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The Cost of Mupirocin Resistance in *Staphylococcus*.

Susan D. Reynolds
East Tennessee State University

The Cost of Mupirocin Resistance in *Staphylococcus*

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by
Susan D. Reynolds
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Foster Levy, Ph.D., Co-Chair
Elaine Walker, Ph.D., Co-Chair
Burt Lampson, Ph.D.
Felix Sarubbi, M.D.

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ABSTRACT

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Control of antibiotic resistance in bacteria is based on the concept that resistance incurs a fitness cost in non-selective conditions. Fitness costs were assessed for low- and high-level mupirocin resistance in locally-derived *Staphylococcus aureus* and *S. epidermidis*. Costs of resistance were assessed in pure cultures by comparing growth curve characteristics and in mixed culture as the proportion of resistant cells surviving. Costs were not present in comparisons of growth rates among groups of naturally-occurring isolates from the different resistance categories. However, in *S. aureus*, growth rates within resistance categories differed by approximately 30 – 90%. Among near-isogenic pairs of strains, fitness costs ≥10% were present in three of eleven pairs under pure culture and in six of eleven pairs under competition in mixed culture. Differences in intrinsic growth rates could easily mask fitness costs of the magnitudes observed. Thus, clinical outcomes also depend on whether there is a mixed infection and if so, on the growth rates of strains present.

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CHAPTER 1

INTRODUCTION

Staphylococcus aureus is a frequent component of the human microflora being found primarily on the skin and in the nares. In healthy individuals, *S. aureus* does not usually cause infection but, when skin or the membranes in the nares become compromised through trauma such as injury or surgery, *S. aureus* can be introduced into deeper tissues with the potential of becoming pathogenic. *S. aureus* is the causative agent for impetigo and other skin infections; however, it can also manifest as more serious complications such as osteomyelitis and scaled skin syndrome (Holmes et al. 2005). *S. aureus* is also a common nosocomial pathogen (i.e., a pathogen that is acquired during a hospital stay) that tends to infect patients that are already immune compromised. Methicillin-resistant *S. aureus* strains (MRSA) have acquired resistance to a notable portion of the β-lactam class of antibiotics and present a major treatment complication for physicians (Ip et al. 2005). More recently, *S. aureus* has become a public concern with the emergence of community acquired methicillin-resistant *S. aureus* (CA-MRSA) (Takizawa et al. 2005) that is more invasive and genetically different from healthcare associated MRSA.

Antibiotics were first introduced for therapeutic use following the discovery of penicillin by Alexander Flemming in 1928, but penicillin-resistant organisms were soon reported. The emergence of antibiotic resistance has limited the treatment options available. In a 16-year study (1986 – 2002) focusing on the evolution of antibiotic resistance in *Staphylococcus.* Cuevas et al. (2004) reported that resistance levels in *S. aureus* increased with respect to 6 of the 13 antibiotics used in the analysis and resistance to penicillin remained stable throughout the 16 year study at approximately 95%. In coagulase-negative *Staphylococcus*, resistance levels increased with respect to 8 of the 13 antibiotics tested.

Suggested Strategies for Managing Resistance

Major treatment complications can accompany an increase in antibiotic resistance in pathogens, including an increase in morbidity and/or mortality rates. Thus, economic costs of health care interventions to combat resistance can be significant. Carbon et al. (1999) reported that the difference in treating an methicillin-sensitive *S. aureus* (MSSA) infection compared with that of an MRSA infection was $ 40,090 (MSSA $24,280, MRSA $64,370) a 250% increase. A common strategy to combat antibiotic resistance is the development of new antibiotics, thereby providing health care professionals with an arsenal of drugs with which to fight infectious diseases.

An alternative strategy to combat resistance is to actively manage the resistance to the antibiotics already in use. There are three common practices that have been implemented. The first strategy is referred to as "judicious usage" (Gordts et al. 2000). Judicious usage is implemented by individual physicians and is contingent upon the physicians abiding by the assumptions that; 1) the antibiotic will be used for target prophylaxis versus general prophylaxis, 2) the infection being treated is bacterial in nature and, 3) the bacteria responsible for the infection is susceptible to the antibiotic being used. The second management strategy is referred to as "administrative control" (Walker et al. 2004). This policy can be very effective when implemented in an environment such as a hospital where there is a hierarchical administrative structure or in socialized countries in which medicine in under government control. Physicians are prohibited from prescribing an antibiotic in question unless given permission from an infectious disease physician. The third strategy is termed "antibiotic cycling" in which an antibiotic is used until resistance becomes apparent, at which time usage will be decreased and replaced with an alternative antibiotic until resistance to the first antibiotic is no longer detected allowing that antibiotic to once again be used (Brown et al. 2005).

All three management strategies are based on the assumptions that there is a fitness "cost" to the acquisition and maintenance of the resistance trait. The "cost" associated with

antibiotic resistance may be due to either the altering of basic cell processes such as cell wall synthesis, DNA replication, or protein synthesis but may also be attributed to the maintenance of extra-chromosomal DNA as in the case of a plasmid. The cost of antibiotic resistance is most typically manifested and assayed as a reduction in growth rate (Lenski 1997). When patients, infected with sensitive bacterial strains are treated with an appropriate antibiotic, the antibiotic action results in eradication of the infection. However, widespread antibiotic therapy also introduces selective pressure that provides an environment capable of facilitating the acquisition of resistance by means of spontaneous mutation, horizontal gene transfer via conjugation (transfer of genetic material from cell to cell), natural transformation (uptake and incorporation of homologous DNA into the host genome), or transduction (introduction of genetic material via a bacteriophage) (Neidhardt et al. 1990). If the acquisition of resistance incurs a cost, once the selective pressure is removed, sensitive strains would be able to outgrow resistant strains and therefore, eliminate the resistant sub-population. While mutation/selection balance may allow for effective management of resistance, it has been shown that with continued selection (i.e., exposure to antibiotics) bacteria may have the ability to acquire compensatory mutations that mitigate the cost of resistance (Schrag and Perrot 1997). To compound the problem, there is evidence to suggest that in many cases where multiple mutations result in the same resistance phenotype, those mutations incurring either a low or no–cost will be selected for and proliferate in the population (Hurdle et al. 2004). In each of these cases, management strategies would not be effective. Because of the key role of fitness costs in influencing the outcome of antibiotic selection, an investigation into the fitness cost of mupirocin resistance was the focus of my research.

Mupirocin

The antibiotic mupirocin occurs naturally as a fermentation product of *Pseudomonas fluorescens* (Chain et al. 1977). Mupirocin intercalates into the Rossman fold domain of the t-RNA synthetase enzyme, preventing isoleucine from binding to the isoleucyl t-RNA synthetase and thereby inhibiting protein synthesis (Hughes et al. 1978).

Bacteria can acquire resistance to mupirocin by two genetic pathways. One is via a chromosomal mutation in the native *ileS* gene that encodes for the isoleucyl t-RNA synthetase. Resistance occurs when there is a conformational change in the Rossman fold domain that prevents the intercalation of the mupirocin but allows for the binding of the amino acid isoleucine, thereby allowing protein synthesis to proceed. The chromosomal mutation confers low-level resistance, as defined by an MIC, minimum inhibitory concentration (i.e., the minimum concentration of an antibiotic that prevents visible growth). Chromosomal mutations can lead to MICs of >8 μg/mL - < 256 μg/mL. It has been suggested that low-level resistance increases the failure rate for nasal decolonization of methicillin-resistant *S. aureus* (MRSA) (Hurdle et al. 2004). High–level resistance is conferred by the acquisition of the *mupA* gene, most often reported as plasmid-borne (Morton et al. 1995). The *mupA* gene encodes an additional isoleucyl t-RNA synthetase gene originally derived from an unknown species but not historically found in staphylococci (Gilbart et al. 1993). The additional gene encodes for a different protein from that of the native protein but one that is functionally similar, that is, the enzyme is an isoleucyl-tRNA synthetase. The *mupA* encoded isoleucyl-tRNA synthetase confers resistance to mupirocin but allows the binding of the amino acid isoleucine, thereby allowing protein synthesis. Clinically, high-level mupirocin resistance allows for the proliferation of a MRSA infection in the presence of the antibiotic.

Mupirocin provides a particularly appropriate system for the study of the cost of resistance because it was recently introduced clinically, (in the 1980's) and because of the novel target. Thus, it is most likely that any resistance observed in the study isolates is most likely due to the selection pressure of this particular antibiotic and not the result of previous antibiotic exposure or cross resistance.

Previous Studies

A fitness cost associated with high-level mupirocin resistance has yet to be studied however, Hurdle et al. (2004) investigated the fitness cost of low-level mupirocin resistance in *S. aureus* using both *in vitro* and *in vivo* methods. Mutations $V_{588}F$ and $V_{631}F$ are chromosomally encoded in the *ileS* gene and are found in the ATP-binding domain of the Rossman fold region. These mutations are first-step mutations that are achieved by plating strains onto media containing the antibiotic at a concentration that lies between the MIC and the MPC, mutant prevention concentration (i.e. the lowest concentration of an antibiotic that prevents bacterial colony formation from a culture containing 10^{10} CFU). This method of mutant selection produced mutants at a frequency ($7.2 +/- 0.9 \times 10^{-8}$) that is consistent with single mutational events (i.e. first-step mutations). Mutations $V_{588}F$ and $V_{631}F$ confer low-level resistance and are widely distributed among clinical isolates. A mixed culture competition assay and a mouse wound abscess infection model were used to assess the fitness cost. There was no significant fitness cost associated with either of these mutations either *in vitro* or *in vivo.*

Mupirocin was introduced locally at the James H. Quillen Veterans' Affairs Medical Center (VAMC) in 1990. Many patients at this facility have chronic health problems and many are immune compromised. Because of the at-risk patient population, carriage of methicillin-resistant *S. aureus* (MRSA) can be considered a potential source of infection. Therefore, eradication of colonizing MRSA in patients in the facility was deemed desirable. Mupirocin was used prophylatically to decolonize the nares of those patients who were carriers of MRSA upon their admission to the VAMC. In the four years after the introduction of mupirocin, high–level resistance increased from less than 10% to > 30% of the MRSA recovered from VAMC patients (Walker et al. 2004). At that point, the management policy of "judicious usage" was implemented, but this was not effective in notably reducing resistance. Both the proportion of low-level and high-level resistant MRSA continued to increase. Isolates that were collected during the time period in which mupirocin was being used were categorized into eras according the mupirocin usage policy and incidence of resistance to mupirocin. Era 1 represents the introduction of mupirocin. Eras 2 and 3 correspond to when resistance was highest and Eras 4

and 5 correspond to when mupirocin usage had dramatically declined due to administrative control. Era 6 represents November to December of 2002, which followed the study of administrative control. At the end of Era 3, when resistance was highest, the management strategy of "administrative control" was introduced, and was very successful in reducing the incidence of both low- and high-level resistance. By 2001, (Era 5) both forms of resistance had been reduced to ≤ 10% (Walker et al. 2004). The reduction observed would suggest that there is a cost to both low-level and high-level mupirocin resistance.

To determine whether mupirocin could effectively be used again at the VAMC we looked at possible reservoirs for mupirocin resistance that might provide a means of transfer back into the *S. aureus* population. The most logical source of resistance would be another *Staphylococcus* species. One of the most common species of *Staphylococcus* is S. *epidermidis*, which is also a typical component of human microflora primarily colonizing the skin and is less pathogenic than *S. aureus*. *Staphylococcus epidermidis* has the potential to serve as a reservoir because it fulfills the following criteria. *S. epidermidis* shares a common niche with *S. aureus* in that both may be found in the human nares (Shobha et al. 2005) and *S. epidermidis* possesses antibiotic resistance diversity that is not present in *S. aureus*. Finally, in order to serve as a reservoir, *S. epidermidis* must have the ability to transfer the mupirocin resistance to *S. aureus*. To determine if local *S. epidermidis* could serve as a reservoir, a series of conjugative experiments was performed (Udo et al. 1998; Morton et al. 1995).

A proportion of healthcare workers at the VAMC also work at the Johnson City Medical Center (JCMC). To determine whether similar trends in resistance would be observed at the JCMC, isolates from both facilities were analyzed over time for level of resistance. Mupirocin resistance declined at the JCMC as well as at the VAMC in MRSA. The question remained whether or not mupirocin resistance had been eradicated from the population to a point that the drug could be effectively prescribed again. The current level of mupirocin resistance in *S. epidermidis* was determined from both the VAMC and the JCMC and low-level and high-level resistance was significantly greater in *S. epidermidis* than in the current populations of *S. aureus*. Unfortunately, we did not have historical data for *S. epidermidis* and were, therefore, unable to

infer the trend in resistance for this species. However, the observation that mupirocin resistance had not been effectively eradicated from the current *S. epidermidis* population at either facility suggests *S. epidermidis* is not incurring a significant cost of mupirocin resistance..

In the first experimental design, *S. aureus* was used as a donor to transfer its high-level resistance to other strains of *S. aureus* as well as to strains of *S. epidermidis*. It was possible to transfer high-level resistance to both *S. aureus* and *S. epidermidis* strains. The second experimental design used a strain of *S. epidermidis* as the donor and other strains of *S. epidermidis* or *S. aureus* as potential recipients. There was not evidence to support the transfer of high-level mupirocin resistance to either *S. aureus* or *S. epidermidis* from *S. epidermidis*. The third experimental design used a *S. epidermidis* transconjugant, which had just received the *mupA* gene from *S. aureus,* to serve as a donor. The transconjugant strain of *S. epidermidis* was capable of transferring the gene to both *S. aureus* and *S. epidermidis* strains (Barnard 2006). Possible explanations would be that perhaps the high-level resistance in *S. epidermidis* may be encoded on the chromosome or that it may exist on a non-transmissible plasmid (Udo et al. 2003)

With continued exposure to selective pressure bacteria will maintain the resistance genes and often may acquire compensatory mutations that mitigate the "cost" associated with resistance. Besier reported in 2005 that a second-site mutation in elongation factor G compensated for the fitness burden incurred by fusidic acid resistance (due to a mutation occurring in the *fusA* gene of elongation factor G) in *S. aureus.*

In *Salmonella typhimurium* the acquisition of streptomycin resistance results in a decreased rate of protein synthesis, bacterial growth, and virulence. However, in 77 out of 81 low-level resistant strains analyzed, compensatory mutations in the target genes had occurred without any loss in resistance (Maisnier-Patin et al. 2002). Even more surprising was the discovery that when rifampin-resistant *E.coli* was analyzed under antibiotic selection both the fitness level increased and the resistance level increased (Reynolds, 2000). In contrast,

Wichelhaus (2002) that a variety of mutations in the *rpoB* gene conferring high-level rifampin resistance were associated with varying levels of cost. However, the mutation occurring at amino acid position 481 exchanging histidine for asparagines and conferring low-level resistance, was not associated with a cost in *S. aureus*. Thus, a review of the literature revealed that fitness costs are not universal and are specific to particular antibiotics and genera combinations.

Proposed Research

The goal of the study was to assess the "cost" of mupirocin resistance in *Staphylococcus*, with an emphasis on *S. aureus*. Several questions were addressed, 1) does the acquisition of the *mupA* gene, conferring high-level resistance, have a detrimental affect on the fitness of the bacteria? 2) has the cost of mupirocin resistance changed over time in locally-derived strains? 3) is the cost of resistance different in *S. epidermidis.*

The primary hypothesis, based on a previous study (Walker et al. 2004), was that the *mupA* gene, which is usually carried on a plasmid (Gilbart et al. 1993), causes a decrease in growth rate in staphylococci and, therefore, imparts a fitness cost to the organism. The second hypothesis was that when *S. aureus* first acquired the *mupA*-mediated resistance there was a cost but over time, compensatory mutations have accumulated to mitigate the cost of the resistance. Therefore, a decrease in the cost can be expected over time. The third hypothesis is that there will be a cost associated with the acquisition of the *mupA* gene in *S. epidermidis.*

The first prediction is that the growth rates of the strains that have high-level mupirocin resistance will be significantly lower compared to those strains that are mupirocin-sensitive when analyzed in an environment with no selective pressure. The second prediction is that a significant decrease in growth rate of the strains that have high-level resistance compared to those that are sensitive will be observed when analyzing those strains isolated when the *mupA* plasmid was first acquired by the organism. The third prediction is that this same phenomenon will be observed in *S. epidermidis*.

To test these hypotheses the growth rates of a group of naturally-occurring sensitive, low-level, and high-level resistant strains of *S. aureus* and *S. epidermidis* were analyzed *in vitro* in pure liquid culture using fitness estimators based on growth curves. The sample of isolates spanned a 14-year time period that ranged from the introduction of mupirocin through 2004 at VAMC. This sample provided an opportunity for studying changes in the cost of resistance over time that may have occurred. In addition, a head to head paired mixed liquid culture, competition assay was used to assess the cost of the *mupA* gene in near-isogenic pairs of strains. There were two types of near isogenic pairs generated for the assay. First were *mupA* gene cures generated from naturally-occurring, high-level resistant *S. aureus* and *S. epidermidis* strains that were cured of their high-level resistance. Transconjugants were the second type of isogenic pairs generated using naturally-occurring, sensitive *S. aureus* and *S. epidermidis* strains into which high-level resistance was transferred via filter matings with high-level resistant strains.

CHAPTER 2
MATERIALS AND METHODS

Curing of *mupA* Gene

A group of naturally-occurring, locally-derived, clinical, high-level mupirocin-resistant strains of *S. aureus* and *S. epidermidis* were subjected to a heat-curing procedure in an attempt to generate a group of near-isogenic pairs. They are considered near isogenic because in some pairs there is evidence that other antibiotic resistances cured in conjunction with mupirocin resistance. Since prior research (Udo et al. 1998; Morton et al. 1995) reported that the *mupA* gene was plasmid-borne, a method designed to cure plasmids through heat stress was used to generate near-isogenic pairs. When successful, these isogenic pairs consisted of a high-level mupirocin-resistant progenitor strain and its sensitive derivative that had been cured of its mupirocin resistance. Each strain that was used in the heat-cure procedure was initiated from -80°C freezer stocks of high-level resistant strains. Clinical cultures of MRSA sometimes represent mixtures of strains. Therefore, strain purity was confirmed as follows. Aliquots of freezer stocks were spread on tryptic soy agar (TSA) plates and incubated overnight at 37°C. Ten isolated colonies of each strain were transferred first to TSA plates and then to plates containing mupirocin at 125 μg/mL (mup^{125}) to prevent carryover of the antibiotic to the non-selective plate. Mupirocin powder was a gift from Glaxo SmithKline, Research Triangle, Raleigh, N.C. to Dr. Elaine Walker. All plates containing antibiotics were incubated at 35°C unless otherwise noted and all non-antibiotic (non-selective) plates were incubated at 37°C. Resultant colonies that grew on both selective and non-selective plates, (potential high-level resistant progenitors) were expanded on a second TSA plate to be tested for mupirocin susceptibility using the Etest method. Colonies that had an MIC ≥ 256μg/mL indicated high-level resistance presumably conferred by the presence of the *mupA* gene. After confirmation of high-level mupirocin resistance, cultures were stored frozen at -80°C to be used for future experiments.

Sensitive derivatives of mupirocin-resistant strains were generated by serial passage of liquid cultures grown under heat stress, as follows. Aliquots of frozen cultures of each

progenitor strain were used to inoculate 5 mL of tryptic soy broth (TSB) and incubated overnight at an elevated temperature of 42°C. This temperature places the bacteria under heat stress and that can enhance plasmid loss (May et al. 1964). Each strain was cultured at 42°C for a total of either 4 or 10 days. Following each overnight incubation, an aliquot of culture of each strain was Gram stained to ensure purity of the cultures. Prior to the next overnight incubation, 100 µl of each strain was transferred to fresh 5 mL of TSB. Following the final day of incubation, a loopful of broth culture was spread on TSA and incubated overnight. Between 20 and 100 individual, isolated colonies were transferred to both TSA and mup^{125} plates and incubated overnight at 37°C and 35°C, respectively. Colonies that did not grow on the mup^{125} plates (potential cured derivative strains) were rescued and expanded on TSA plates. Colonies can be rescued when the antibiotic has a bacteristatic effect on the bacteria verses a bactericidal effect. Rescuing is accomplished by swabbing the area in which the colony was inoculated (area may appear to have no or very slight growth) and then transferring those cells to a non-selective media. Following incubation, each potential derivative was tested for mupirocin susceptibility by Etest to confirm sensitivity. Strains with an MIC $\leq$ 32µg/mL were considered cured of the high-level resistance gene and were frozen at -80°C to be used for fitness cost analysis assays.

Transconjugants

Near isogenic progenitor/transconjugant pairs of isolates were created by conjugative transfer of the *mupA* gene from a mupirocin-resistant donor to a sensitive recipient. This procedure resulted in near isogenic pairs of strains (i.e. there was evidence that other antibiotic resistances co-transferred with mupirocin resistance) comprised of the sensitive progenitor strain and the resistant recipient. Five such pairs were used for cost analysis assays. Prior to the conjugation procedure the resistance phenotypes of prospective donor and recipient strains were confirmed by spreading each strain on a plate containing combinations of antibiotics. All donors were mupirocin-resistant, rifampin-sensitive, and fusidic acid-sensitive (mup^{R}, rif^{S}, FA^{S}). All recipient strains were marked with rifampin and fusidic acid resistances. After confirming donor and recipient phenotypes, each was used to inoculate 10 mL of TSB, followed by incubation overnight at 37°C. Donor cultures were grown in TSB with mupirocin at a concentration of 150

μg/mL to ensure retention of the mupirocin resistance gene. The recipient cultures, following incubation, were diluted $10^{-6, -7,}$ and $^{-8}$ to estimate the number of potential recipient cells in each mixture. Excess mupirocin was removed from the donor cells prior to mating, as follows. Following incubation, donor cells were washed two times in TSB by centrifugation 1500 x g for 5 minutes, the supernatant removed, and cells resuspended in fresh TSB to remove excess mupirocin. The donor cultures and recipient cultures were mixed at a 1:3 ratio respectively, resulting in a final volume of 4 mL. The donor/recipient mixture was then transferred to a Millipore® filter (0.45μm) using a vacuum filtration device. Filters were placed on a TSA plate and oriented with bacteria on top, without inversion, overnight at 37°C. Following incubation, the bacteria were washed off of the filter into 1 mL TSB by vortexing. A 25 μl aliquot of each potential transconjugant strain was spread onto a selective Mueller-Hinton plate containing mupirocin at (32 μg/mL), rifampin (32 μg/mL), and fusidic acid (15μg/mL). This three antibiotic combination would eliminate donors and recipients and therefore select only for transconjugants. Following incubation at 35°C potential transconjugants were single colony picked to another triple antibiotic plate to confirm resistance. This selection step would minimize any false transconjugants resulting from antibiotic depletion caused by background growth. One transconjugant colony from each successful cross was then grown on TSA and its purity was confirmed using Gram stain, catalase, and coagulase tests.

Cost Analysis Assay

Each strain analyzed originated from freezer stocks of strains showing varying levels of susceptibility to mupirocin (susceptible < 4 μg/ml; low-level resistance 4-256 μg/ml; high–level resistance > 256 μg/ml; Table 3). Aliquots of each strain were spread on TSA plates and incubated overnight at 37°C. The resultant growth from the TSA plates was used to inoculate 10 mL of TSB the following day and was incubated overnight at 37°C. Following incubation, 5 mL of 0.85% NaCl was inoculated with cells until a density equal to that of a 0.5 McFarland Standard had been reached. A 0.5 McFarland standard is considered equivalent to1 x 10^8 cells/mL.

The cost of resistance was estimated from growth curves generated by microtiter plate cultures in a dynamic spectrophotmetric analyzer (Bioscreen C). Each sample well contained a total of 200 μL. Each strain was run in triplicate at each of three different mupirocin concentrations: [0, 8, and 1024 μg/mL]. One well of only TSB was used as a negative control. The Bioscreen C microtiter plate analyzer was programmed to shake every 3 minutes for 10 seconds and the optical density of each sample was recorded using a 600 nm filter every 10 minutes for a total of 48 hours. A graphical depiction of the growth curve for each sample was extrapolated by the program using the absorbance readings. Strains that were marked with rifampin and fusidic acid (i.e., resistances that are chromosomally-encoded and reported to incur a fitness cost) (Nagaev et al. 2001, Wichelhaus et al. 2002) were compared to their corresponding susceptible strains and served as a reference for the sensitivity of the assay (Table 3).

The doubling time for each strain was estimated from individual growth curves that were generated from optical density readings. Strain-specific doubling times were calculated as: doubling time = log10(final OD) – log10(initial OD) / log2 (time in minutes) from the linear portion of the curve when visualized on a semilog plot. The linear portion of the growth curve was determined by maximization of the linear regression coefficient (R^2). In addition, the area under the curve was recorded for each strain at 24 hours. This measurement provided a second measure of fitness because it takes into account the length of the lag phase, slope of the log phase, and the time at which stationary phase was reached, factors that are not accounted for in fitness estimates from doubling times.

Paired Competition Assay

The near-isogenic pairs of strains used for the competition experiments originated from frozen stock cultures that were generated from the heat cure procedures and conjugation experiments. Aliquots from corresponding freezer stocks were spread onto TSA and incubated overnight at 37°C. Following incubation, cells were added to 0.85% NaCl solution until the density was equivalent to a # 3 McFarland standard ($9x10^8$ cells/mL). Using Bioscreen C, a single optical density reading was taken for each bacteria solution to ensure similar cell densities. An aliquot of 100µL from each isogenic pair was combined in a microfuge tube and vortexed to mix. An aliquot of 100 µL from the above mixture (i.e. resistant progenitor and respective sensitive derivative) was used to inoculate 10 mL of TSB and was incubated overnight in a 37°C water bath. The proportion of each population within the mixed culture was determined from CFU counts of serial dilution plates. To estimate the proportions of resistant and sensitive cells, each dilution was spread on both selective media, containing mupirocin 32 µg/mL and on non-selective TSA media. For each mixed culture, colony counts were taken at 0, 24 hours, and 72 hours. The resistant population was determined as a proportion of the total population by dividing the total number of resistant colonies by the number of colonies in the total population (i.e. resistant population / total population x 100 = percentage of resistant bacteria in the population).

Statistical Analysis

All statistical analysis was conducted using Minitab software (Minitab, Inc., State College, PA). The data pertaining to the naturally-occurring *S. aureus* and *S. epidermidis* strains were analyzed using a one-way ANOVA. A one-way ANOVA analysis is used to compare categories of data and was used specifically to assess differences between resistance categories. Then, separate one-way ANOVA analyses were conducted to test for variance among strains within resistance categories. The heat cured strains, transconjugants, and control strains were analyzed using a paired t-test.

CHAPTER 3

RESULTS

Curing of *mupA* Gene

Heat curing of mupirocin resistance was attempted on a total of 34 *Staphylococcal* ssp. strains (29 *S. aureus* and five *S. epidermidis*). After an initial round of attempted cures, two out of nine (22%) strains were cured. Therefore, both the length of time and the total number of colonies from each strain screened were increased from four to ten days and from 20 to at least 50 colonies as noted (Table 1). In spite of the increase in curing time and total colonies screened the cure rate remained relatively stable, with cures of four of 20 (20%) subsequent strains attempted. A total of six *S. aureus* strains were successfully cured of their *mupA* gene to generate sensitive derivatives. One *S. epidermidis* strain was successfully cured of its *mupA* gene; however, it was found to have low-level resistance (MIC = 128 μg/mL), which has been shown to be chromosomally encoded (Hurdle, et al. 2004). The cured *S. epidermidis* strain was not used in the cost analysis assay. Table 2 gives a complete list of the resistance phenotypes (other than mupirocin) that were lost through the curing process. Gentamicin resistance was most commonly lost (three of seven) in association with curing of the *mupA* gene.

An effect of isolate time-of-recovery was apparent when the curing data were analyzed. Curing was significantly more successful during the era 1 with a cure rate of 75%. Era 1 corresponds to the introduction of mupirocin into the population. The other eras had a combined cure rate of 12% (Table 1). A heterogeneity chi-square analysis showed the cure rate for Era 1 was significantly greater ($\chi^2 = 9.46$, df = 3, p = 0.024) than the cure rate of eras 2-4.

Table 1. Cures of Mupirocin Resistance. Bacterial strains, cure conditions, and cure rates for which mupirocin curing was attempted. Era 1: August 1990 – August 1993; Era 2: September 1993 – December 1995; Era 3: June 1996 – February 1999; Era 4: March 1999 – April 2000; Era 5: May 2000 – May 2001; Era 6: November – December 2002. "Sa" denotes *S. aureus.* All *S. aureus* that lost high-level resistance were cured to sensitivity. ***** *Staphylococcus epidermidis* 17 was cured to low-level resistance with an MIC = 128 μg/mL.

Strain	Era	Number of Treatment Days	Number of Colonies Screened	Number of Sensitive Colonies	% Cured
Sa 1593	1	4	20	1	
Sa 1480	1	10	50	0	75%
Sa 1484	1	10	50	2	
Sa 1589	1	10	50	1	
Sa 1858	2	4	20	0	
Sa 2549	2	4	20	0	
Sa 1680	2	10	50	0	
Sa 1770	2	10	50	4	
Sa 1706	2	10	50	0	
Sa 1724	2	10	50	0	
Sa 1754	2	10	50	0	
Sa 3475	3	4	20	0	
Sa 3780	3	4	20	0	
Sa 4323	3	4	20	0	
Sa 5519	3	4	20	0	
Sa 2825	3	10	50	0	12%
Sa 2885	3	10	50	0	
Sa 3147	3	10	50	0	
Sa 3388	3	10	50	0	
Sa 3431	3	10	50	0	
Sa 3447	3	10	50	0	
Sa 3651	3	10	50	6	
Sa 4431	3	10	50	0	
Sa 4907	3	10	50	0	
Sa 5068	3	10	50	0	
Sa 5554	3	10	50	0	
Sa 5592	4	4	20	0	
Sa 5965	4	4	20	20	
Sa 5579	4	10	50	0	
S. epidermidis 7	6	10	50	0	
S. epidermidis 17	6	10	50	1*	
S. epidermidis 27	6	10	50	0	20%
S. epidermidis 34	6	10	50	0	
S. epidermidis 35	6	10	50	0	

Table 2. Antibiotic Resistances and Susceptibilities for Each Isogenic Pair. Changes in resistance attributed to either acquisition of the *mupA* gene via conjugation or loss of the *mupA* gene via curing are highlighted. Progenitor / derivative pairs are shown on adjacent rows. "Sa" denotes *S. aureus* and "Se" denotes *S. epidermidis*. "TC" denotes transconjugant. Antibiotics: Mupirocin (MUP), Tetracycline (TE), Erythromycin (E), Gentamicin (GM), Rifampin (RA), Sulfamethoxazole/Trimethoprim (SXT), Chloroamphenicol (C), Clindamycin (CC), and Ciprofloxacin (CIP).

Strain	MUP	TE	E	GM	RA	SXT	C	CC	CIP
Sa 1589-1	R	S	R	R	S	S	S	R	R
Sa 1589-48	S	I	R	S	S	S	S	R	S
Sa 1480-1	R	S	R	R	S	S	S	R	R
Sa 1480-4	S	I	R	R	S	S	S	R	R
Sa 3651-9	R	S	R	R	S	S	S	R	R
Sa 3651-37	S	S	R	S	S	S	S	R	R
Sa 1770-1	R	S	R	S	S	S	S	R	S
Sa 1770-24	S	S	R	S	S	S	S	R	S
Sa 5965-10	R	S	I	R	S	S	S	I	S
Sa 5965-1	S	S	I	S	S	S	S	S	S
Sa 1858-8	R	S	S	S	S	R	S	S	I
Sa 1858-13	S	S	S	S	S	R	S	S	R
Se 17	R	S	R	S	S	R	S	R	R
Se 17-10	S	S	R	S	S	I	S	R	R
Sa 2782-22	S	S	R	S	R	S	S	R	R
Sa 2782-22 TC	R	S	R	R	R	S	S	R	R
Se 22B1	S	S	S	S	R	S	S	S	S
Se 22B1 TC	R	S	S	R	R	S	S	S	S
Se 5A1	S	S	S	S	R	S	S	S	S
Se 5A1 TC	R	S	S	R	R	S	S	S	S
Se 27A2	S	S	S	S	R	S	S	S	R
Se 27A2 TC	R	S	S	R	R	S	S	S	I
Se 30B1	S	S	E	S	S	I	S	S	R
Se 30B1 TC2	R	S	E	I	S	R	S	S	R

Transconjugants

In filter-mating assays of locally-derived strains, *S. aureus* donors could transfer the *mupA* gene to both *S. aureus* and *S. epidermidis* in several cases. In contrast, when *S. epidermidis* was used as a donor, it was not possible to transfer the *mupA* gene to either a *S. aureus* or *S. epidermidis* recipient. However, if a *S. epidermidis* transconjugant (i.e. a strain that had received the *mupA* gene from an *S. aureus* strain) was used as a donor, the *mupA* gene could be transferred to both *S. aureus* and *S. epidermidis* recipients (Barnard 2006). Table 2 gives a complete list of antibiotic resistance phenotypes transferred in association with mupirocin. Gentamicin resistance was most commonly transferred with mupirocin resistance. For both mupirocin-resistance-cured strains and transconjugants there was a strong, but not absolute association between gentamicin resistance and mupirocin resistance. Three of four cured strains lost both mupirocin resistance and gentamicin resistance and four of five transconjugants gained both mupirocin resistance and gentamicin resistance.

Cost Analysis Assays

Naturally-Occurring Strains

A total of 36 naturally-occurring strains comprised of 25 *S. aureus* and 7 *S. epidermidis* of varying levels of mupirocin resistance were included in the cost analysis assay. The cost analysis was based on growth curve characteristics of pure strain cultures. The doubling time for each strain was inferred from the linear-most portion of the log phase of the growth curve. Each growth curve was generated using optical density readings. The average doubling times and area under the curve measurements at 24 hours are given in Table 3. The mean and standard deviation for each resistance category within each species are given in Table 4. Three strains resistant to both rifampin and fusidic acid were used to determine sensitivity of the assay. Only one, Se 5A1, showed a significant cost of resistance when assessed using the growth curve assays.

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Strains were assayed under no mupirocin as well as 8 μg/mL and 1024 μg/mL mupirocin. Sensitive strains were inhibited by both 8 μg/mL and 1024 μg/mL mupirocin. High-level-resistant strains grew equally well under no mupirocin and 8 μg/mL. At 1024 μg/mL the lag phase and log phases were longer compared to growth under no mupirocin and 8 μg/mL. Low-level-resistant strains were inhibited by mupirocin at 1024 μg/mL, while their growth was arrested (i.e., a longer lag phase and more gradual log phase) under mupirocin 8 μg/mL (Figures 2 and 3).

When naturally-occurring *S. aureus* strains were compared among resistance categories, there was not a significant difference in growth rates based on doubling times and areas under the curve. However, in *S. epidermidis,* the low-level-resistant strains were marginally significantly slower growing than the high-level-resistant and sensitive strains (Tables 5 and 6). In general, there was a significant difference among *S. aureus* strains within resistance categories based on doubling times and areas under the curve. Among *S. epidermidis* strains there was a significant difference among high-level-resistant strains based on doubling times and among low-level-resistant strains based on areas under the curve (Table 7). However, the magnitudes of these differences in *S. epidermidis* were very small (< 5% difference among strains) when compared to ~ 30%-100% inter-strain differences evident in *S. aureus*. (Figures 4 and 5).

Table 3. Results of the Pure Culture Assays of Growth Rates. Doubling time and area under the curve (AUC) at 24 hours are given in the table as mean values of the replicates of each strain. Era 1: August 1990 – August 1993; Era 2: September 1993 – December 1995; Era 3: June 1996 – February 1999; Era 4: March 1999 – April 2000; Era 5: May 2000 – May 2001; Era 6: November – December 2002. "Sa" denotes *S. aureus* and "Se" denotes *S. epidermidis.* * = ATTC Strain.

Strain	Era	Resistance [a] Category	Doubling Time (min)	AUC 24 hours
Naturally-Occurring *S. aureus*				
Sa 1965	2	S	103.6	1112.0
Sa 5371	3	S	86.5	1016.0
Sa 5894-L	4	S	114.6	1102.0
Sa 7A2	6	S	93.9	1107.0
Sa 8C1	6	S	103.7	1106.0
Sa 15A1	6	S	88.2	1109.5
Sa 37A1	6	S	93.5	1162.0
Sa 44B1	6	S	89.7	1047.0
Sa 1864	2	L	130.3	1006.5
Sa 2365	2	L	89.7	1071.0
Sa 5791-1	4	L	108.9	916.0
Sa 8B1	6	L	101.1	1127.0
Sa 1484-H	1	H	86.2	1038.5
Sa 1680-5	2	H	81.7	1109.5
Sa 1754-1	2	H	122.9	982.0
Sa 5579-1	4	H	106.6	946.0
Sa 5588-L	4	H	82.3	1106.0
Sa 44A1	6	H	103.9	1062.5
Sa 44A3	6	H	90.9	1103.0
Naturally-Occurring *S. epidermidis*				
Se 30B1	6	S	99.7	990.5
Se 40A1	6	S	100.6	962.5
Se 4C3	6	L	111.1	1030.0
Se 8B2	6	L	112.4	956.0
Se 9A1	6	H	105.8	1080.5
Se 50A1	6	H	98.6	1108.5

Table 3 (continued)

***mup A* Cure Isogenic Pairs**				
Sa 1480-1	1	H	86.3	1061.0
Sa 1480-4	1	S	81.3	1107.0
Sa 1589-1	1	H	103.1	1012.0
Sa 1589-48	1	S	86.3	895.0
Sa 1770-1	2	H	84.2	959.5
Sa 1770-24	2	S	82.0	965.0
Sa 1858-8	2	H	156.3	1017.0
Sa 1858-13	2	S	152.8	1023.5
Sa 3651-9	3	H	93.7	1144.0
Sa 3651-37	3	S	90.9	1073.5
Sa 5965-10	4	H	110.0	869.5
Sa 5965-1	4	S	96.8	761.0
Se 17	6	H	95.6	995.5
Se 17-10	6	L	107.0	1051.0
Transconjugant Isogenic Pairs				
Sa 2782-22 RF	3	S	101.1	1015.3
Sa 2782-22 TC	3	H	98.4	989.3
Sa 22B1 RF	6	S	104.5	1099.7
Sa 22B1 TC	6	H	101.7	1059.7
Se 5A1 RF	6	S	131.1	884.7
Se 5A1 TC	6	H	127.8	763.7
Se 27A2 RF	6	S	122.1	960.3
Se 27A2 TC	6	H	124.0	977.0
Se 30B1	6	S	98.4	919.7
Se 30B1 TC2	6	H	100.1	921.3

Table 3 (continued)

Rif/FA "Controls" Isogenic Pairs				
Se 5A1	3	S	93.8	1049.0
Se 5A1 RF	3	R	124.1	787.5
ATCC 35556	*	S	105.4	972.5
ATCC 35556 RF	*	R	108.7	652.5
Sa 2782-22	3	S	100.2	1113.5
Sa 2782-22 RF	3	R	101.1	1014.5

[a] "S" signifies sensitivity to mupirocin, "L" signifies low-level resistance, and "H" signifies high-level resistance.

Table 4. Mean and Standard Deviation for Naturally-Occurring *S. aureus* and *S. epidermidis*. Standard deviation is given in parentheses. N= number of strains.

	Doubling Time		**AUC 24 Hours**		
	S. aureus	**S. epidermidis**	**S. aureus**	**S. epidermidis**	**S. aureus**
Sensitive	96.72 N = 8 (9.71)	100.18 N = 2 (0.658)	1095.2 N = 8 (44.5)	976.5 N = 2 (19.8)	208.3 N = 8 (44.5)
Low	107.48 N = 4 (17.11)	111.17 N = 2 (0..926)	1030.1 N = 4 (90.6)	993.0 N = 2 (52.3)	251.3 N = 4 (34.1)
High	100.63 N = 13 (20.93)	99.97 N = 2 (5.28)	1031.6 N = 13 (78.0)	1094.5 N = 2 (19.8)	268.4 N = 13 (205.0)

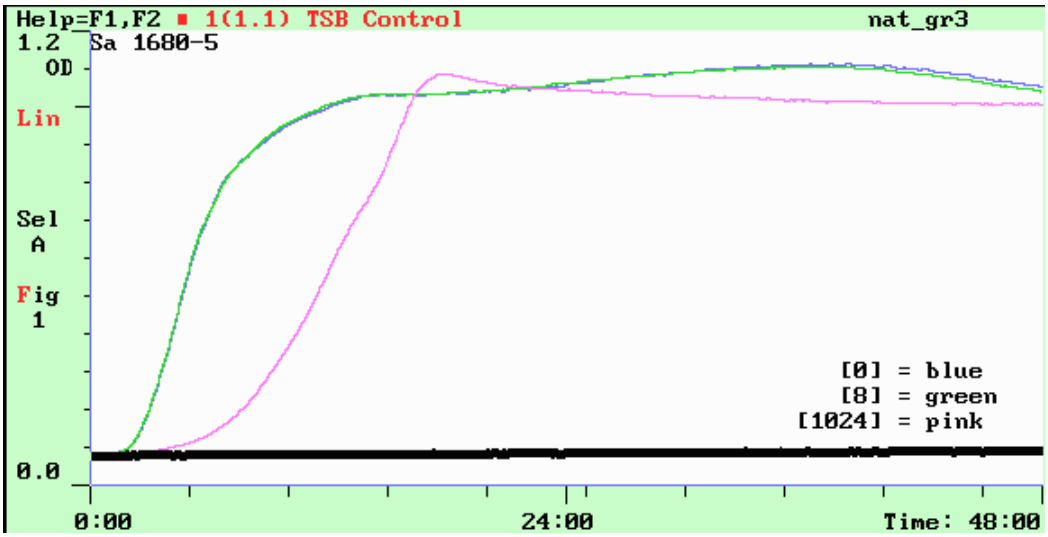

Figure 1. Growth Curves of Strain Sa1680-5, a Naturally-Occurring High-Level Resistant *S. aureus* Strain. Assayed under no mupirocin (blue) and mupirocin at concentrations 8 µg/mL (green) and 1024 µg/mL (pink).

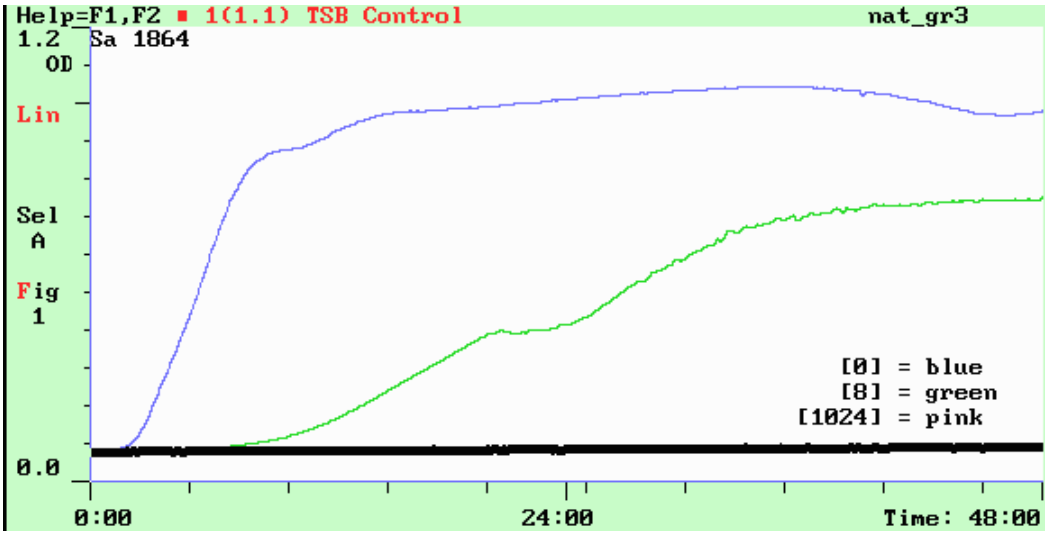

Figure 2. Growth Curves of Strain Sa 1864, a Naturally-Occurring Low-Level Resistant *S. aureus*. Assayed under no mupirocin (blue) and mupirocin at concentrations of 8 µg/mL (green) and 1024 µg/mL (pink).

Table 5. Results of a One-Way ANOVA Analysis Comparing the Doubling Times among Resistance Categories within Naturally-Occurring *S. aureus.*

Naturally-Occurring *S. aureus*					
Source	**DF**	**SS**	**MS**	**F**	**P**
Resistance Category	2	309	155	0.50	0.613
Error	22	6794	309		
Total	24	7103			

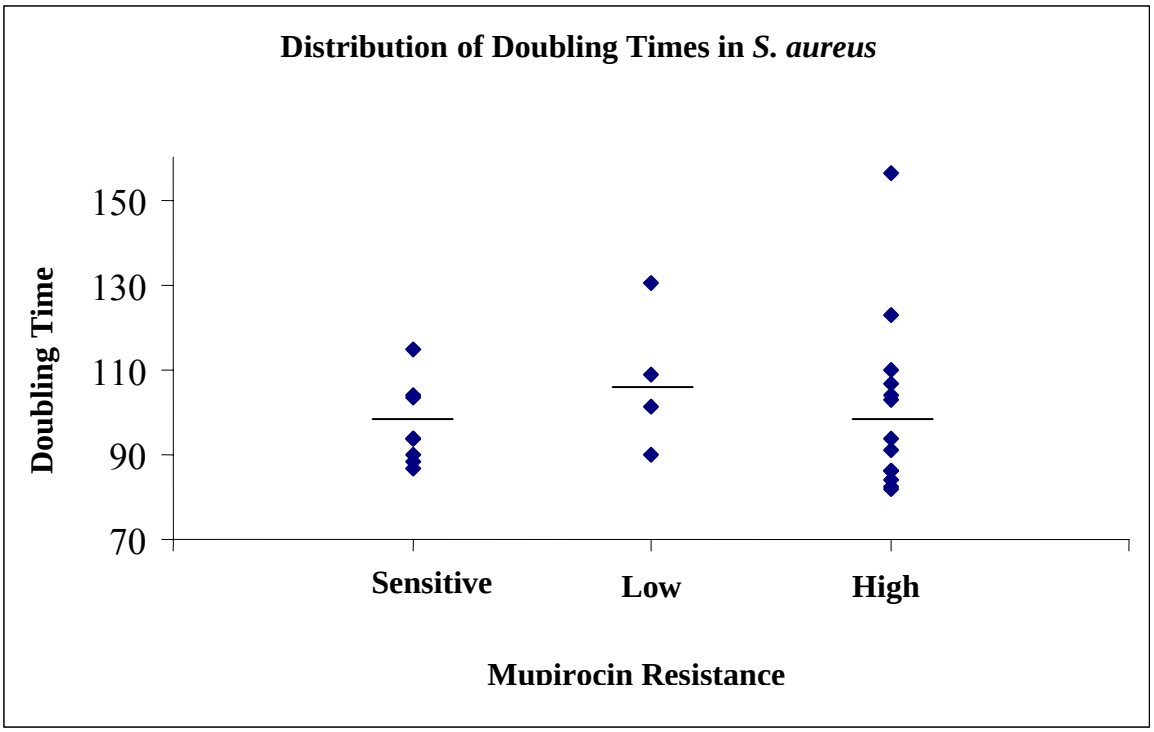

Figure 3. Distribution of Doubling Times within Resistance Categories in *S. aureus*. The mean for each group is represented by the horizontal line.

Table 6. Results of a One-Way ANOVA Analysis Comparing the Doubling Times among Resistance Categories within Naturally-Occurring *S. epidermidis*.

Naturally-Occurring S. epidermidis					
Source	**DF**	**SS**	**MS**	**F**	**P**
Resistance Category	2	196	98	6.87	0.051
Error	4	57.1	14.3		
Total	6	253			

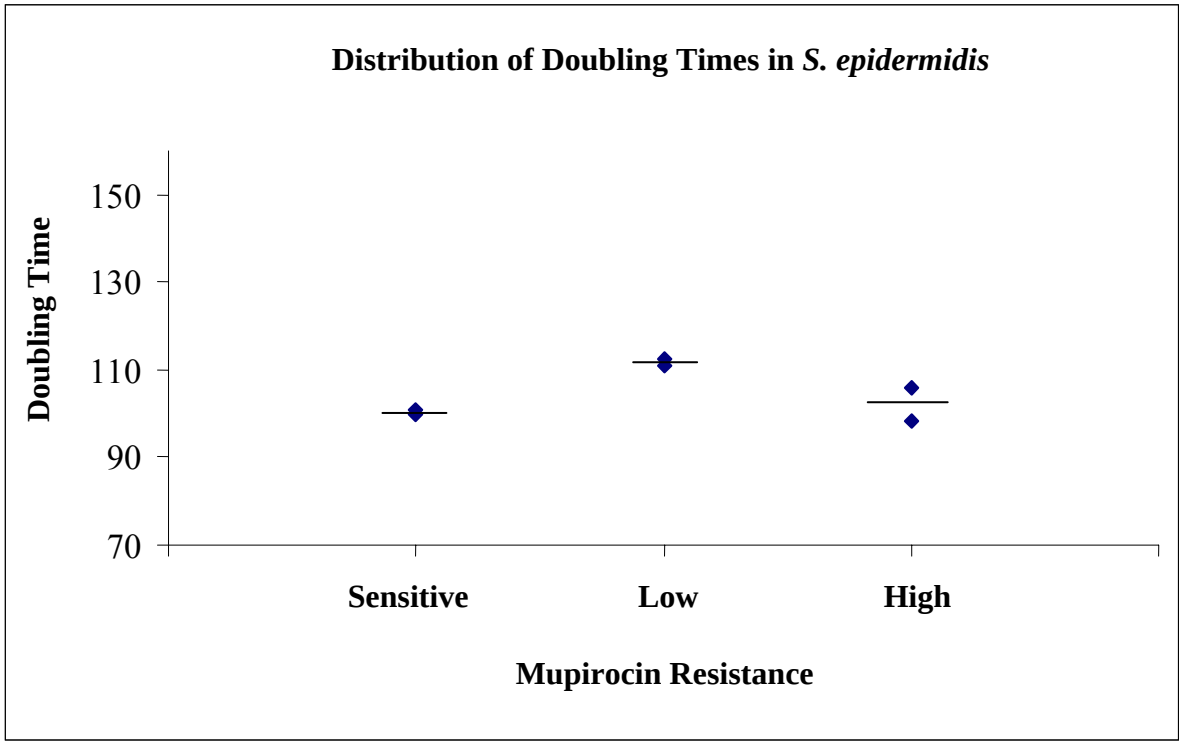

Figure 4. Distribution of Doubling Times within Resistance Categories in *S. epidermidis*. The mean for each group is represented by the horizontal line.

Table 7. Summary of Analysis of Variance Analyses among Strains within Mupirocin Categories in *S. aureus* and *S. epidermidis.* Entries are P-values for separate analyses.

Strain Variation	**Doubling Times**		**AUC 24 Hours**	
Resistance Category	**S. aureus**	**S. epidermidis**	**S. aureus**	**S. epidermidis**
Sensitive	0.001	0.928	<0.001	0.745
Low	0.3	0.657	<0.001	0.007
High	<0.001	0.043	<0.001	0.04

Pure Culture Assay

Strains that were cured of mupirocin resistance were compared with their high-level-resistant progenitors in both the pure culture assays and a mixed culture competition assay for a more direct comparison of the cost incurred by the acquisition or loss of mupirocin resistance. In pure cultures, cost was calculated as [(doubling time of the sensitive strain – doubling time of the resistant strain) / doubling time of the sensitive strain]. A difference of ≥ 10%was considered significant. Doubling times were significantly slower in resistant progenitors compared to their cured sensitive derivatives but there was no significant difference based on area under the curve (Table 8). Each strain showed a decrease in doubling time, with the loss of the mupirocin resistance (Figure 6). Only one *S. epidermidis* strain was able to be cured and this strain was cured to low-level resistance (MIC = 128 μg/mL). Therefore, it was not used for cost analyses.

Mixed Culture Competition Assay

Except for strain Sa 5965, whose cost estimates were varied across replicates and strain Sa 1480, all paired competition assay comparisons of the high-level mupirocin-resistant strains and their sensitive derivative strains showed evidence of a fitness cost. Cost was inferred by a reduction of ≥ 10 % in the percent of resistant bacteria present in the population (Table 9).

Table 8. Results from Paired t-Test Analysis of Isogenic Pairs. P-values are given for the *mupA* cures (C), strains which have been cured of the *mupA* gene; transconjugants (TC), strains which have acquired the *mupA* gene via conjugation; and "control" strains, those strains marked with chromosomally-encoded rifampin and fusidic acid resistances. Separate tests were conducted for doubling times and area under the curve (AUC).

	Doubling Times			**AUC 24 Hours**		
	C	TC	Controls	C	TC	Controls
P-value	0.035	0.422	0.347	0.213	0.232	0.075

Table 9. Cost of Mupirocin Resistance in the Isogenic Pairs. Individual cost or benefit for each isogenic pair is given below and is based on differences in doubling time (DT). "Sa" denotes *S. aureus* and "Se" denotes *S. epidermidis*. A "-" indicates cost, while "+" indicates a benefit. Cost was calculated as $(DT_S - DT_R)/ DT_S$, where "$_S$" = mupirocin-sensitive and "$_R$" = mupirocin-resistant. A difference of ≥10% indicates a significant cost/benefit associated with mupirocin resistance.

Strain	**Era**	**Resistance Category**	**Doubling Time (min)**	**Cost/ Benefit**
Sensitive Progenitor/ Cured Derivative				
Sa 1480-1	1	H	86.3	-6%
Sa 1480-4	1	S	81.3	
Sa 1589-1	1	H	103.1	-19%
Sa 1589-48	1	S	86.3	
Sa 1770-1	2	H	84.2	-2.60%
Sa 1770-24	2	S	82.0	
Sa 1858-8	2	H	156.3	-2.20%
Sa 1858-13	2	S	152.8	
Sa 3651-9	3	H	93.7	-3%
Sa 3651-37	3	S	90.9	
Sa 5965-10	4	H	110.0	-13.60%
Sa 5965-1	4	S	96.8	

Table 9 (continued)

Sensitive Progenitor / Resistant Derivative				
Sa 2782-22 RF	3	S	101.1	+2.60%
Sa 2782-22 TC	3	H	98.4	
Sa 22B1 RF	6	S	104.5	+2.60%
Sa 22B1 TC	6	H	101.7	
Se 5A1 RF	6	S	131.1	+2.50%
Se 5A1 TC	6	H	127.8	
Se 27A2 RF	6	S	122.1	-1.50%
Se 27A2 TC	6	H	124.0	
Se 30B1	6	S	98.4	-1.70%
Se 30B1 TC2	6	H	100.1	
Rif/FA "Controls"				
Se 5A1	3	S	93.8	-32.30%
Se 5A1 RF	3	R	124.1	
ATCC 35556	*	S	105.4	-3%
ATCC 35556 RF	*	R	108.7	
Sa 2782-22	3	S	100.2	-0.80%
Sa 2782-22 RF	3	R	101.1	

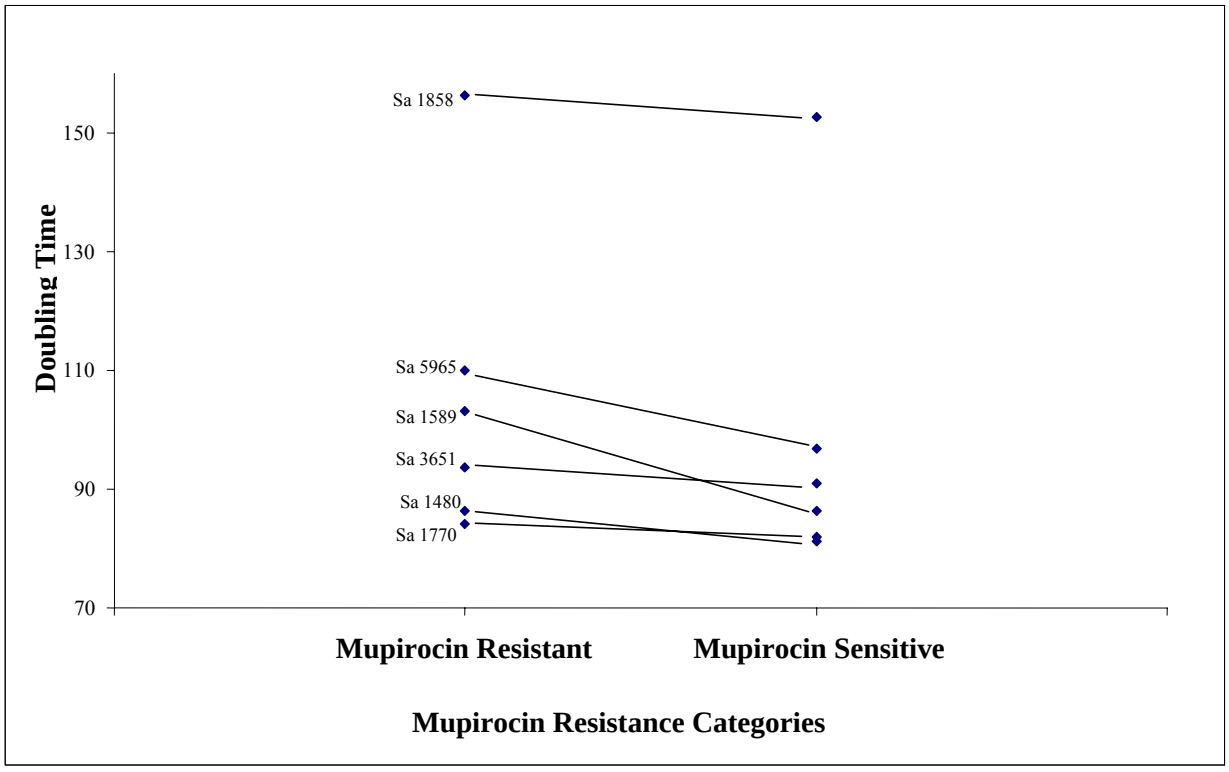

Figure 5. Doubling Time by Mupirocin Resistance Status Plot. Strains cured of high-level mupirocin resistance are represented in a graph depicting the change in doubling time attributed to the loss of the *mupA* gene.

Transconjugants

Strains that received high-level mupirocin resistance in filter mating experiments were compared to their sensitive progenitor strains in pure culture assays as well as in paired competition assays. Two of the five pairs tested (Se 27A2 and Se 30B1) showed a slight increase in doubling times associated with the presence of the *mupA* gene, while the other three strains (Se 5A1, Sa 2782-22, and Sa 22B1) showed a slight decrease in doubling time when analyzed in pure cultures (Figure 7). However, these changes in growth rate were not significant, nor were the differences in area under the curve (Tables 8 and 9). Transconjugant strains Se 5A1, Sa 2782-22, and Se 27A2 however, showed a significant cost in paired competition experiments (Table 10).

Controls

When analyzed as a group the rifampin and fusidic acid resistant strains did not show a significant difference in growth rates in pure culture assays. However, when they were analyzed as isogenic pairs a cost was evident in Se 5A1 based on doubling time and area under the curve using the pure culture assay and in both Se 5A1 and Sa 2782-22 using the paired competition assay.

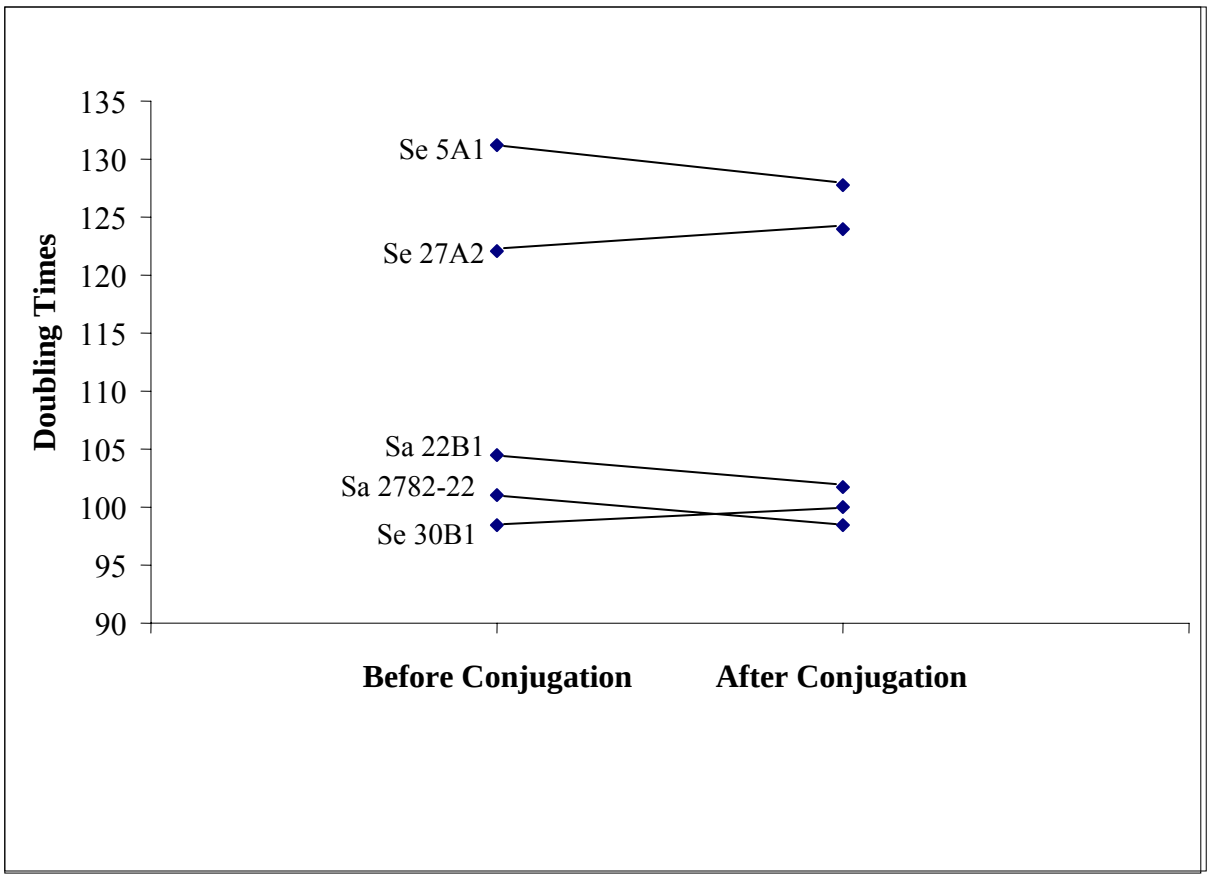

Figure 6. Doubling Time by Mupirocin Resistance Status Plot. Graph depicting the change in doubling times attributed to the acquisition of the *mupA* gene via conjugation.

Table 10. Paired Competition Experiment Results. "Sa" denotes *S. aureus* and "Se" denotes *S. epidermidis*. A difference of ≥10% indicates a significant cost/benefit. "-" indicates cost , "+" indicates a benefit. T = time in hours. "I" denotes the data was inconclusive because one replicate showed a cost and the other a benefit. The percent change (% change) is calculated as the average percent of the resistant population at T = 0 minus the average percent of the resistant population at T= 72. "TSA" represents the total population (number of resistant and sensitive colonies) while "Antibiotic" represents only the resistant sub-population. All assays compare isogenic pairs of strains. "Cures" refer to a comparison of mupirocin-resistant progenitors and cured sensitive derivatives. "Transconjugants" refer to sensitive progenitors and mupirocin-resistant derivatives. "Controls" refer to sensitive progenitors and rifampin- and fusidic acid-resistant derivatives.

		T = 0			**T = 24**			**T = 72**			
Strain	**Replicate**	**TSA**	**Antibiotic**	**Resistant Population**	**TSA**	**Antibiotic**	**Resistant Population**	**TSA**	**Antibiotic**	**Resistant Population**	**% Change**
Control											
Se 5A1	1	609	231	38%	162	30	18.50%	250	0	0%	-39%
	2	571	229	40%	529	96	18%	122	0	0%	
Sa 2782-22	1	608	324	53%	632	85	13%	36	3	8%	-39.5%
	2	784	332	42%	622	138	22%	215	18	8%	
ATCC 35556	1	138	41	30%	336	141	42%	236	53	22%	0%
	2	233	51	22%	159	67	42%	132	39	30%	

Table 10 (continued)

***mup A* Cures**											
Sa 1589	1	481	~368	76%	161	50	31%	268	34	12.7%	-71.8%
	2	263	242	92%	139	33	24%	411	48	11.7%	
Sa 5965	1	165	131	79%	46	34	74%	28	50	178%	I
	2	162	112	69%	100	91	91%	98	54	55%	
Sa 1858	1	206	78	37.90%	~1700	~988	58%	401	128	32%	-22%
	2	630	321	50.90%	~1700	~784	46%	368	48	13%	
Sa 3651	1	471	222	47%	243	81	33%	506	32	6%	-32.5%
	2	686	218	32%	~614	253	41%	430	35	8%	
Sa 1480	1	460	113	25%	76	55	72%	146	46	32%	-5.5%
	2	415	182	44%	102	30	29%	87	40	46%	
Sa 1770	1	759	388	51%	117	53	45%	78	13	16%	- 37%
	2	614	354	58%	105	44	42%	113	21	19%	
Transconjugants											
Se 5A1	1	414	187	45%	368	95	26%	38	32	84%	+18.5%
	2	394	265	67%	301	123	41%	91	59	65%	
Sa 2782-22	1	355	101	28%	726	120	16%	115	33	29%	-10%
	2	416	143	34%	168	14	8%	126	16	13%	
Se 27A2	1	224	146	65%	78	35	45%	151	32	21%	-33.3%
	2	278	141	51%	89	25	28%	70	20	28.50%	
Se 30B1	1	150	77	51%	47	21	45%	87	29	33%	-7%
	2	203	87	43%	125	62%	50%	106	50	47%	
Sa 22B1	1	717	230	32%	99	31	31%	110	40	36%	- 2%
	2	944	363	38%	311	102	33%	117	45	38%	

Table 11. Qualitative Summary of Fitness Cost Assays using Isogenic Pairs of Strains. "Sa" denotes *S. aureus* and "Se" *S. epidermidis.* "–" denotes that the analysis was not done. "NS" denotes difference was not significant. "IC" denotes results were inconclusive. "DT" denotes doubling time. "R" denotes resistant. "I" denotes intermediate resistance. Erythromycin (E), Gentamicin (GM), Sulfamethoxazole/Trimethoprim (SXT), Clindamycin (CC), and Ciprofloxacin (CIP).

		Fitness Assay		**Transferable**		**Other Resistances**				
	Strain	**D.T.**	**Competition**	**Donor**	**Recipient**	**E**	**GM**	**SXT**	**CC**	**CIP**
	Sa 1858	NS	cost	yes	-			R		I-->R
	Sa 5965	cost	IC	yes	no	I	R		I	
	Sa 3651	NS	cost	yes	-	R	R		R	R
	Sa1480	cost	NS	yes	-	R	R		R	R
	Sa 1589	cost	cost	yes	-	R	R		R	R
***mupA* Cures**	Sa 1770	NS	cost	yes	-	R			R	
	Se 5A1	cost	cost	-	yes					
	Sa 2782-22	NS	cost	-	yes					
Controls	ATCC 35556	NS	NS	-	yes					
	Se 22B1 TC	NS	NS	-	yes		R			
	Se 5A1 TC	NS	benefit	-	yes		R			
	Se 27A2 TC	NS	cost	-	yes		R			I
Transconjugant	Sa 2782-22 TC	NS	cost	-	yes		R			
s	Se 30B1 TC2	NS	NS	-	yes		I	R		R

CHAPTER 4

DISCUSSION

Administrative control of mupirocin prescriptions was successful in reducing the incidence of high-level and low-level resistance in the *S. aureus* population at the James H. Quillen Veteran's Affairs Hospital from approximately 30% at its peak to less than 10% following implementation (Walker et al. 2004). The apparent response to relaxed selection on mupirocin resistance suggested there was a fitness burden associated with the mupirocin resistance. The observation that both high- and low-level resistance decreased further suggests there were costs associated with the acquisition of the *mupA* gene conferring high-level resistance as well as the mutant *ileS* gene conferring low-level resistance. However, in the current study widespread costs were not immediately evident in naturally-occurring strains when the growth rates of high-level and low-level strains of *S. aureus* and *S. epidermidis* were compared to sensitive strains *in vitro*. There was no evidence of a fitness cost associated with the *mupA* gene in either species. However, in *S. epidermidis,* low-level mupirocin-resistant strains were marginally significantly slower growing compared to sensitive and high-level resistant strains. This is consistent with a prior report for low-level mupirocin resistance in *S. aureus*. Hurdle et al. reported(2004) that chromosomal mutations conferring low-level mupirocin resistance in clinical strains were associated with low or no-cost. Hurdle also generated mutations *in vitro* that did incur a cost and this led to the conclusion that there is selective pressure *in vivo* for those mutations that do not incur a cost.

While it has been reported that maintaining a plasmid incurs a fitness cost to the bacteria (Zünd and Lebek 1980) it has also been reported that the cost is quickly compensated for over subsequent generations (Bouma and Lenski 1988, Schrag and Perrot 1996). There appears to be a consensus that there is often a cost associated with antibiotic resistance genes, and that costs have been demonstrated both *in vivo* and *in vitro.* However, bacteria can quickly evolve to mitigate this cost by means of compensatory mutations, reversion of resistance, selection for no- or low-cost mutations, or suppression of resistance under non-selective conditions (Nguyen 1989, Reynolds 2000, Hurdle et al. 2004).

In the current study, comparison of growth rates based on naturally-occurring strains highlighted significant inter-strain variation among resistance categories in *S. aureus*. All three resistance phenotypes showed significant inter-strain variation with the high-level resistant strains showing the greatest strain variation (i.e. the slowest growing strain was 91% slower than the fastest growing strain). The extreme magnitude of this strain variation could easily mask a fitness cost that may exist. Consequently, it is necessary to more closely examine costs based on comparisons of isogenic pairs of strains (*mupA* cures and transconjugants) that were generated for the purpose of a more direct comparison of the *mupA* gene.

It was difficult to cure the high-level mupirocin-resistant *S. aureus* strains and even more difficult to cure the *S. epidermidis* strains. Only one *S. epidermidis* strain was successfully cured, suggesting that in most cases the *mupA* gene is in a non-curable form in this species. Curing can be interpreted as strong evidence that the *mupA* gene was maintained on a plasmid (Cookson 1998, Caierao et al. 2006). Conversely, the inability to cure suggests that the *mupA* gene has been incorporated into the chromosome. In addition, *S. epidermidis* reportedly has the ability to maintain plasmids more faithfully than *S. aureus*. Cookson reported in 1998 that the initial discovery of the *mupA* plasmid was in a form that was easily transferred and cured. However, he noted that subsequently discovered *mupA* plasmids were of varying molecular weight and had varying transfer and curing capabilities. The inability to cure is consistent with the fact that administrative control had not eradicated mupirocin resistance from *S. epidermidis.* While the historical data to discern a trend in mupirocin resistance in *S. epidermidis,* is not available, the lack of eradication supports the hypothesis that there is not a cost of resistance in *S. epidermidis.*

Curing was most successful in *S. aureus* in strains from era 1, the era that corresponds to when mupirocin was first introduced locally. Although the era 1 sample size is small, there was a 63% difference between the curing rate of era 1 and the combine rate for eras 2-4. The difference in cure rates suggests the *mupA* gene was initially maintained on a plasmid but it has since become incorporated into the chromosome or is now on a plasmid harboring essential gene(s). If the *mupA* gene had changed forms, such a transition would be expected to have been

accompanied by a change in the fitness cost. Specifically, a higher fitness burden would be expected to be associated with a plasmid-borne *mupA* gene because of widespread costs of maintaining plasmids.

Gentamicin resistance was most commonly associated with either the loss through curing or gain through filter-mating transfer of the *mupA* gene. In *S. aureus, g*entamicin is often maintained on the plasmid pGO1 (Thomas and Archer 1989). In addition, Morton (1995) characterized a pGO1-like plasmid that had transferable mupirocin resistance but had lost gentamicin resistance. Thus, the association of gentamicin and mupirocin resistance further suggests the *mupA* gene was being maintained on a plasmid as well, with either *mupA* and *Gmr* (encoding gentamicin resistance) carried on the same plasmid or on different plasmids. Although prescription rates for gentamicin did not change over time at the VAMC, gentamicin resistance experienced a similar decline as mupirocin resistance and both were low in recent surveys. The similar decline of gentamicin resistance supports the hypothesis of a transition in the form of the *mupA* gene and the difference in cure rates.

While in pure cultures, the doubling times of strains cured of mupirocin resistance was significantly lower than their derivatives, the individual costs were, for the most part small, with the exception of the 16% cost borne by strain Sa 1589. However, competition experiments using cured derivatives and their respective progenitors demonstrated the potential biological importance of even small differences in doubling times. In *S. aureus,* four of the five pairs of cured derivative-progenitor strains showed a significant reduction (>10%) in the resistant population when grown in competition with their respective sensitive derivatives. Thus, among strains capable of curing, there was evidence of a fitness cost associated with the *mupA* gene. Moreover, these analyses demonstrate the competition assay must be considered the more sensitive of the two methods of detecting a fitness cost.

Only two of the five transconjugant isogenic pairs showed evidence of a significant fitness cost. It was expected that a strain that had just received the *mupA* gene would show the strongest evidence of a fitness cost having not had ample time to mitigate the cost through either,

compensatory mutations (Schrag and Perrot, 1996), incorporation into the chromosome, or reversion of the resistance (Hurdle et al. 2004). It is not clear why there was not a widespread fitness cost associated with the transconjugants. One possible explanation is that when the *mupA* gene is in a transferable form that compensatory mutations may be maintained on the same plasmid thus being co-transferred. Another possible explanation would be that mupirocin resistance is being maintained on a transposon as reported by (Cookson 1998).

One of the most clinically relevant implications of this study derives from the significant strain variation and the low rate of plasmid curing in *S. aureus*. Consider the case of a pure-culture infection. If the *mupA* gene is in a non-curable form, there is not opportunity for competition with a cured sensitive sub-population. In a pure culture, a fitness cost becomes irrelevant regardless of the selective pressure. Therefore, only if the *mupA* gene is curable does a fitness cost influence the selective outcomes.

In an alternative scenario, consider mixed strain infections with large strain differences in intrinsic growth rates. For example, Figure 6 highlights the case of a patient infected with both strains A and B. Strain A is an intrinsically fast growing sensitive strain, while B is an intrinsically slow growing sensitive strain. Strain A acquires the *mupA* gene to generate strain A^R. Relative to strain A, strain A^R shows a significant fitness cost manifested as a reduction in the growth rate of 16%. However strain A^R, although significantly slower than its sensitive progenitor, remains significantly faster growing (10%) than strain B. Therefore, when mupirocin is withdrawn or in components of the body not subjected to treatment, the resistant strain would outgrow the sensitive strain.

While there is evidence to suggest that there is a significant fitness cost associated with the *mupA* gene in some cases, the cost is typically small. Therefore, resolution of the question why administrative control was so effective in reducing the incidence of mupirocin resistance may lie in quantifying the extent of inter-strain interactions where differences in growth rates influence selective outcomes and also in understanding the genomic location of the *mupA* gene.

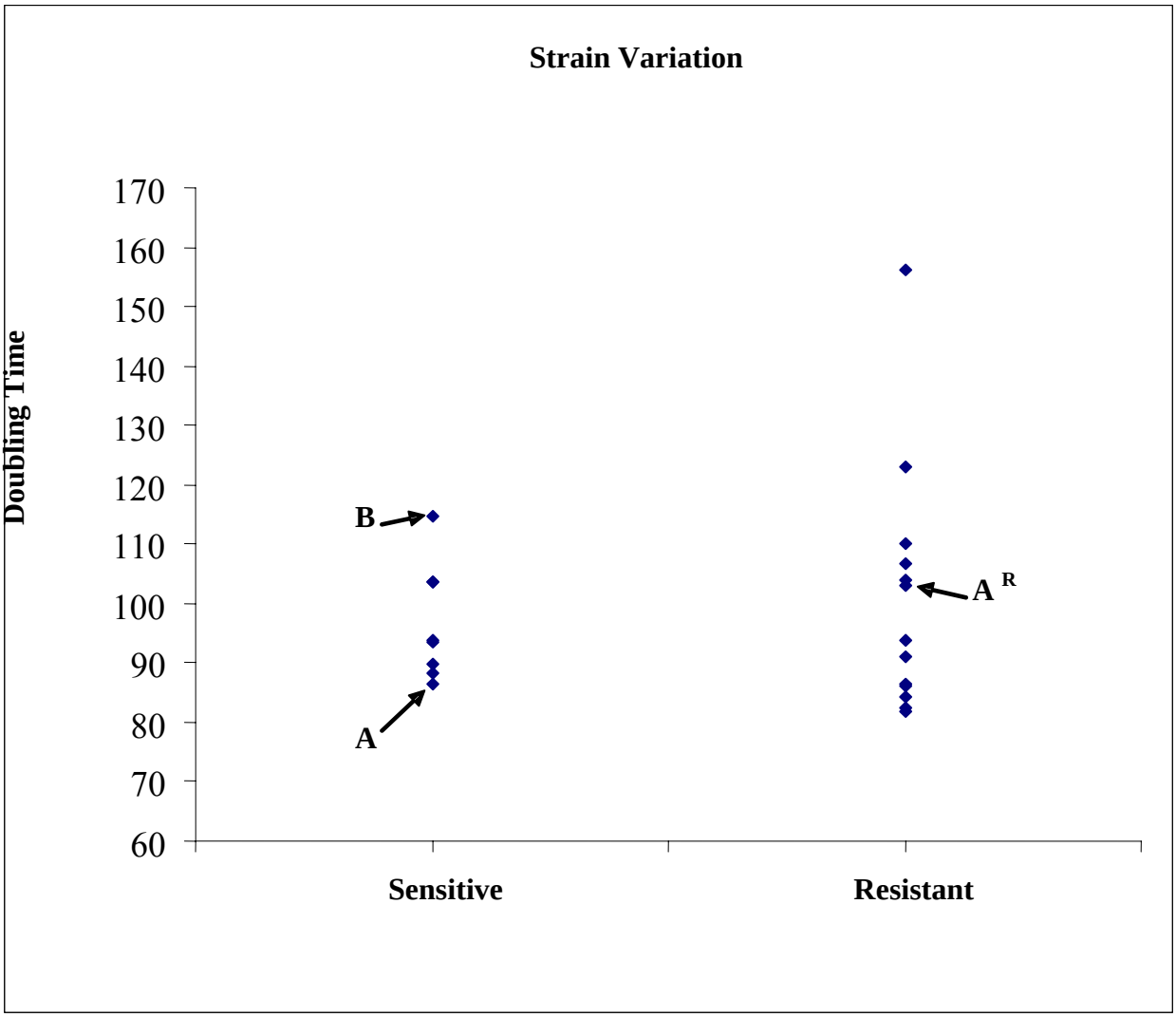

Figure 7. Effect of Strain Variation on Selective Outcomes. "A" represents an intrinsically fast growing, naturally-occurring, mupirocin-sensitive *S. aureus* strain. "A^R" represents a mupirocin-resistant form of "A" following acquisition of the *mupA* gene. "B" represents a significantly slower growing, naturally-occurring, mupirocin-sensitive *S. aureus* strain compared to strain A and A^R for which "B" is 24% and 10% slower, respectively.

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APPENDIX

Expanded Data Tables

Table 1. Complete Doubling Time Data for Growth Curve Assay. "Sa" signifies *S. aureus*. "Se" signifies *S. epidermidis*. Era 1: August 1990 – August 1993, Era 2: September 1993 – December 1995, Era 3: June 1996 – February 1999, Era 4: March 1999 – April 2000, Era 5: May 2000 – May 2001, Era 6: November – December 2002. Doubling times are given in minutes for each replicate of each strain (R#). Average is the mean of the replicates of each strain. "ND" indicates that a third replicate was not done.

			Doubling Times			
Strain	**Era**	**Resistance Category**	**R1**	**R2**	**R3**	**Average**
Naturally-Occurring *S. aureus*						
Sa 8B1	6	L	99.01	103.09	ND	101.05
Sa 8C1	6	S	101.01	106.38	ND	103.7
Sa 44A1	6	H	99.01	108.7	ND	103.86
Sa 44A3	6	H	89.28	92.59	ND	90.94
Sa 37A1	6	S	91.74	95.24	ND	93.5
Sa 7A2	6	S	93.46	94.34	ND	93.9
Sa 44B1	6	S	88.49	90.91	ND	89.7
Sa 15A1	6	S	85.47	90.91	ND	88.19
Sa 5791-1	4	L	86.21	131.58	ND	108.9
Sa 5588-L	4	H	84.03	80.65	ND	82.34
Sa 5894-L	4	S	108.7	120.48	ND	114.6
Sa 1484-H	1	H	86.21	86.21	ND	86.21
Sa 1754-1	2	H	117.65	128.21	ND	122.93
Sa 1680-5	2	H	83.33	80	ND	81.67
Sa 5579-1	4	H	109.08	104.17	ND	106.63
Sa 1864	2	L	117.65	142.86	ND	130.26
Sa 2365	2	L	90.91	88.5	ND	89.71
Sa 5371	3	S	84.75	88.33	ND	86.54
Sa 1965	2	S	103.09	104.17	ND	103.63

Table 1 (continued)

Naturally-Occurring *S. epidermidis*						
Se 9A1	6	H	105.26	106.38	ND	105.82
Se 50A1	6	H	97.09	100	ND	98.55
Se 8B2	6	L	109.89	114.94	ND	112.42
Se 4C3	6	L	111.11	111.11	ND	111.11
Se 40A1	6	S	92.59	108.69	ND	100.64
Se 30B1	6	S	95.24	104.17	ND	99.71
***mupA* Cures**						
Sa 1858-8	2	H	153.85	158.73	ND	156.3
Sa 1858-13	2	S	156.25	149.25	ND	152.75
Sa 5965-10	4	H	106.38	113.64	ND	110.01
Sa 5965-1	4	S	92.59	101.01	ND	96.8
Sa 3651-9	3	H	98.04	89.29	ND	93.67
Sa 3651-37	3	S	92.59	89.29	ND	90.94
Sa 1480-1	1	H	89.29	83.33	ND	86.31
Sa 1480-4	1	S	82.64	80	ND	81.32
Sa 1589-1	1	H	103.09	103.09	ND	103.1
Sa 1589-48	1	S	77.42	95.24	ND	86.33
Sa 1770-1	2	H	77.52	90.91	ND	84.22
Sa 1770-24	2	S	76.34	87.72	ND	82.03
Se 17	6	H	101.01	90.09	ND	95.55
Se 17-10	6	L	109.89	104.17	ND	107.03

Table 1 (continued)

Transconjugants						
Se 5A1 RF	6	S	136.99	128.21	128.21	131.14
Se 5A1 TC	6	H	133.33	125	125	127.78
Se 27A2 RF	6	S	128.21	119.05	119.05	122.1
Se 27A2 TC	6	H	128.21	121.95	121.95	124.04
Sa 2782-22 RF	3	S	104.17	100	99.01	101.06
Sa 2782-22 TC	6	H	96.15	100	99.01	98.4
Sa 22B1 RF	6	S	104.17	105.26	104.17	104.53
Sa 22B1 TC	6	H	100	103.09	102.04	101.71
Se 30B1	6	S	95.24	100	100	98.41
Se 30B1 TC2	6	H	98.04	104.17	98.04	100.08
Rif/FA Controls						
Se 5A1	3	S	88.5	99.09	ND	93.8
Se 5A1 RF	3	R	136.99	111.11	ND	124.05
Sa 35556	ATCC Strain	S	102.04	108.7	ND	105.37
Sa 35556 RF	ATCC Strain	R	108.7	108.7	ND	108.7
Sa 2782-22	3	S	89.29	111.11	ND	100.2
Sa 2782-22 RF	3	R	98.04	104.17	ND	101.11

Table 2. Complete Area under the Curve Data for Growth Curve Assay. "Sa" signifies *S. aureus*. "Se" signifies *S. epidermidis*. Era 1: August 1990 – August 1993, Era 2: September 1993 – December 1995, Era 3: June 1996 – February 1999, Era 4: March 1999 – April 2000, Era 5: May 2000 – May 2001, Era 6: November – December 2002. Doubling times are given in minutes for each replicate of each strain (R#). Average is the mean of the replicates of each strain. "ND" indicates that a third replicate was not done.

			AUC 24 hours			
Strain	**Era**	**Resistance Category**	**R1**	**R2**	**R3**	**Ave.**
Naturally-Occurring *S. aureus*						
Sa 8B1	6	L	1133	1121	ND	1127
Sa 8C1	6	S	1108	1104	ND	1106
Sa 44A1	6	H	1064	1061	ND	1062.5
Sa 44A3	6	H	1105	1101	ND	1103
Sa 37A1	6	S	1133	1191	ND	1162
Sa 7A2	6	S	1102	1112	ND	1107
Sa 44B1	6	S	1054	1040	ND	1047
Sa 15A1	6	S	1114	1105	ND	1109.5
Sa 5791-1	4	L	918	914	ND	916
Sa 5588-L	4	H	1090	1122	ND	1106
Sa 5894-L	4	S	1108	1096	ND	1102
Sa 1484-H	1	H	1038	1039	ND	1038.5
Sa 1754-1	2	H	980	984	ND	982
Sa 1680-5	2	H	1117	1102	ND	1109.5
Sa 5579-1	4	H	943	949	ND	946
Sa 1864	2	L	1009	1004	ND	1006.5
Sa 2365	2	L	1070	1072	ND	1071
Sa 5371	3	S	1020	1012	ND	1016
Sa 1965	2	S	1118	1106	ND	1112
Naturally-Occurring *S. epidermidis*						
Se 9A1	6	H	1083	1078	ND	1080.5
Se 50A1	6	H	1114	1103	ND	1108.5
Se 8B2	6	L	962	950	ND	956
Se 4C3	6	L	1029	1031	ND	1030
Se 40A1	6	S	971	954	ND	962.5
Se 30B1	6	S	916	1065	ND	990.5

Table 2 (continued)

***mupA* Cures**						
Sa 1858-8	2	H	1025	1009	ND	1017
Sa 1858-13	2	S	1013	1034	ND	1023.5
Sa 5965-10	4	H	871	868	ND	869.5
Sa 5965-1	4	S	770	752	ND	761
Sa 3651-9	3	H	1139	1149	ND	1144
Sa 3651-37	3	S	1060	1087	ND	1073.5
Sa 1480-1	1	H	1046	1076	ND	1061
Sa 1480-4	1	S	1105	1109	ND	1107
Sa 1589-1	1	H	1010	1014	ND	1012
Sa 1589-48	1	S	895	895	ND	895
Sa 1770-1	2	H	946	973	ND	959.5
Sa 1770-24	2	S	974	956	ND	965
Se 17	6	H	1001	990	ND	995.5
Se 17-10	6	L	1031	1071	ND	1051
Transconjugants						
Se 5A1 RF	6	S	889	881	884	884.67
Se 5A1 TC	6	H	763	766	762	763.67
Se 27A2 RF	6	S	967	964	950	960.33
Se 27A2 TC	6	H	977	974	980	977.00
Sa 2782-22 RF	3	S	999	1030	1017	1015.33
Sa 2782-22 TC	6	H	998	991	979	989.33
Sa 22B1 RF	6	S	1118	1105	1076	1099.67
Sa 22B1 TC	6	H	1063	1045	1071	1059.67
Se 30B1	6	S	916	924	919	919.67
Se 30B1 TC2	6	H	917	913	934	921.33
Rif/FA Controls						
Se 5A1	3	S	1048	1050	ND	1049.00
Se 5A1 RF	3	R	791	784	ND	787.50
Sa 35556	ATCC Strain	S	977	968	ND	972.5
Sa 35556 RF	ATCC Strain	R	640	665	ND	652.5
Sa 2782-22	3	S	1107	1120	ND	1113.5
Sa 2782-22 RF	3	R	999	1030	ND	1014.5

Table 3. Complete ANOVA Data for Naturally-Occurring *S. aureus* Strains.

S. aureus	**Doubling Time**					**AUC 24 hours**					
Source	DF	SS	MS	F	P	Source	DF	SS	MS	F	P
Resistance Category	2	309	155	0.5	0.613	Resistance Category	2	22255	11128	2.19	0.135
Error	22	6794	309			Error	22	111547	5070		
Total	24	7103				Total	24	133803			

Table 4. Complete ANOVA Data for Naturally-Occurring *S. epidermidis* Strains.

S. epidermidis	**Doubling Time**					**AUC 24 Hours**					
Source	DF	SS	MS	F	P	Source	DF	SS	MS	F	P
Resistance Category	2	196	98	6.87	0.051	Resistance Category	2	16332	8166	6.96	0.075
Error	4	57.1	14.3			Error	3	3522	1174		
Total	6	253				Total	5	19854			

Table 5. Strain Variation among Naturally-Occurring *S. aureus* Resistance Categories Comparing Doubling Times

Strain Variation of Doubling Times among S. aureus Resistance Categories

Source	DF	SS	MS	F	P
Low	3	1756	585	1.72	0.3
Error	4	1358	340		
Total	7	3114			

Source	DF	SS	MS	F	P
Sensitive	7	1319.6	188.5	13.11	0.001
Error	8	115	14.4		
Total	15	1434.6			

Source	DF	SS	MS	F	P
High	6	2824.9	470.8	25.06	<0.001
Error	7	131.5	18.8		
Total	13	2956.4			

Table 6. Strain Variation among Naturally-Occurring *S. aureus* Comparing Area under the Curve at 24 Hours Data

Strain Variation of AUC 24 among S. aureus Resistance Categories

Source	DF	SS	MS	F	P
Low	3	49276.4	16425.5	695.26	<0.001
Error	4	94.5	23.6		
Total	7	49370.9			

Source	DF	SS	MS	F	P
Sensitive	7	27694	3956	15.41	<0.001
Error	8	2055	257		
Total	15	29748			

Source	DF	SS	MS	F	P
High	12	146124	12177	94.88	<0.001
Error	13	1669	128		
Total	25	147792			

Table 7. Strain Variation among Resistance Categories in Naturally-Occurring *S. epidermidis* Comparing Doubling Times

Strain Variation of Doubling Times among S. epidermidis Resistance Levels

Source	DF	SS	MS	F	P
Low	1	1.7	1.7	0.27	0.657
Error	2	12.75	6.38		
Total	3	14.45			

Source	DF	SS	MS	F	P
Sensitive	1	0.9	0.9	0.01	0.928
Error	2	169.5	84.7		
Total	3	170.4			

Source	DF	SS	MS	F	P
High	1	52.93	52.93	21.77	0.043
Error	2	4.86	2.43		
Total	3	57.79			

Table 8. Strain Variation among Resistance Categories in Naturally-Occurring *S. epidermidis* Comparing Area Under the Curve at 24 Hours

Strain Variation of AUC @ 24 Among S. epidermidis Resistance Levels

Source	DF	SS	MS	F	P
Low	1	5476	5476	148	0.007
Error	2	74	37		
Total	3	5550			

Source	DF	SS	MS	F	P
Sensitive	1	784	784	0.14	0.745
Error	2	11245	5623		
Total	3	12029			

Source	DF	SS	MS	F	P
High	1	784	784	21.48	0.04
Error	2	73	36.5		
Total	3	857			

VITA

Susan D. Reynolds

Personal Data: Date of Birth: November 8, 1977
Place of Birth: Bristol, TN.
Marital Status: Single

Education: Public Schools, Bristol, Tennessee
University of Tennessee, Knoxville, TN;
Biology, concentration Microbiology, B.S., 2000
East Tennessee State University, Johnson City, Tennessee;
Biology, M.S., 2006

Professional Experience: Graduate Assistant
East Tennessee State University, College of Arts and Sciences, 2004-2006

Ecology Teacher
Upward Bound, ETSU, 2005

Research Associate
ETSU, Biological Sciences, 2004

DNA Technician/ Technical Writer
Microbial Insights, Inc., Nov. 01 – Dec. 03

Forensic Intern, Drug Chemistry / Serology
Tennessee Bureau of Investigation Forensics Laboratory – Knoxville, TN.
Sept. 01 – Oct. 01

Research Assistant
East Tennessee State University–Johnson City, TN
June 98 – Aug. 98
June 99- Aug. 99

Research Experience:

Thesis research – testing the hypothesis that resistance to the antibiotic mupirocin incurs a fitness cost to the host bacteria and that cost is similar among host species of *Staphylococcus*. Cost of fitness was determined through the analysis of growth curves.
Jan. 04 – present

Research Associate – collected strains of *Staphylococci* from hospitals, healthcare workers, and community members to understand transmission pathways
May 04 – Aug. 04

Independent Research – investigation of transfer of antibiotic resistance genes via natural transformation in *Moraxella catarahlis.* Although *M. catarahlis* was thought to be naturally competent some strains were not and some would only take up DNA from specific donor strains.
Summer 1999

Independent Research – Study of adherence as a virulence factor in *Moraxella catarahlis*
Summer 1998

Presentations:

Seminar; April 2006: ETSU "The Cost of Antibiotic Resistance in *Staphylococcus"*

Poster; April 2005: "Bacterial Fitness and Conjugative Transfer of Antibiotic Resistance in *Staphylococcus*", Appalachian Student Research Forum

Presentation, April 2004: "*Staphylococcus epidermidis*- A Potential Reservoir for Antibiotic Resistance in *S. aureus*", Appalachian Student Research Forum (First Prize)

Seminar; November 2004: ETSU "The Cost of Antibiotic Resistance in *Staphylococcus*"

Presentation, September 1999: Reynolds, S., Levy, F., Walker, E., "DNA Exchange Pathways in *Moraxella catarahlis*.