initial Iberian clone and subsequent healthcare-associated MRSA lineages bearing SCC*mec* types II and III have functioning *mecI* and *mecR1*; whereas more recent community-associated MRSA bear cassettes with *mecI* deletions and rely on *blaI*/*blaR1* to regulate *mecA* expression.[87-89]

In addition to *mec*-specific regulatory genes, staphylococci also carry the more generalized accessory gene regulator (*agr*) complex, which produces one of four potential quorum-sensing auto-inducing peptides (AIP) characteristic of its lineage. The peptides are seven to nine amino acids in length, and are constitutively expressed and deposited into the surrounding environment by the bacteria. Once a threshold density of AIP is reached, the expression of RNAIII is triggered, and a cascade of potential virulence-enhancing genes is activated.[66] Recent expression studies with MRSA have suggested that *mecA* may fall under the broader control of *agr*.[68] The *agr* locus of MRS(P)I has been described[67], but its relationship to *mecA* has yet to be investigated.

The emergence of MRSP is recent relative to that of MRSA and thus far restricted to two known geographic clones bearing distinct cassette types. These SCC*mec* types differ in the *mecI*/*mecR1* regulatory apparatus of *mecA*, and are analogous with the two types of cassette structures found in HA and CA-MRSA. Here we inventory the *mecA*/*mecI*/*mecR1*, *blaZ*/*blaI*/*blaR1*, and *mecA* promoter region status of both major and minor MRSP lineages and apply a real-time RT-PCR *mecA* expression assay to correlate regulatory gene presence or absence with speed and abundance of transcript production.

Materials and Methods

Bacterial Isolate Selection and Identification

S. pseudintermedius isolates were obtained from clinical samples submitted to the University of Tennessee College of Veterinary Medicine Clinical Bacteriology Laboratory as well as from European and North American collaborators through previous clonality studies.[45,77] A total of 17 non-duplicate isolates *from dogs* were selected representing

nine MLST lineages associated with methicillin resistance as an initial pool to screen for *blaI*/*blaR1* and *mecI*/*mecR1* variation. Bacterial isolation and identification procedures were those routinely used in the laboratory as previously described.[8] Isolates phenotypically identified as ''*S. intermedius*'' were presumed to be *S. pseudintermedius*. Multilocus sequence typing provided definitive species identity of *S. pseudintermedius* with partial 16S rRNA and *pta* gene sequencing. The type strain of *S. intermedius* isolated from a pigeon (ATCC 29663) and *S. pseudintermedius* (ATCC 51874, isolated from a dog and originally designated *S. intermedius*) served as reference strains for this study.

DNA Extraction

Isolates were grown on blood agar plates overnight at 37°C and bacteria derived from a single colony were suspended in 0.5 ml of TE buffer mixed with an equal volume of glass beads and vortexed for ten minutes. Supernantant in the centrifuged cell lysate was used as template DNA for traditional PCR amplification of *mec* and *bla* regulatory genes.

PCR Primers and Conditions

Olignucleotide primers specific for *S. pseudintermedius mecA* promoter and *blaI*/*blaR1* were based upon MRSP SCC*mec* type V cassette (GenBank accession no. FJ544922.1) and unpublished ST68 genomic data respectively (TABLE 3.1), and designed using IDT SciTools application (Integrated DNA Technologies, Coralville, IA).[90] Primers for *mecI*/*mecR1* were adapted from Hiramatsu et al.[91] Primers for RNAIII were adapted from Sung et al.[67] Conventional PCR was performed using the following parameters: 95º for 90 seconds followed by 35 cycles of 55° for 30 seconds, 68º for 120 seconds, and 94º for 30 seconds.

***mecA* promoter**	
forward	5’-CGGACGTTCAGTCATTTCTACTTC-3’
reverse	5’-ACACCTTCTACACCTTATCAC-3’
mecI[a]	
forward	5’-AATGGCGAAAAAGCACAACA-3’
reverse	5’-GACTTGATTGTTTCCTCTGTT-3’
mecR1	
forward	5’-TGGTATTTGGTTTAGTGAA-3’
reverse	5’-GATTAGGTTTAGGCATTGA-3’
***blaI* 1**	
forward	5’-AGATGGAAAGCCATCTGGGA-3’
reverse	5’-GTCTCGCAATTCTTCAATTTCTTTG-3’
***blaI* 2**	
forward	5’-TCTATGGCTGAATGGGATGTTATG-3’
reverse	5’-GTCTCGCAATTCTTCAATTTCTTT-3’
blaR1	
forward	5’-CCCAGACGGCTTTCCATCTGATAA-3’
reverse	5’-GCCCACAGTTGAAAGTAGGTCACA-3’
RNAIII[b]	
forward	5'-GCAGCAGATATCATTAGC-3'
reverse	5'-TGCTACAATGGCTTCA-3'
[a] Hiramatsu et al.	
[b] Sung et al.	

TABLE 3.1. **Primers used with conventional PCR.**

Sequence analysis

PCR products of expected sizes were treated to destroy single-stranded DNA (ExoSap-IT, USB Corp., Cleveland, OH) and submitted to the University of Tennessee Molecular Biology Resource Facility for DNA sequencing. PCR primers were used for direct DNA sequencing of PCR amplification products. Open reading frames for *blaI*, *blaR1*, *mecI*, *mecR1* and *mecA* promoter were compared between isolates using Lasergene® SeqMan Pro software (DNASTAR, Inc., Madison, WI).

MLST and PFGE

Genetic diversity of *S. pseudintermedius* was determined by MLST of five genes (16S rDNA, *tuf*, *cpn60*, *pta* and *agrD*) and by SmaI-PFGE as previously described.[33,45] MLST sequences were compared with allele sequences present in the NCBI nucleotide database in order to determine the allele number. Sequence type numbers were assigned using the key table for MLST typing of *S. intermedius* group isolates. New sequence types were assigned by the curator, Vincent Perreten (vincent.perreten@vbi.unibe.ch).[77]

Bacterial Growth and RNA Extraction – Time Point *mecA* Expression Assay

For each isolate a 5 ml vial of BBL™ Trypticase™ soy broth (TSB, Becton, Dickinson and Co., Sparks, MD) was inoculated with a single colony grown on blood agar and then incubated overnight at 37°C and 225 rpm. Fifty microliters of overnight growth was used to inoculate four additional 5 ml soy broth vials, which were subsequently incubated at 37°C and 225 rpm for four hours. Oxacillin (Sigma Aldrich, St. Louis , MO) was added to a single vial at four, two, and one hours prior to RNA extraction. The oxacillin concentration was 0.01 µg/ml for all induction vials; this concentration was chosen because in preliminary testing it represented the minimum amount at which the type strain ST68 (06-3228) reproducibly increased *mecA* expression greater than five-fold over baseline. One of the four vials was incubated without oxacillin induction to serve as the baseline for *mecA* expression.

Following four hours of growth RNA was extracted using UltraClean™ Microbial RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Contaminating DNA was removed using TURBO DNA-free™ DNase (Ambion, Inc., Austin, TX). The resulting RNA template was stored in aliquots suspended in DNase RNase free water at -80.0°C.

Bacterial Growth and RNA Extraction – Oxacillin Curve *mecA* Expression Assay

For each isolate a 5 ml vial of BBL™ TSB (Becton, Dickinson and Co., Sparks, MD) was inoculated with a single colony from growth on blood agar and was then incubated overnight at 37°C and 225 rpm. Fifty microliters of overnight growth was used to inoculate seven additional 5 ml soy broth vials, which were incubated at 37°C and 225 rpm for four hours. Oxacillin (Sigma Aldrich, St. Louis , MO) was added to six vials in a fivefold dilution series at concentrations 0.0016, 0.0080, 0.0400, 0.2000, 1.0000 and 5.0000 µg/ml. One of seven vials was incubated without oxacillin induction to serve as a *mecA* expression baseline.

Following four hours of growth RNA was extracted using UltraClean™ Microbial RNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Contaminating DNA was removed using TURBO DNA-free™ DNase (Ambion, Inc., Austin, TX). Resulting RNA template was stored in aliquots suspended in DNase RNase free water at -80.0°C.

Realtime RT-PCR – Measurement of *mecA* Induction and Copy Number

Primers and probes (TABLE 3.2) for target *mecA* and endogenous control 16S were designed using Primer Express® software and constructed as custom TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA). Applied Biosystems reagents TaqMan® 2X Universal PCR Master Mix and Multiscribe™ Reverse Transcriptase were used in a 10 µl one-step RT-PCR protocol in the following amounts per well: 5.00 µl Taq polymerase, 2.75 µl water, 1.50 µl template RNA, 0.50 µl primer and probe mix, and 0.25 µl reverse transcriptase. Each sample was analyzed in triplicate. Template RNA was diluted either 1 to 100 or 1 to 1000. All time point or differing oxacillin concentration samples for each isolate were analyzed together in one run per isolate on a 48-well plate in a StepOne™ Real-Time PCR System (Applied Biosystems, Foster City,

mecA	
forward	5’-GGCATGAGTAACGAAGAATATAATAAATTAACCG-3’
reverse	5’-TTGAGTTGAACCTGGTGAAGTTGT -3’
probe	5’-CTGCTCAACAAGTTCC-3’
16S	
forward	5’-CCCTTGAACTTAGTTGCCATCATTC-3’
reverse	5’-CACCTTCCTCCGGTTTGTCA-3’
probe	5’-CCGGCAGTCAACTTA-3’
***mecA* with T7 promoter**	
forward	5’-TAATACGACTCACTATAGGGGCATGAGTAACGAAGAATATAATAAATTA
reverse	5’-TTGAGTTGAACCTGGTGAAGTTGT-3’
RNAIII	
forward	5'-AGTAAGGAAAAAGATTCTAACAAATACTT-3'
reverse	5'-GCAGCAGATATCATTAGCACAATCG-3'
probe	5'-CTGTTTCTGCGATAAGTTT-3'

TABLE 3.2. **Realtime RT-PCR primers and probes.**

CA). Thermocycler parameters were as follows: 48°C for 30 minutes, 95°C for 10 minutes, 40 cycles: 95°C for 15 seconds and 60°C for 1 minute.

The *mecA* mRNA for standard curve analysis was produced by adding a T7 promoter to the sequence of the *mecA* forward primer utilized in the real-time assay (TABLE 3.2). This primer coupled with the reverse primer from the real-time assay was applied to genomic DNA of 06 3228 under the following thermo-cycling conditions: 95º for 90 seconds followed by 35 cycles of 55º for 30 seconds, 68º for 120 seconds, and 94º for 30 seconds. The resulting T7 mecA DNA template was electrophoresed on an 0.8% agarose gel. The single band 117 bp product was excised and extracted (QIAquick® Gel Extraction Kit, Germantown, MD). Following manufacturer's recommendations for DNA <500 bp in length, 1.0 µg served as template in a 16 hour transcription reaction (MEGAscript® T7, Ambion Inc., Austin, TX). The 97 bp RNA product was treated with 4 U TURBO DNase (Ambion Inc., Austin, TX) at 37ºC for 30 min, and filtered to remove all non-RNA elements (MEGAclear™, Ambion Inc., Austin, TX). The copy number per µl was calculated using µg/ml measurements from a NanoDrop 3300 fluorospectrometer (ThermoScientific Inc., Wilmington, DE). The transcripts were used to create standard curves for absolute quantitation of *mecA* mRNA transcripts isolated from the samples. The numbers of RNA copies were estimated based on the molecular weights of the RNA standards and the RNA concentrations. Ten-fold serial dilutions were prepared, and aliquots of each dilution were stored at –80°C and used only once.

The efficiency of the real-time RT-PCR assay to amplify the *mecA* standard mRNA was evaluated with the equation: Efficiency = $[10^{(-1/\text{slope})}] - 1$.[92] Slope is a value derived from the constructed standard curves. To insure equal amplification efficiency in tested isolates, four ten-fold serial dilutions of RNA extracted from wild-type MRSP were tested against the standard curve. The efficiencies were comparable. Quantitiation of *mecA* copy number was calculated by transforming Ct values to the relative number of RNA molecules. The quantity of cDNA for each experimental gene was normalized to the quantity of 16S cDNA in each sample as previously described.[93]

Estimation of 16S Endogenous Control Copy Number

Multiple 16S genes appear in the bacterial chromosome of *S. aureus*, usually ranging from six to eight copies.[94] To insure that *mecA* transcript measurement was not compromised by significant disparities in 16S copy number between *S. pseudintermedius* sequence types, comparison of 16S DNA relative to that of single copy *mecA* was made using real-time PCR.

Genomic DNA from one isolate each representing STs 68, 71, 73, and 116 was extracted using UltraClean™ Microbial DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA). The purified DNA amount was measured and adjusted to equal µg/ml concentrations. Real-time PCR was performed as previously described without reverse transcriptase using 16S rRNA and *mecA* primers and probes. Based upon the assumptions that both primer sets are equally efficient (achieved with validation for ΔΔCt *mecA* expression assay) and that *mecA i*s represented in the genome as a single copy, 16S rRNA Ct values arising before that of *mecA* indicate that multiple 16s genomic copies are present. Fold change calculations can then show 16s copy number relative to that of *mecA.*

RNAIII Expression in Conjunction with *mecA* Expression

For each isolate a 5 ml vial of BBL™ TSB (Becton, Dickinson and Co., Sparks, MD) was inoculated with a single colony from growth on blood agar and was then incubated overnight at 37°C and 225 rpm. Fifty microliters of overnight growth was used to inoculate five additional 5 ml soy broth vials, which were incubated at 37°C and 225 rpm for one, three, five, seven, and nine hours. RNA extraction and realtime RT-PCR was performed as previously described using *mecA* and RNAIII specific primers and probes. Each reaction was performed in triplicate with one primer set per well. ΔΔCt fold-change over baseline comparisons were made against the one hour measurement.

Statistical Analysis

A mixed model ANOVA (SAS, version 9.2, SAS Institute Inc, Cary, NC) was used to estimate the effect of sequence type (68 verses 71) and time on inverse log of *mecA* expression. Isolate, sequence type and time were modeled as class variables and isolate was included as a random factor in the model. Independent variables sequence type, time and the interaction of sequence type and time were evaluated for an effect on the dependent variable log of *mecA* copy number. A second and similar model was used to assess the effect of *blaI* regulation alone verses functional *mecI* regulation, time and the interaction of *blaI* and *mecI* status on the inverse log of *mecA* copy number. In the second model isolate, gene status (*blaI* vs. *mecI*) and time were included as class variables. A multiple range test according to the method of Tukey was used to adjust for multiple comparisons in both models. The fit of the both models to the data was evaluated by assessing the degree to which the residuals from the models fit a normal distribution using the Shapiro-Wilk test statistic. An a priori adjusted p-value of < 0.05 was used to determine statistical significance for all tests.

Results

Screening for *mecA*, *mecI*, *mecR1*, *blaZ*, *blaI*, and *blaR1* Initial *mecA* Expression Assay

Twelve isolates representing nine sequence types comprised the initial *mec* and *bla* regulatory element survey of MRSP isolates (TABLE 3.3). All were phenotypically methicillin resistant and confirmed *mecA* positive. All but one isolate (ST73) were phenotypically beta-lactamase positive and contained *blaZ*, *blaI* and *blaR1* elements. Sequence types 71 and 73 were the only MRSP backgrounds that screened positive for the repressor *mecI*. The two ST71 isolates (one European and one North American) contained both repressors *mecI* and *blaI*. No American isolates other than NA18 (ST71) had a complete *mecI* sequence.

The initial twelve isolates chosen for *mec* and *bla* regulatory element detection were grown with and without 0.01 µg/ml oxacillin for four hours and the relative level of *mecA* transcript production was measured by realtime RT-PCR using the ΔΔCt method

isolate identifier	sequence type	*blaI*	*blaR1*	*blaZ*	*mecI*	*mecR1*	*mecA*	SCC*mec* type
na 4	26	pos	nd	pos	neg	neg	pos	III[a]
na 6	26	pos	nd	pos	neg	neg	pos	III[a]
na 11	118	pos	nd	pos	neg	neg	pos	III[a]
na 12	118	pos	nd	pos	neg	neg	pos	III[a]
na 31	100	pos	nd	pos	neg	neg	pos	untypeable[a]
07 5066	105	pos	nd	pos	neg	neg	pos	untypeable[a]
e 44	69	pos	nd	pos	neg	pos	pos	untypeable[b]
e 138	116	pos*	pos*	pos	neg	pos	pos	VI[b]
06 3228a	68	pos	pos	pos	neg	neg	pos	V[c]
06 3187	68	pos	pos	pos	neg	neg	pos	V[a]
08 494	68	pos	pos	pos	neg	neg	pos	V[a]
08 1043	68	pos	pos	pos	neg	neg	pos	V[a]
na 18	71	pos	nd	pos	pos	pos	pos	untypeable[a]
e 64	71	pos	pos	pos	pos	pos	pos	II-III[d]
e 50	71	pos	pos	pos	pos	pos	pos	II-III[e]
e 55	71	pos	pos	pos	pos	pos	pos	II-III[e]
e 69	73	neg	neg	neg	pos	pos	pos	VII[c]

[a] isolate characterized in-house using multiplex pcr Zhang et al.
[b] isolate characterized by Perreten et al.
[c] GenBank accession no. FJ544922
[d] GenBank accession no. AM904732
[e] GenBank accession no. AM904731

nd = not determined
* sequence difference in comparison with *bla* of STs 68 and 71

TABLE 3.3. Isolates used in study – presence/absence of *mec* and *bla* elements. Shaded rows represent isolates that were further characterized by time point *mecA* expression assay.

(FIGURE 3.1). Error bars indicate the standard error of the mean and relative quantitation of *mecA* expression is represented on a log scale.

Those isolates screening positive for *blaI* only (represented by North American STs 26, 68, 69, 105, 116, and 118) increased *mecA* expression to a greater extent than the ST71s containing both *mecI* and *blaI*. Sequence type 73 bearing *mecI* alone increased *mecA* expression at four hours to a level similar to that of the *blaI*-only isolates.

Sequence Type Selection for Further Analyses

Based upon the initial *mecA* expression assay and the presence or absence of *mec* and *bla* elements, STs 73 and 116 were selected to contrast the regulatory apparatuses of the predominant geographic clones ST68 (North America) and ST71 (Europe). Sequence type 73 lacks a *bla* operon but has a complete *mec* operon resulting in a *-blaI/-blaR1 +mecI/+mecR1* configuration with regard to *mecA* expression control. Whereas ST68 has *+blaI/+blaR1 -mecI/*^{mut}*mecR1* (absent *mecI* and mutation/truncation of *mecR1*) and ST71 has both complete repressor/sensor systems in *+blaI/+blaR1 +mecI/+mecR1*. Sequence type 116 was chosen because the original *blaI* 1 forward and reverse primers (TABLE 3.1) were unable to elicit a PCR product. A region internal to the previous *blaI* target was amplified using *blaI* 2 forward and reverse primers. Sequence type 116 *blaI* genomic sequence compared with that of STs 68 and 71 had multiple nucleotide and translational dissimilarities (FIGURE 3.2). Therefore ST116 was classified as ^{mut}*blaI/+blaR1 -mecI/+mecR1* (the mutation designation for *blaI* is relative to that of STs 68 and 71).

Sequence types 73 and 116 contain only one known characterized isolate each. However, additional wild-type clinical ST68 (n=3) and ST71 (n=2) isolates were added to subsequent analyses to show reproducibility of *mecA* expression within sequence type background. In total four North American ST68s, three European ST71s, and one each of STs 73 and 116 were fully characterized by MLST, PFGE (FIGURE 3.3), *mec* and *bla* regulatory gene sequencing, and mecA expression analysis at four time points.

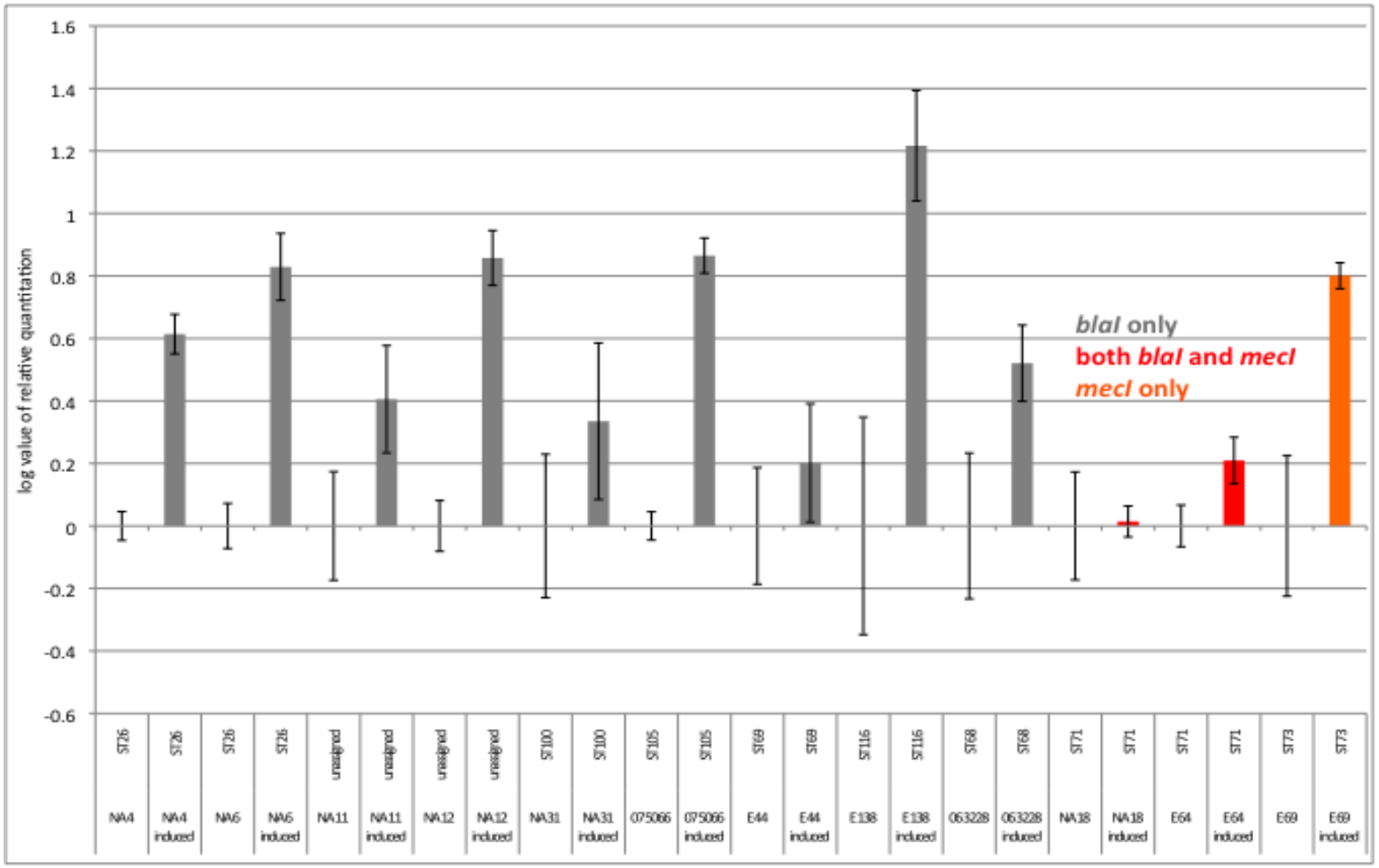

FIGURE 3.1. **Relative *mecA* expression measured with and without 0.01 µg/ml oxacillin induction at 4.0 hours growth.**

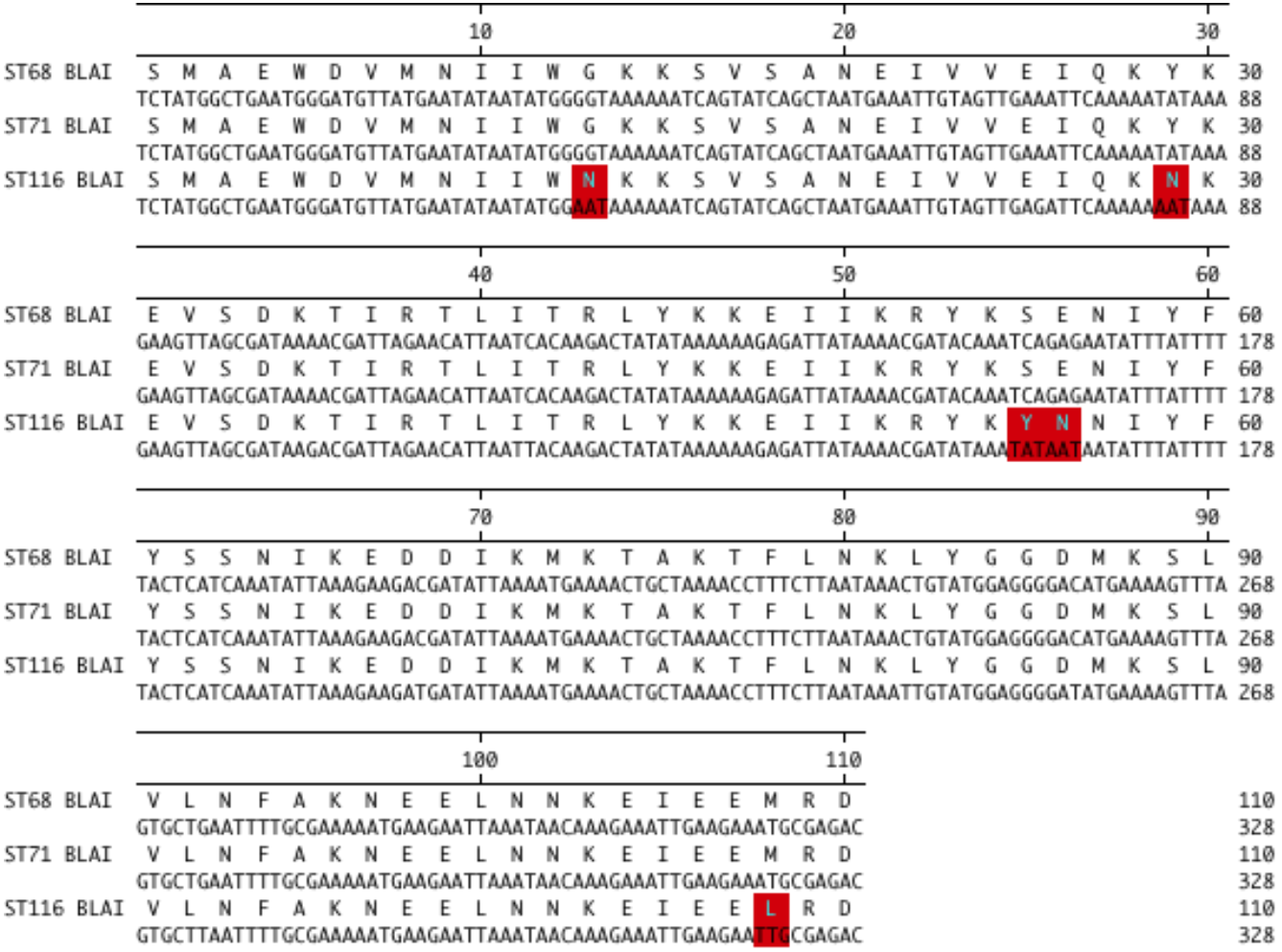

FIGURE 3.2. ***blaI*** **nucleotide and peptide variation between ST116 and STs 68 and 71.**

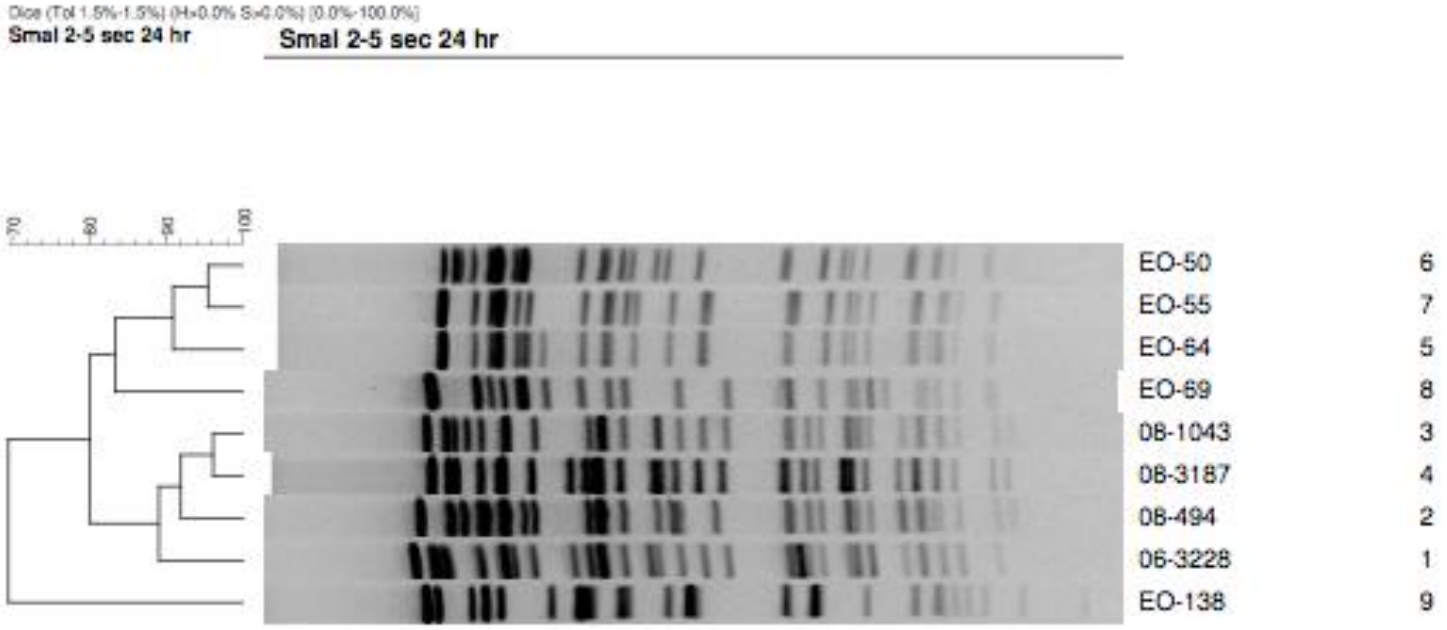

FIGURE 3.3. **PFGE of nine MRSP isolates. EO-50, EO-55, and EO-64 are European ST71 and closely related by the Tenover et al. criteria. 08-1043, 08-3187, 08-494, and 06-3228 are southeastern US ST68 and closely related by the Tenover et al. criteria. EO-69 and EO-138 are sequence types 73 and 116 respectively.**

mec Sequence Analyses

Promoter sequences for ST68 and ST71 were identical. There was a *mecA* promoter single bp polymorphism between ST73 and STs 68 and 71 at *mecA* -10; this was adjacent to a *mecI*/*blaI* TACA binding motif (solid boxes FIGURE 3.4). Sequence type 116 differs from STs 68 and 71 by a single base pair difference at the *mecA* ribosomal binding site (dashed box FIGURE 3.4).

Repressor *mecI* sequence was complete and presumed functional in only STs 71 and 73, SCC*mec* types II-III and VII respectively.[47] In the isolates sequenced for this study, there was complete homology between ST71 and ST73 in the *mecI* open reading frame (data not shown).

Sensor *mecR1* sequence was similar between STs 71, 73, and 116, sharing 97% nucleotide homology. The significance of the slight dissimilarity is unknown. However, because only *mecR1* and not *blaR1* can inactivate *mecI*, *mecR1* is presumably a non-essential remnant in ST116. *mecR1* in ST71 differs from ST73 by a single bp deletion, resulting in a frame shift mutation at the C-terminal end of the peptide sequence (FIGURE 3.5).

bla Sequence Analyses

As was previously reported in "Sequence type selection for further analyses", ST116 *blaI* genomic sequence compared with that of STs 68 and 71 had multiple nucleotide and translational dissimilarities (FIGURE 3.2). The sensor *blaR1* was 100% homologous between STs 68 and 71. However, peptide composition of BlaR1 between STs 68 and 71 and ST116 was as different as that observed for peptide composition of BlaI (FIGURE 3.6).

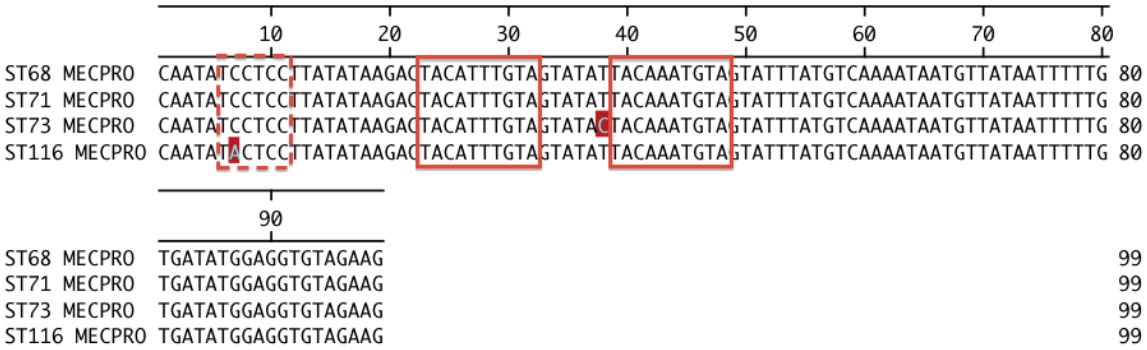

FIGURE 3.4. ***mecA*** **promoter region polymorphisms of MRSP sequence types.** ***blaI/mecI*** **binding motifs represented within solid boxes,** ***mecA*** **ribosomal binding site in dashed box.**

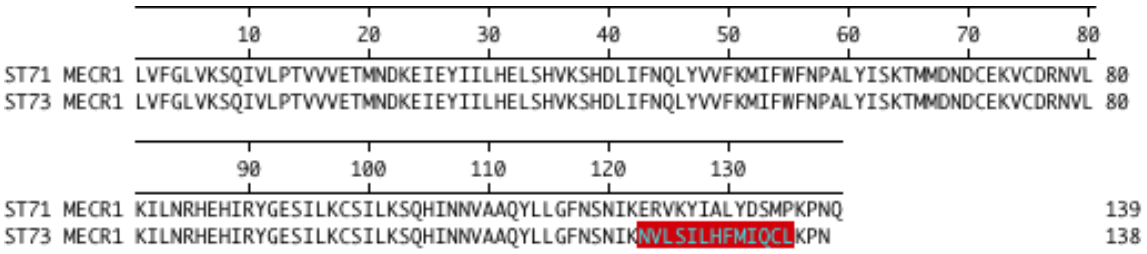

FIGURE 3.5. ***mecR1*** **peptide dissimilarity between STs 71 and 73.**

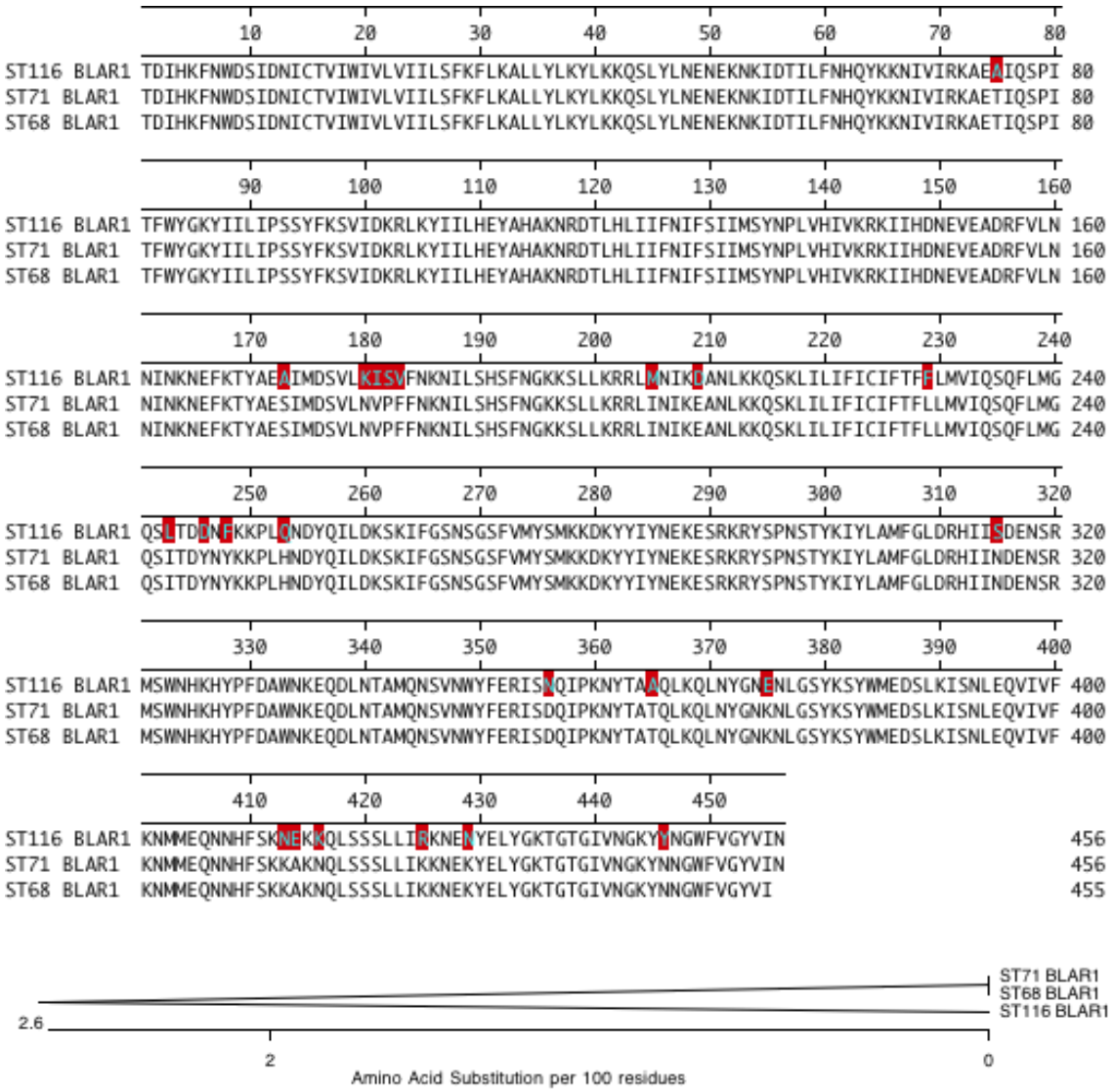

FIGURE 3.6. ***blaR1*** **peptide dissimilarity between ST 116 and STs 71 and 73.**

Relative Quantitation of *mecA* Expression – ΔΔCt Method

Each RT-PCR value for the nine isolates below was the result of the analysis of a single RNA sample. However, growth experiments with RNA extraction and RT-PCR were performed at least twice with each isolate.

Measurements of *mecA* mRNA copy number relative to that of the 16S RNA endogenous control allowed intra-isolate comparisons at differing time points. This method was useful in illustrating the direction of *mecA* expression (FIGURE 3.7).

Sequence type 73, which lacked the *blaI* repressor, showed a stepwise increase in *mecA* expression over four hours. Sequence types 68 and 116, each lacking the *mecI* repressor, showed induction immediately at one hour, and then maintained elevated *mecA* expression at two and four hours. Sequence type 71, which possessed both *blaI* and *mecI* repressors, did not increase *mecA* expression at any time point at the 0.01 µg/ml oxacillin level.

Quantitation of *mecA* Expression – Standard Curve

Transcript copy number tied to absolute measurements of known *mecA* mRNA in a standard curve make possible inter-isolate comparisons at the different time points tested. In addition to illustrating the direction of *mecA* expression, the method allows a copy production number to be applied to the population of cells producing *mecA* under varying oxacillin exposures (FIGURE 3.8).

At four hours growth in 5.0 ml TSB with no oxacillin, *mecA* copy numbers were 5.39 million to 1.12 million for ST 68s and 71s respectively; though a 4.81 fold difference, this was not statistically significant (p = 0.6475). However, significance in *mecA* copy number disparity between ST68 (+*blaI*/+*blaR1* -*mecI*/^{mut}*mecR1*) and ST71 (+*blaI*/+*blaR1* +*mecI*/+*mecR1*) isolates were noted at all subsequent induced time points. At one hour induction, *mecA* expression was 34.71 million to 0.97 million for ST 68s and 71s respectively (~35 times greater *mecA* expression, p = 0.0114). At two hours induction, *mecA* expression was 23.76 million to 0.71 million for ST 68s and 71s respectively (~33 times greater *mecA* expression, p = 0.0158). At four hours induction, *mecA* expression

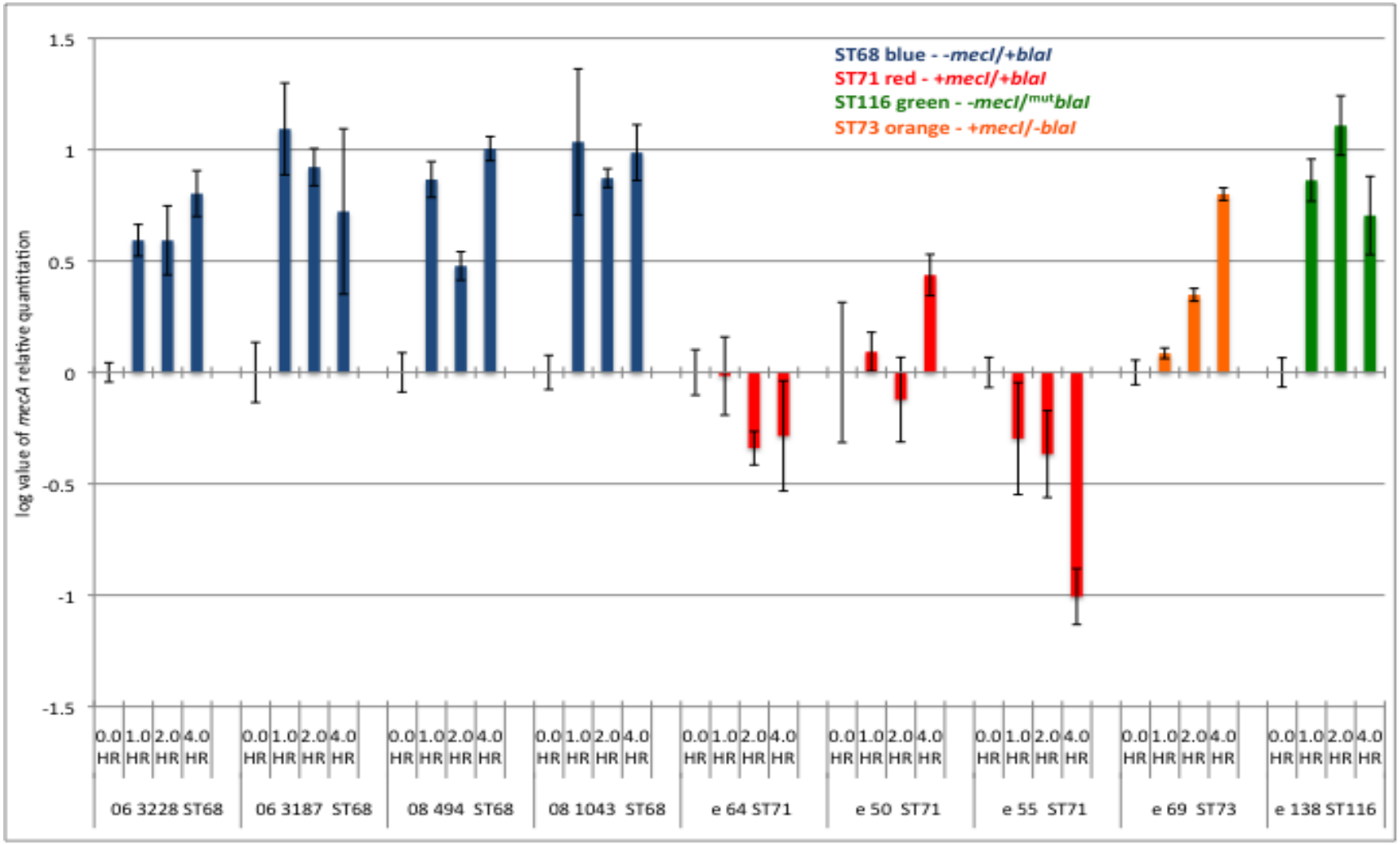

FIGURE 3.7. **Influence of repressor gene presence or absence on relative *mecA* expression, measured at one, two and four hours of growth following exposure to 0.01 µg/ml oxacillin.**

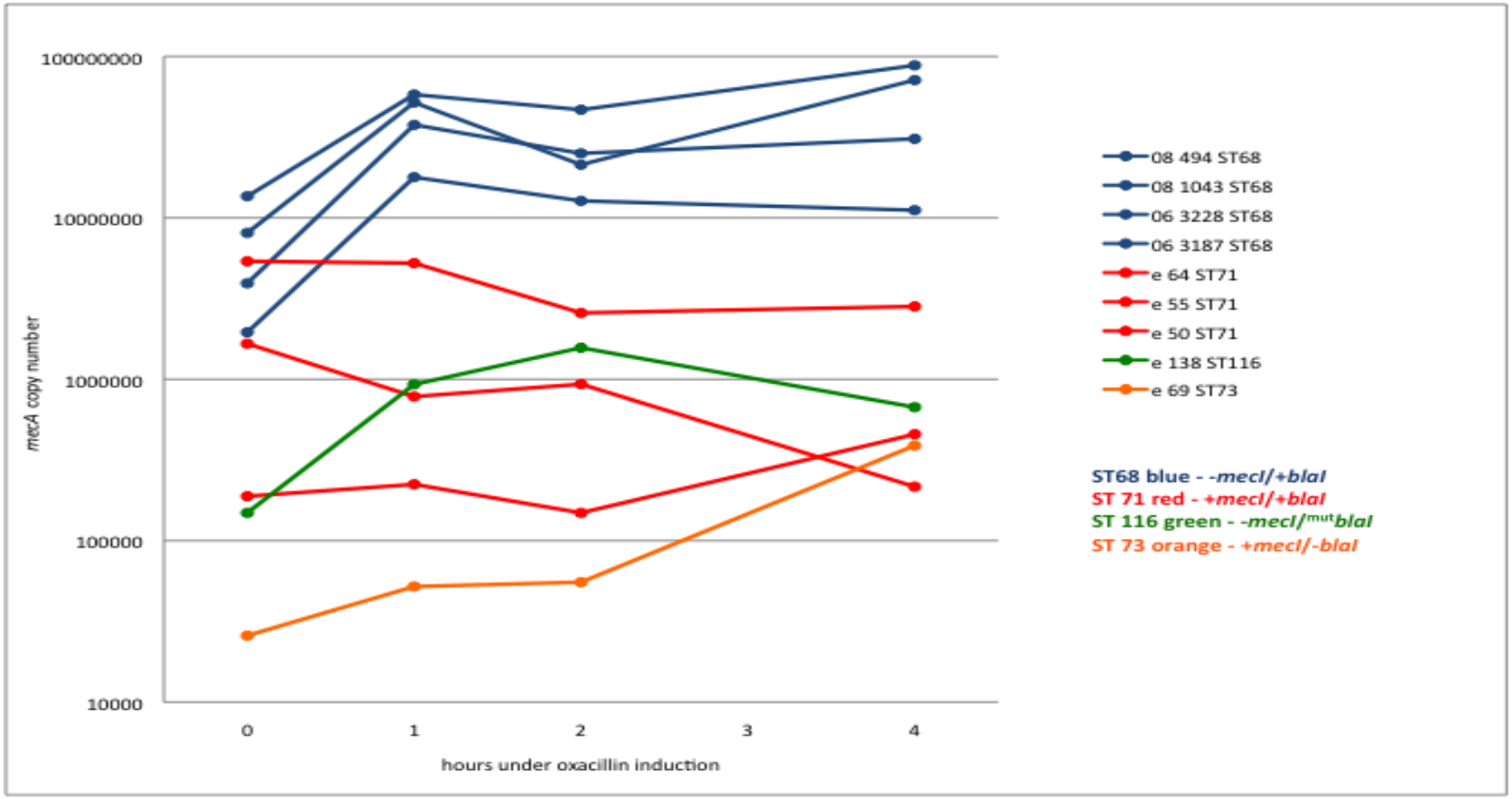

FIGURE 3.8. Influence of repressor gene presence or absence on absolute *mecA* expression, measured at one, two and four hours of growth following exposure to 0.01 µg/ml oxacillin.

was 38.34 million to 0.65 million for ST 68s and 71s respectively (~59 times greater mecA expression, p = 0.0046).

Sequence type 116 (^{mut}blaI/+blaR1 -mecI/+mecR1) produced *mecA* transcript in amounts equivalent to ST71 (10^5-10^6) at all time points. Whereas ST73 (*-blaI/-blaR1 +mecI/+mecR1*) while increasing *mecA* output in a time-dependant manner, maintained the tightest control of *mecA* expression of the isolates tested.

Effect of Increasing Oxacillin Concentration on *mecA* Expression

One isolate from each sequence type was exposed to increasing amounts of oxacillin to determine if and at what level *mecA* copy number induction occurred (FIGURE 3.9). Absolute measurement of *mecA* copy number revealed a one to two log difference between ST68 (06 3228) and ST71 (e 64) at every oxacillin concentration at which both isolates grew. No clear *mecA* induction level was noted for ST71; *mecA* copy number or expression did not increase with increased oxacillin concentration. Sequence type 73 showed no *mecA* induction until a two-log change in copy number at 1.0 µg/ml oxacillin. Sequence type 116 exhibited a dose-dependent *mecA* copy increase in response to increasing oxacillin pressures.

RNAIII Expression in Conjunction with *mecA* Expression

Measurement of RNAIII at two-hour intervals during log phase growth revealed an expected sequential increase in RNAIII as population density within the growth chamber intensified. *mecA* expression for the ST68 isolate shown (FIGURE 3.10) also increased with increasing population density for the first five hours, and then reached a plateau with high-level expression that was maintained through hours seven and nine.

Discussion

The pattern of *mecA* expression observed in this MRSP study in part parallels previous reports of how *mec* and *bla* regulatory elements contribute to *mecA* repression in MRSA

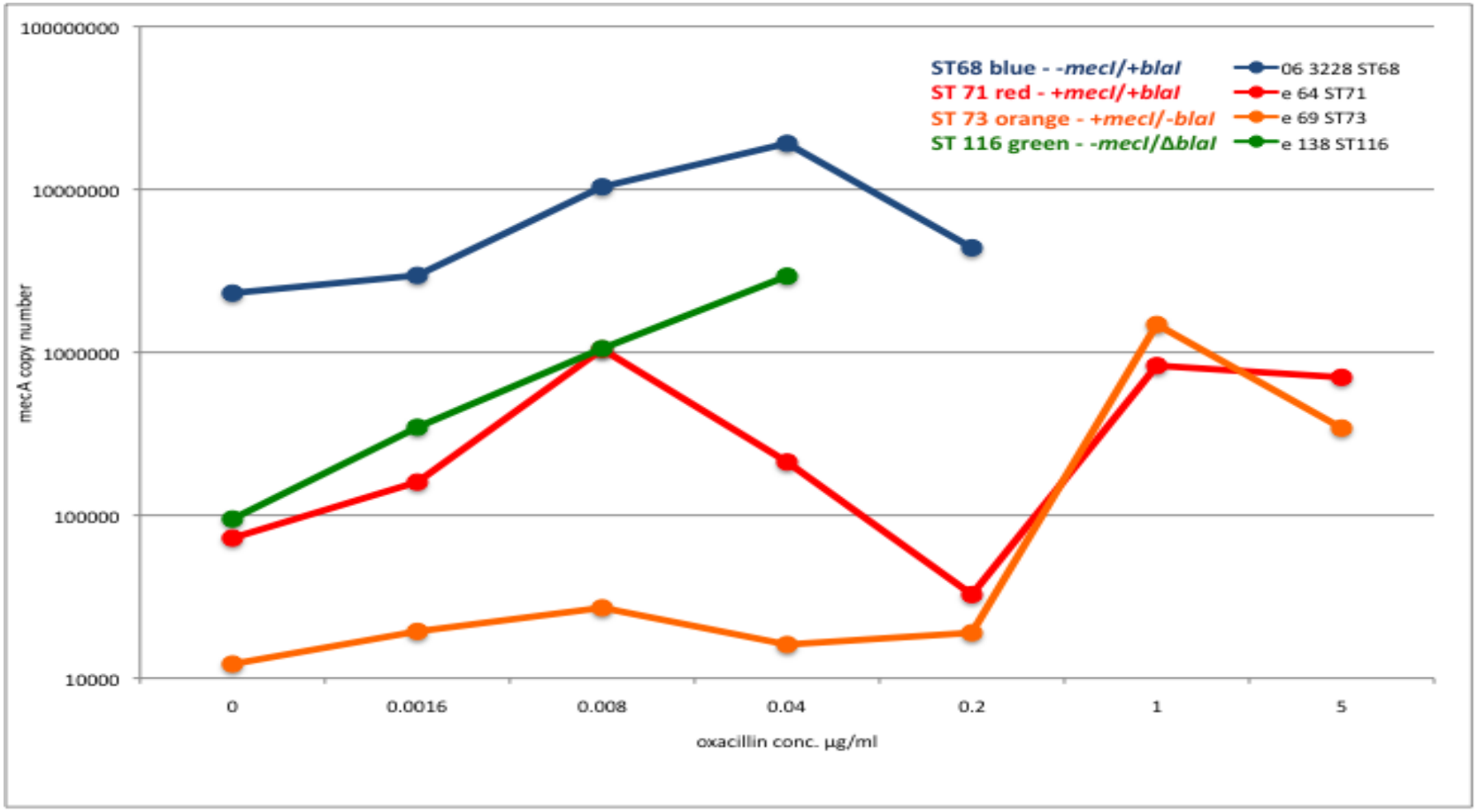

FIGURE 3.8. **Absolute *mecA* transcript production over four hours at increasing concentrations of oxacillin.**

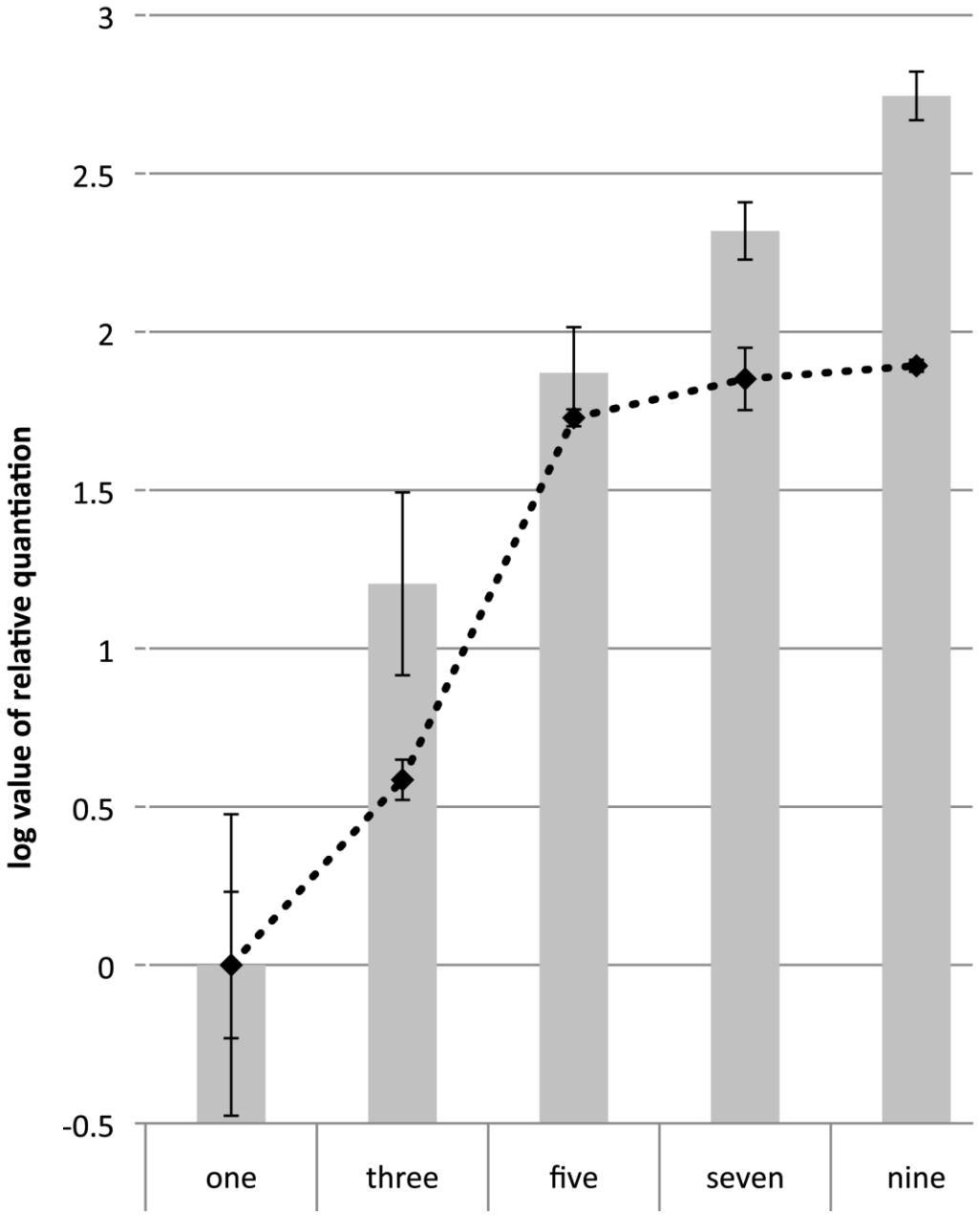

FIGURE 3.10. **Gray columns represent RNAIII expression. The black dashed line represents *mecA* expression over nine hours. Error bars for columns and dashed line represent standard error of the mean of technical replicates.**

– BlaI is more permissive at the *mecA* promoter than MecI.[84,85] However, because the acquisition of *mecA* by *S. pseudintermedius* was recent, relative to that which occurred in *S. aureus*, the identification of prevailing *mec* regulatory strategies in regional clones provides more convincing evidence as to the level of *mecA* regulation is required for successful clonal expansion. For MRSP it appears that some level of *bla* regulatory control is needed to efficiently express methicillin resistance.

The exclusively MecR1-controlled system (ST73) was slower than the exclusively BlaR1-controlled systems (STs 68 and 116) to respond to oxacillin, taking hours to achieve a comparable response. Sequence type 73 also had the lowest level of constitutive *mecA* expression in the absence of oxacillin exposure. It is difficult to extrapolate these and other *in vitro* assessments (*mecA* expression, antimicrobial susceptibility tests, etc. Kirby-Bauer disc diffusion, MIC) to how infections caused by these isolates will behave in the patient. However, tight control of *mecA* by MecI-only systems likely puts these MRSP and MRSA lineages at a disadvantage in the clinical setting, as induction may not be rapid enough to prevent cell death when faced with therapeutic levels of beta-lactam antibiotics.[81,95] The paucity of successful *mec*-only controlled methicillin-resistant lineages for both *S. aureus* and *S. pseudintermedius* suggests that some level of *bla* control in *mecA* expression is necessary for geographic clonal expansion.

Despite having similar *bla* and *mec* regulatory apparatuses (*blaI* repression without *mecI*), sequence types 68 and 116 are genetically dissimilar when analyzed by MLST and PFGE, and contain the distinct SCC*mec* types V and VI respectively. BLAST analysis of *blaI* and *blaR1* sequence reveals differing origins for beta-lactamase. Sequence type 116 *bla* sequence is identical to *S. aureus* plasmid pWBG748 (GenBank accession no. GQ915265); whereas ST68 *bla* sequence matches most closely with beta-lactamase-containing *S. aureus* plasmid pWBG756 (GenBank accession no. GQ900472). Both sequence types respond to oxacillin induction at one-hour post induction and maintain increased *mecA* expression at two and four hours. However, *mecA* transcript copy number differs between the two sequence types by one to two logs at all time points and at all oxacillin concentrations measured, suggesting that either the *blaI*/*blaR1* of ST68 is more permissive than the *blaI*/*blaR1* of ST116, or that additional factors are affecting *mecA* expression.

With the singleton comparator strains (STs 73 and 116) removed from the analysis, the one to two log difference in *mecA* expression between the North American MRSP ST68s and European ST71s at all induced time points and at all levels of antibiotic exposure suggests there exists at least two divergent pathways to geographic clonal success with

regard to control of PBP2a metabolism. Sequence types 68 and 71 are not genetically similar when analyzed with MLST and PFGE.[77] However, sequence analysis revealed complete homology in all shared *mec* and *bla* regulatory elements – *mec* promoter, *blaI* and *blaR1*. Sequence type 68 induces at one hour and maintains induction throughout the assay. Whereas ST71 does not induce in the presence of 0.01 µg/ml oxacillin and maintains essentially the same level of constitutive *mecA* expression at all time points measured. Presumably BlaI and MecI compete for access to the *mec* operator in ST 71. This competition could account for the variability observed in the direction of *mecA* expression. With increasing oxacillin concentrations ST 71 appears to have a biphasic response with increased *mecA* copy number at 0.08 µg/ml and then again at 1.0 µg/ml. This pattern should be further explored to determine whether BlaR1 and MecR1 differ in response to oxacillin concentration as well as time.

As of yet there is no known overlap in the geographic areas affected by STs 68 and 71. These predominant sequence types emerging in North America and Europe with their distinct *mec* regulatory strategies are either the product of chance, or are a reflection of unique environmental pressures. Sequence type 68 has only BlaI/BlaR1 regulation of *mecA*, has high constitutive *mecA* expression, and responds rapidly to small concentrations of oxacillin. Sequence type 71 has both BlaI/BlaR1 and MecI/MecR1 regulation of *mecA*, and has less constitutive and induced expression of *mecA* than ST68. Because uncontrolled PBP2a production is deleterious[86], it has been suggested that MRSA lineages require some level of *bla* regulatory control to stabilize the *mecA* gene in the absence of beta-lactam antibiotic exposure.[85, 91] MecI/MecR1 alone is likely too restrictive of *mecA* expression to adequately respond to beta-lactam treatment. MecI/MecR1 coupled with BlaI/BlaR1 represents a middle ground with ST71 where a lineage could call on *mecA* expression if needed; this conformation may indicate that these isolates evolved in an environment in which antibiotic perturbations were less frequent. The BlaI/BlaR1 of ST 68 represents the least stringent *mecA* control, and the apparent success of this lineage in the United States may indicate a constant need for some level of PBP2a, possibly due to presence of low-level beta-lactam in the environment in which they evolved.

Chapter 4: Conclusions and Application of Findings

The molecular characterization study is now part of a collection of similar investigations[33,45,46,77] that have firmly established ST71 and ST68 MRSP clonal expansion in Europe and US respectively. The follow-up study is the first to utilize comparative regulatory gene sequencing and gene expression in MRSP to compare the two regional clones with regard to differences in gene expression. The approach used in measuring *mecA* copy number gave unique insight into not only fold change over baseline expression, but absolute copy number of *mecA* mRNA produced in MRSP.

Sequence type 68 exhibited a significantly different *mecA* response relative to ST71 in quickness, duration, and number of transcripts when exposed to very small amounts of oxacillin. This presumably translates to greater PBP2a in ST68 relative to ST71 within the first few hours of oxacillin exposure. Earlier MRSA studies have not been able to detect a correlation between beta-lactam mean inhibitory concentration (phenotype) and cellular concentrations of PBP2a.[96,97] We too observed that high and low *mecA* production response did not correspond to phenotype. Isolates of ST68 and ST71 exhibited a full range of phenotypic oxacillin resistance, from low-level measurements near 17 mm by Kirby-Bauer disc diffusion and 0.5 µg/ml by broth microdilution to high-level resistance ≤ 10 mm and ≥ 4.0 µg/ml respecitively.[77,98] This discrepancy between reproducible amounts of *mecA* transcript production (measured at one, two and four hours of oxacillin exposure) and variable disc diffusion phenotype (typically measured at 24 hours of bacterial growth) suggests that expression analysis may be more indicative of *in vivo* MRSP response to antibiotic therapy than more traditional measurements. Clinicians select antibiotics and dosage based upon phenotypic measurements. But these finding perhaps indicate more aggressive empirical beta-lactam protocols could be beneficial against clonal lineages like ST71 in Europe that demonstrate more stringent *mecA* regulatory control. Further research would be required to bear this out.

In 2003 Rosato et al performed a similar analysis of clinical MRSA isolates, sequencing regulatory genes and the *mecA* promoter, and performing *mecA* expression analysis. They reported a synergism in isolates with both MecI and BlaI, with lower *mecA* transcription than isolates containing either one alone.[84] Our observations of MRSP in Chapter 3 conflict with the Rosato assessment – ST71 with both BlaI and MecI was intermediate to the high *mecA* production of ST68 and the low *mecA* production of MecI-only ST73. The less-diversified MRSP genetic evolutionary picture may explain this difference. Heterogeneous resistance is a strategy in many successful MRSA lineages,

whereby a small subset of bacterial cells in pure culture express high-level methicillin resistance in response to selection. Multiple genetic factors (in addition to those examined in these studies) have been shown to affect *mecA* production in MRSA. We have been unable to demonstrate heterogeneous methicillin resistance thus far in MRSP. Perhaps this early less complicated stage of *mecA* regulatory evolution in MRSP can be exploited. We've demonstrated that some level of beta-lactamase control likely contributes to the success of STs 68 and 71. Our findings suggest that the membrane-bound receptor BlaR1 as a prime target for therapeutic control of methicillin-resistant isolates.

The most lasting aspect of this work will likely be the development of a highly reproducible MRSP *mecA* expression assay as an *in vitro* model to analyze novel antibiotic approaches. New antibiotics from the traditional development pipelines are slow in coming and quick to select for resistance. Our model can be used in conjunction with any approach that could hinder the production of *mecA* and reinstate the effectiveness of synthetic beta-lactam antibiotics. The development of a BlaR1- and/or PBP2a-specific vaccine, the effect of non-cognate auto-inducing peptides, and the activity of synthetic antisense oligonucleotides on *mecA* regulatory genes could all readily be assessed using the *mecA* expression assay.

MRSP was first identified in 1999.[5] UTCVM has been at the forefront of research institutions in tracking the proliferation of this emerging pathogen from phenotypic, molecular, and clinical perspectives. At the beginning of this project in 2007 the first publication hinting at regional clonality had just been published.[33] In the time since, this dissertation project has contributed to the MRSP knowledge-base with the identification of ST68 as the regional clone associated with our collection and the United States, and has brought to light the significant differences in *mecA* expression strategies between the European and American regional clones.

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Vita

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