Abstract

The genus staphylococcus was given its name by Sir Alex Ogston, a Scottish surgeon, in 1881 when he observed grape-like clusters of bacteria associated with clinical infections. Three years later, the German physician Anton Rosenbach isolated and grew these microorganisms in a pure culture. He named them *Staphylococcus aureus* because of their golden color. *S. aureus* remains one of the most important pathogens in clinical settings largely due to the rapidity of its evolutionary response to treatment. The first antibiotic-resistant strains of *S. aureus* were isolated only several years after penicillin was introduced commercially, and the subsequent history of antibiotics has been driven by the evolving emergence of antibiotic resistance.

Our ability to control the rate at which resistance develops and spreads is predicated on our understanding of the evolutionary biology of pathogens and the basic features of bacterial populations, and while hospital acquired strains of *S. aureus* have received a great deal of attention, the distribution and virulence (ability to cause infection) of community-associated strains of *S. aureus* are not well-defined and few studies have assigned isolates of the bacteria to known strains.

The objectives of this study were to collect, isolate and characterize samples of *S. aureus* from areas utilized by midshipman athletes at the United States Naval Academy and patients at the Naval Medical Clinic, Annapolis. These samples were classified in several steps including their gross morphology and determinative biochemical activities. Finally, a multilocus sequence typing (MLST) scheme was used to unambiguously characterize the isolates and analyze significant evolutionary trends in the population. The sequences of internal fragments of seven genes were obtained for 36 *S. aureus* isolates and assigned a unique allelic profile. These profiles, like fingerprints, were used to assign the isolates to known strains or to identify them as

Report Documentation Page

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OMB No. 0704-0188

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1. REPORT DATE **06 MAY 2004**	2. REPORT TYPE	3. DATES COVERED **-**
4. TITLE AND SUBTITLE **Classification of clinically relevant microorganisms in non-medical environments**		5a. CONTRACT NUMBER
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S) **Daniel Bowers**		5d. PROJECT NUMBER
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) **U. S. Naval Academy,Annapolis,MD,21402**		8. PERFORMING ORGANIZATION REPORT NUMBER **TRIDENT-2004-320**
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)		10. SPONSOR/MONITOR'S ACRONYM(S)
		11. SPONSOR/MONITOR'S REPORT NUMBER(S)
12. DISTRIBUTION/AVAILABILITY STATEMENT **Approved for public release; distribution unlimited**		
13. SUPPLEMENTARY NOTES		

14. ABSTRACT					
The genus staphylococcus was given its name by Sir Alex Ogston, a Scottish surgeon, in 1881 when he observed grape-like clusters of bacteria associated with clinical infections. Three years later, the German physician Anton Rosenbach isolated and grew these microorganisms in a pure culture. He named them Staphylococcus aureus because of their golden color. S. aureus remains one of the most important pathogens in clinical settings largely due to the rapidity of its evolutionary response to treatment. The first antibiotic-resistant strains of S. aureus were isolated only several years after penicillin was introduced commercially, and the subsequent history of antibiotics has been driven by the evolving emergence of antibiotic resistance. Our ability to control the rate at which resistance develops and spreads is predicated on our understanding of the evolutionary biology of pathogens and the basic features of bacterial populations, and while ho spital acquired strains of S. aureus have received a great deal of attention, the distribution and virulence (ability to cause infection) of community-associated strains of S. aureus are not well-defined and few studies have assigned isolates of the bacteria to known strains. The objectives of this study were to collect, isolate and characterize samples of S. aureus from areas utilized by midshipman athletes at the United States Naval Academy and patients at the Naval Medical Clinic, Annapolis. These samp les were classified in several steps including their gross morphology and determinative biochemical activities. Finally, a multilocus sequence typing (MLST) scheme was used to unambiguously characterize the isolates and analyze significant evolutionary trends in the population. The sequences of internal fragments of seven genes were obtained for 36 S. aureus isolates and assigned a unique allelic profile. These profiles, like fingerprints, were used to assign the isolates to known strains or to identify the m as novel strains via publicly accessible MLST databases. The genetic information provided by MLST was used not only to distinguish between unrelated strains, but to predict relationships in the causes, distribution, and control of infection in populations. Detailed environmental studies are the first and most important step in delineating the population biology of community associated bacteria and will contribute to a viable long term strategy for the treatment of infectious disease.					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE		**44**	
unclassified	**unclassified**	**unclassified**			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std Z39-18

novel strains via publicly accessible MLST databases. The genetic information provided by MLST was used not only to distinguish between unrelated strains, but to predict relationships in the causes, distribution, and control of infection in populations.

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Acknowledgements

Through the interesting turns this project has taken so far, I'm sure that I haven't even met all the people who have given freely of their time to ensure that we have the facilities and resources to pursue our goals. I would like to take the time to graciously thank those who have helped me get this far.

Dr. Lee Weigt, for taking the project to a whole new level.

Ken Shallop and the staff of the Smithsonian Laboratory for Analytical Biology, for their patient and thorough guidance.

Ms. Clarene Mullin, for patiently helping me when we still had a biology lab.

Associate Professor Christine Copper, for kindly sharing her lab with us.

Associate Professor Gary Fowler, for putting numbers into a meaningful context.

The entire staff of the Microbiology Lab at NMCL, for their overwhelming support.

I would like to especially thank Professor Joyce Shade, Dean William C. Miller, Academic Dean and Provost, U.S. Naval Academy, and the Trident Scholar Committee for giving me this wonderful opportunity.

Finally, I'd like to thank my advisor, Dr. Tammy Domanski, for being an excellent mentor as a scientist and as a person.

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Introduction

Staphylococci are gram-positive spherical bacteria that occur in microscopic clusters resembling grapes (Bergey *et al.*, 1994). Although nineteen species of *Staphylococcus* are described in Bergey's Manual of Determinative Bacteriology (1994), only *Staphylococcus aureus* and *Staphylococcus epidermidis* are significant in their interactions with humans. *S. aureus* should always be considered a potential pathogen, while most strains of *S. epidermidis* are nonpathogenic and may even play a protective role in their host as normal flora. *S. aureus* most commonly causes a localized skin infection, although it can also infect the eye, nose, throat, urethra, vagina, and gastrointestinal tract. In addition, *S. aureus* can cause more serious ailments when it enters the bloodstream, such as pneumonia, osteomylitis, arthritism, endocarditis, myocarditis, brain abcesses, and meningitis (Lowry 1998). Human staphylococcal infections are frequent, but usually remain localized at the portal of entry by the normal immune response. The treatment of *S. aureus* infections showing no signs of broad-antibiotic resistance is achieved successfully through many classes of antibiotics such as flucoxacillin, gentamycin, rifamicin, fusidic acid, erythromycin, vancomycin, and cefotaxime. Antibiotics are classified according to their chemical structure and method of production. Each class usually targets a specific cellular structure or process, but classes are often related by mechanism or target. Therefore, bacteria often evolve resistance to multiple classes of antibiotics. For example, methicillin and vancomycin are the only available treatment options for the 90% of isolated strains of bacteria that are penicillin resistant (Mandeil *et al.,* 1995). Vancomycin is the drug of last resort in the treatment of *S. aureus* infections.

Penicillin was produced industrially as early as 1941, but restricted to mostly military and emergency applications. By 1946, it had become the first widely used commercial antibiotic. In that same year, one hospital reported that 14% of the strains of *S. aureus* isolated from sick patients were penicillin resistant. By the end of the decade, the same hospital reported that resistance had been conferred to 59% of the strains studied (Levy, 1992). Bacteria develop resistance to antibiotics through many biological processes (figure 1). New traits, such as resistance, arise from spontaneous mutations in chromosomal genes and are selected for either positively or negatively by environmental pressure.

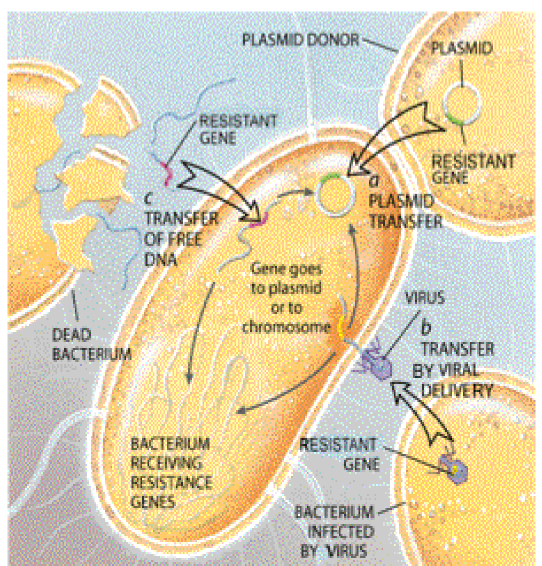
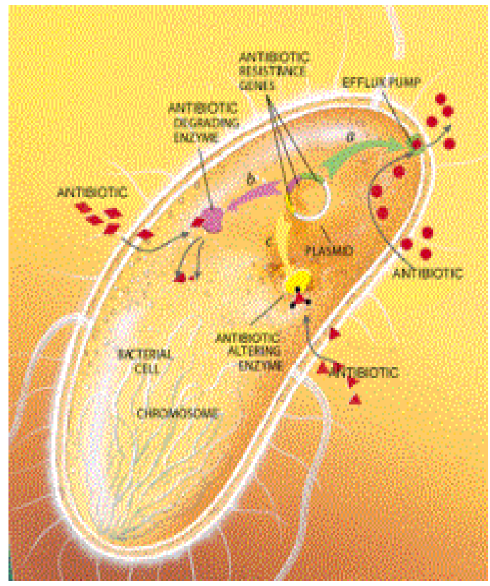


Figure 1. Transfer of resistance factor. The most method employed by bacteria to gain resistance is through a process called conjugation, in which direct contact between bacterial cells allows a copy of a plasmid containing a resistance determinant to be transferred to a formerly sensitive bacterium. In addition, a resistance gene can be picked up from the environment and incorporated into the genome of the once-sensitive bacterium. A virus that has infected a resistant bacterium may also transfer the

For example, when a microbial population is exposed to an antibiotic, more susceptible organisms will succumb, leaving behind only those resistant to the antibiotic. These organisms can then either pass on their resistance genes to their offspring by replication, or to other related bacteria through conjugation, whereby plasmids carrying the genes are transmitted from one organism to another (Brundtland, 2000). This process is a natural, unstoppable phenomenon. The biochemical are transmitted from one organism to another (Brundtland, 2000).

The biochemical mechanisms through which bacteria "resist" the action of antibiotics vary greatly between strains of bacteria and classes of antibiotics (figure 2). Strains of *S. aureus* that are resistant to penicillin produce the enzyme penicillinase, which deactivates the drug by changing its chemical structure and properties (Lowry, 1998). One way that scientists combated this resistance was to create derivatives of penicillin through synthetic chemical modifications that avoided enzymatic degradation. The most widely used penicillin derivative in the treatment of penicillin-resistant *S. aureus* is the drug methicillin, which was introduced in 1961 (Saravolatz, 1982). Strains of methicillin resistant *S. aureus* (MRSA) were isolated less than a year after its introduction. Vancomycin is a glycopeptide antibiotic not related to penicillin. It was introduced in 1956, but was replaced by methicillin until the emergence of clinical methicillin resistance reinstated it as the last resort drug in the treatment of many bacterial infections. In 1991, 29% of *S. aureus* strains were methicillin resistant, and in 1996, there was a Japanese report of a *S. aureus* strain with intermediate vancomycin resistance (CDC, 1997).

Figure 2. Resistance mechanisms employed by bacteria. Resistance genes may encode for proteins that a) cause the antibiotic to be transported out the cell before reaching its target, b) degrade the antiobiotic, or c) alter the antibiotic and render it non-functional.



Since the popular recognition of the germ theory of disease, scientists had investigated a "magic bullet" that would safely and categorically eliminate the microorganisms responsible for infectious disease. Their search for a universal cure culminated with the advent what is now called the golden age of antibiotics in the latter half of the 20th century. Unfortunately, our

Figure 3: Methicillin-resistant *S. aureus* (MRSA) on an agar plate showing heterogeneous resistance to methicillin with resistant colonies in the zone of inhibition around methicillin (MET 5).

simplified understanding of bacterial evolution and interaction led to an exclusive reliance on broadband antibiotics and, perhaps more importantly, a critical lag in the research and characterization of new strains of bacteria which has resulted in the current resurgence of infectious diseases once thought "cured" and a chillingly dwindling reserve of therapeutic options for resistant bacterial infections.

Broadband antibiotics, antibiotics that do not target a specific bacterium or family of bacteria, are designed to damage universal bacterial structures, such as the bacterial cell wall, and interrupt common pathways, such as the transfer of necessary genetic information from DNA to RNA to proteins. Broadband antibiotics are therefore useful against a wide range of bacteria; however, they also naturally select for resistance in those bacteria, effectively accelerating the evolution of resistance in the entire bacterial population. Narrowband antibiotics are engineered for a single bacterium or family of bacteria, which makes them both more effective against those bacteria they are designed to kill and less likely to select for resistance in other bacteria.

An effective long term strategy of narrowband antibiotic treatment requires a thorough understanding of significant strain differentiations within bacterial populations. This study addresses the lack of fundamental research into current and developing strains of bacteria, which is perhaps the most costly error in the history of antibiotic resistance in *S. aureus.* Although penicillinase-producing strains were universally present in hospitals by the early 1950s,

community isolates of *S. aureus* were considered to be largely penicillin susceptible. Penicillin continued to be recommended as an effective anti-staphylococcal agent as late as the early 1970s (Weinstein, 1975). However, then as now, there was no systematic observation or documentation of antibiotic resistance among *S. aureus* isolates circulating within non-medical environments and communities. The first comprehensive description and accurate assessment of the epidemiology of drug-resistant strains of *S. aureus* were not published until 1969 (Jessen *et al*., 1969).

Table 1. Time required for prevalence rates of resistance to reach 25% in hospitals (Chambers 2001)

Drug	Year drug introduced	Years to report of resistance	Years until 25% rate in hospitals	Years until 25% rate in community
Penicillin	1941	1-2	6	15-20
Vancomycin	1956	40	?	?
Methicillin	1961	<1	25-30	40-50 (projected)

Strains of *S. aureus* resistant to methicillin and other antibiotics are endemic in hospitals. However, as with penicillin resistant strains, infection with methicillin resistant strains (MRSA) may be increasing in non-medical settings. In the past few years, sporadic reports of MRSA not associated with the medical environment have been confirmed. Since the summer of 2002, community associated methicillin resistant *S.* aureus (CA-MRSA) outbreaks particularly linked with sports teams have been reported (CDC, 1981). These outbreaks have included wrestling, volleyball, and most frequently, football teams. This Trident project genotypically and phenotypically characterizes significant environmental strains of clinically relevant bacteria in common athletic areas, and performs evolutionary trend analyses on the data that will contribute to our understanding of these bacteria and provide critical insight into their global interaction with the human population. The working hypothesis of this project was that the frequency and

nature of human physical interactions on common athletic surfaces affects bacterial populations and may create reservoirs of bacteria with unique biological properties.

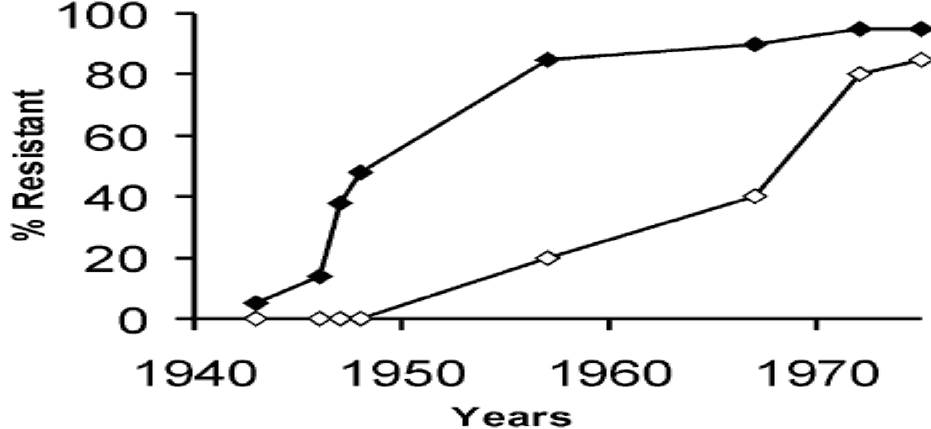


Figure 4. Secular trends of approximate prevalence rates for penicillinase-producing, methicillin-susceptible strains of *Staphylococcus aureus* in hospitals (closed symbols) and the community (open symbols).(Chambers 2001)

It is clear that some of the recently recognized outbreaks of CA-MRSA are associated with strains that have some unique properties compared to the traditional hospital-based MRSA strains, suggesting some biological properties may allow the CA-MRSA strains to spread more or cause more disease (Hiramatsu, 1995). The first step in the molecular characterization of the bacterial population is effectively tracking present strains. The CDC continues to work with state health departments to gather both the organisms and epidemiologic data from all the cases reported in the medical media to determine why certain groups of people are infected. Careful attention must be paid to these emerging bacterial population trends and the genetic drift of clinically relevant bacteria in the community. This project evaluates the relative prevalence of individual strains of *S. aureus* and begins to elucidate the evolutionary trends and molecular relationships between strains, which will contribute to a viable long term strategy for the treatment of infectious disease.

Experimental Section

Materials. Tryptic soy agar and broth and were obtained from Remel (Lenexa, KS). The Slidex Staph ID kit was purchased from BioMerieux (France). Antibiotic Sensi-Discs were obtained from Becton Dickinson (Sparks, MD). Blood agar plates were kindly provided by the Naval Medical Clinic (NMCL). All other reagents and supplies were obtained from standard sources.

Environmental Sampling and Isolation. Using sterile cotton-tipped applicators, samples were collected from locations described in Table 1. The samples were incubated overnight at 37º C on tryptic soy agar (TSA) plates. The plates were observed for gross colony morphology and different colony types were identified. A small amount of bacteria from each unique colony was transferred to a new TSA plate and incubated overnight at 37º C.

Gram Staining. A drop of sterile distilled water was placed on a microscope slide and a small amount of bacteria was transferred from one colony into the water. The mixture was stirred well and dried at 43º C for 5-10 minutes. The slide was flooded with crystal violet, allowed to stand for 60 seconds, then rinsed with distilled water. The smear was flooded with iodine and allowed to stand for 60 seconds, then rinsed with distilled water. The slide was briefly rinsed with an isopropanol/acetone decolorizing solution, then flooded with safranin, allowed to stand for 60 seconds, rinsed with distilled water, and patted dry. The cells were observed at 100X magnification under the oil immersion lens. Cells were characterized according to their color; gram-positive cells appearing violet and gram-negative cells appearing red; the shape of the cells, rods or cocci; and the arrangement of cells, single, clumped or filaments.

Catalase Test. A small amount of bacteria was transferred to a glass microscope slide containing a drop of water and mixed well. Hydrogen peroxide was added to the mixture, and a positive catalase test was indicated by the appearance of bubbling.

Hemolysis Test. A small amount of bacteria was transferred to sheep's blood agar plates and incubated overnight at 37° C. The colonies were characterized according to the type of hemolysis they displayed.

Latex and Red Blood Cell Agglutination. The reagents in the Slidex Staph-Kit were allowed to come to room temperature and re-suspended by mixing. One drop of anti-*Staphylococcus* reagent, R1, and one drop of negative control reagent, R2, were added separately to a glass slide. A small amount of bacteria was transferred from suspected colonies and added to each drop, then mixed well using a sterile wooden stick for 10 seconds. The slide was gently rocked for 20 seconds and the reaction was read under normal lighting. The Slidex kit tests for the production of protein A, indicated by white clumping of latex particles, and coagulase, indicated by a red clumping of blood cells. A positive test for either indicates the presence of *S aureus*.

Antimicrobial Susceptibility Tests. A small amount of bacteria was transferred to test tube containing tryptic soy broth (TSB) and incubated overnight at 37° C in a shaking incubator. The optical densities of the liquid cultures were recorded and the volume of inoculum sample was adjusted for concentration. A sample of the liquid culture was spread on a TSA plate. Antibiotic discs were applied to the plate and allowed to incubate overnight at 37° C. The zones of clearance were recorded.

Chromosomal DNA Extraction. Chromosomal DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). A small amount of bacteria was

transferred to test tube containing tryptic soy broth (TSB) and incubated overnight at 37ºC in a shaking incubator. Pellet cells were prepared by centrifuging 1.5 ml of the liquid culture for 2 min. at 14000 rpm in a 1.5 ml Eppendorf tube. The supernatant was discarded and the pellet cells were resuspended in 480 µl of 50 µM EDTA. Lysostaphin (60 µl, 10 ng/µl) and lysozyme (60 µl, 10 ng/µl) were added and the mixture was incubated at 37ºC for 30 min. The mixture was centrifuged for 2 min at 14000 rpm and the supernatant was removed. Nuclei Lysis solution (600 µl) was added and the mixture was pipetted gently. The reaction mixture was incubated at 80ºC for 5 min, and then cooled to room temperature. RNase solution (3 µl) was added, and the mixture was incubated at 37ºC for 30 min with shaking, then cooled to room temperature. Protein Precipitation Solution (200 µl) was added and the reaction mixture was vortexed and incubated on ice for 5 min. The reaction mixture was centrifuged 3 min at 14000 rpm, and the supernatant was transferred to a clean tube containing 600 µl of room temperature isopropanol. The solution was mixed well and centrifuged 2 min at 14000 rpm. The supernatant was decanted and ethanol (600 µl, 70%) was added. The solution was mixed well and centrifuged 2 min at 14000 rpm. Then the ethanol was aspirated and the pellets were allowed to air-dry for 10 min. The DNA pellet was rehydrated in Rehydration Solution (100 µl) overnight at 4ºC. Optical densities of the resulting solutions were measured and recorded.

Polymerase Chain Reaction and Purification. Polymerase chain reactions (PCR) were carried out using 25 µl reaction volumes containing 1 µl of chromosomal DNA (10-100 ng), 2 µl of each primer, and puRe Taq Ready-To-Go PCR Beads (Amersham Biosciences, Stockholm, Sweden). The PCR was performed in a Peltier Thermal Cycler-225 Tetrad with 5 cycles of denaturation at 94ºC for 20 s, annealing at 48ºC for 5 s, and extension at 72ºC for 30s, followed by 30 cycles of denaturation at 94ºC for 20 s, annealing at 52ºC for 5 s, and extension at 72ºC for

30s, followed by a final extension step of 72ºC for 2 min. The amplified products were purified using a Performa DTR 96-Well gel filtration system (Edge BioSystems, Gaitherburg, MD).

DNA Sequencing Reaction and Purification. The sequences of both strands of the PCR products were determined with an ABI Prism 3100 Genetic Analyzer with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the primers used in the initial PCR amplification.

Results and Discussion

Initial Characterization of Environmental Samples. Environmental samples obtained on 4 NOV were grown on mixed cultures and distinct colonies were categorized based on their appearance and morphology. The results are described in Table 2.

Table 2: Gross Colony Morphology

Location	Number[a] Designation	Gross Colony Morphology[b]					
		Relative Size	Form	Optical Characteristics	Pigmentation	Margin	Elevation
Dewey Field	1,1	medium	irregular	glossy	gray	undulate	flat
	1,2[c]	medium	circular	glossy	yellow	entire	raised
	1,3	medium	circular	glossy	yellow	entire	raised
	1,4	large	irregular	dull	yellow	undulate	flat
Farragut Field	2,1	medium	circular	glossy	gray	entire	raised
	2,2	medium	circular	glossy	yellow	circular	flat
	2,3	small	circular	glossy	yellow	circular	flat
	2,4	large	filamentous	dull	gray	filamentous	flat
Ingram Track	3,1	medium	irregular	dull	gray	undulate	flat
	3,2	medium	irregular	dull	gray	undulate	raised
	3,3	medium	circular	glossy	yellow	entire	raised
Lejeune Karate Room	4,1	medium	circular	dull	gray	entire	flat
	4,2	small	circular	glossy	gray	entire	raised
	4,3	medium	circular	glossy	yellow	entire	raised
MacDonough Volleyball Courts	5,1	small	circular	glossy	white	entire	raised
	5,2	medium	irregular	glossy	yellow	undulate	flat
Rip Miller Field	6,1	medium	circular	glossy	gray	entire	raised
	6,2	medium	circular	dull	yellow	entire	flat
Hospital Point Fields	7,1	medium	circular	dull	gray	undulate	flat
	7,2	medium	circular	glossy	yellow	entire	raised
	7,3	large	filamentous	dull	gray	filamentous	flat
	7,4	small	circular	dull	white	entire	flat
MacDonough Boxing Ring	8,1	small	circular	glossy	white	entire	raised
	8,2	medium	circular	glossy	yellow	entire	raised
	8,3	large	filamentous	dull	gray	filamentous	flat
Boxing Equipment	9,1	small	circular	glossy	white	entire	raised
Lejeune Wrestling Mats	10,1	small	circular	glossy	white	entire	raised
	10,2	medium	circular	glossy	yellow	entire	raised
Halsey Basketball Courts	11,1	medium	irregular	dull	gray	undulate	flat
	11,2	medium	circular	glossy	yellow	entire	raised
	11,3	medium	irregular	dull	gray	undulate	flat
	11,4	medium	circular	dull	gray	undulate	flat
	11,5	small	circular	glossy	white	entire	raised

[a]The first number of the number description indicates the location that the sample was obtained from. The second number indicates a distinct colony type from that location.
[b]Colony morphology is consistent with standard microbiological method.
[c]Colony morphologies that correspond to classical descriptions of *S. aureus* are highlighted in yellow.

S. aureus agar colonies are typically circular in form, with a smooth, glistening colony surface, butyrous consistency, and an entire margin. Typical *S. aureus* cultures produce a characteristic yellow pigmentation, but readily develop variants that grow as gray or dirty white colonies. Therefore, colonies matching this description (highlighted in yellow on Table 2) were transferred to sterile media for cultivation of pure cultures and further description using differential stains.

Differential stains allow for distinguishing certain characteristics of cells, and commonly use two or more stains applied in succession to resolve important microbiological features. The gram stain, which divides most clinically significant bacteria into two main groups, is the first step in bacterial identification. *S. aureus* is a gram-positive coccus. The deep violet clumps of small spheres illustrated in Figure 5 are highly specific for *Staphylococcus.*

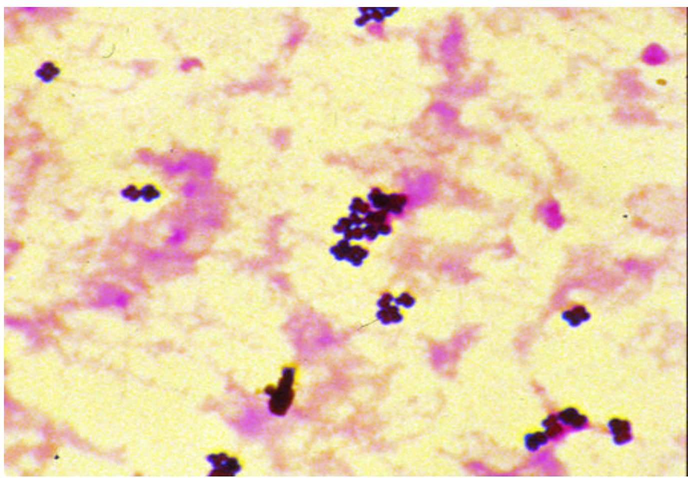


Figure 5: Gram stain of *S. aureus* (Freundlich, 2001)

All of the initial samples were gram-stained and described under an oil immersion lens at 100X magnification. The descriptions are summarized in Table 3. Samples 1,3; 4,1; 4,2; 5,1; 8,1; 10,1; and 11,5 (highlighted in yellow on Table 3) were gram positive cocci that resembled Staphylococci, and they were selected for further characterization.

Table 3: Microbiology Results

Number Designation	Gram Stain Result	Microscopic Description	
		Shape	Arrangement
1,1	positive	rod	filaments
1,2	positive	rod	clumps
1,3 [a]	positive	cocci	clusters
1,4	positive	rod	filaments
2,1	negative	rod	filaments
2,2	negative	rod	single
2,3	negative	rod	filaments
2,4	positive	rod	filaments
3,1	negative	rod	filaments
3,2	positive	rod	filaments
3,3	positive	rod	filaments
4,1	positive	cocci	clusters
4,2	positive	cocci	clusters
4,3	positive	rod	filaments
5,1	positive	cocci	clusters
5,2	negative	rod	clumps
6,1	negative	rod	single
6,2	positive	rod	filaments
7,1	positive	rod	filaments
7,2	positive	rod	clumps
7,3	positive	rod	filaments
7,4	positive	rod	filaments
8,1	positive	cocci	clusters
8,2	positive	rod	clumps
8,3	positive	rod	filaments
9,1	positive	cocci	clusters
10,1	positive	cocci	clusters
10,2	positive	rod	filaments
11,1	positive	rod	filaments
11,2	positive	rod	single
11,3	positive	rod	single
11,4	positive	rod	filaments
11,5	positive	cocci	clusters

[a] Colonies that resemble *S. aureus* are highlighted in yellow.

More discriminating biochemical characterization of those bacteria consistent with the gross and microscopic morphological characteristics of *S. aureus* was conducted via a series of selective reactions classifying determinative biochemical processes of the cell. Specifically, the Slidex Staph-Kit was used to confirm that suspect colonies tested with the classical methods of gram stain, morphology, catalase and hemolysis tests most likely were *S. aureus.* Tests were performed on samples 1,3; 4,1; 4,2; 5,1; 8,1; 10,1; and 11,5 and the results are described in Table 4.

Table 4: Initial Characterization Test Results

Sample	Coagulase	Protein A	Catalase	Hemolysis
1,3	-	-	+	a
4,1A	-	-	-	a
4,1B[a]	+	-	+	a
4,2	-	+	+	ß
5,1	-	-	+	ß
8,1	-	-	+	a
10,1	+	-	+	ß
11,5	-	-	+	a

[a]Samples highlighted in red were positively identified as *S. aureus.*

Coagulase. The coagulase test is a classical method for differentiating between pathogenic and non-pathogenic strains of *Staphylococcus.* Bacteria that produce coagulase use it as a defense mechanism by clotting the areas of plasma around them, thereby enabling themselves to resist phagocytosis by the host's immune system. *S. aureus* is normally coagulase positive (Lally *et al.*, 1984). Protein A is present on the surface of most *S. aureus* strains. It has an affinity for the Fc fragment of most isotype G immunogloblulins (IgG) (Flandrois *et al.*, 1975). The Slidex Staph-Kit enables the identification of *S. aureus* strains from culture media. It is composed of stabilized red blood cells, sensitized with fibrinogen, which will agglutinate if the S. aureus strain produces coagulase. The kit also contains latex particles, sensitized with monoclonal IgG antibodies, which will agglutinate if protein A is present on the surface of the *S. aureus* strain. A positive result is indicated by agglutination. A coagulase result was indicated by red clumps, while a positive result for protein A resulted in white clumping. The sensitivity and specificity of the kit on specimens cultured on TSA is 94.2% and 89.5% respectively. During the initial characterization, positive results for Protein A were observed in sample 4,2 and positive coagulase results were observed in samples 4,1B and 10,1. The differences in agglutination results suggest the presence of at least two phenotypes of *S. aureus*.

Catalase. Hydrogen peroxide and superoxide molecules are toxic to bacteria. Some bacteria, however, possess a defense mechanism which can minimize the harm done by the two compounds. These resistant bacteria use two enzymes to catalyze the conversion of hydrogen peroxide and superoxide back into diatomic oxygen and water. One of these enzymes is catalase and its presence can be detected by a simple test. The catalase test involves adding hydrogen peroxide to a culture sample. If the bacteria in question produce catalase, they will convert the hydrogen peroxide to oxygen gas which will be evolved. The evolution of gas causes bubbles to form and is indicative of a positive test. *S. aureus* is strongly catalase positive. (Bergey *et al.*, 1994) The three samples identified by the Staph-Kit were catalase positive.

Hemolysis. Hemolysis refers to the breakdown of red blood cells. Some diseases and processes can cause the premature breakdown of red blood cells. There are three types of hemolysis on blood agar plates shown on Figure 6: alpha, beta, and gamma. Gamma hemolysis is actually no hemolysis. Alpha hemolysis is characterized by a darkening of the agar to a greenish color, while light will shine through an area of beta-hemolysis. Colonies of *S. aureus* normally produce beta hemolysis on blood agar. (Bergey *et al.*, 1994) However, some strains of *S. aureus* do produce alpha hemolysis.

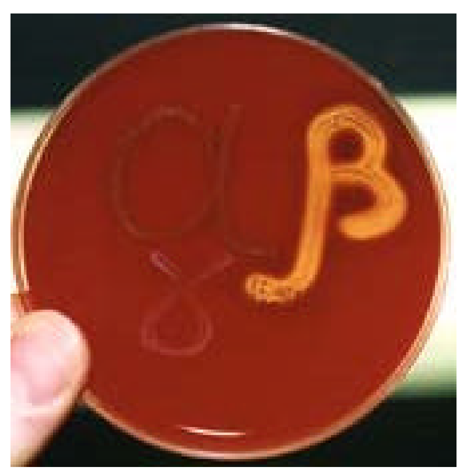


Figure 6: Examples of hemolysis. (Freundlich, 2001)

A red agglutination pattern indicated a positive result for coagulase while a white agglutination pattern indicated a positive result for Protein A. Dual positive results were difficult to interpret because the red agglutination sometimes masked the white. This ambigiuity did not affect the project in a meaningful way because it did not result in missed identifications or false

positives. Positive results for Protein A were observed in sample 4,2 and positive coagulase results were observed in samples 4,1B and 10,1. A positive identification is indicated by an agglutination pattern in either reagent. Positive results for catalase and hemolysis support the identification of those samples as *S. aureus.*

Table 5: Complete Characterization Test Results

Sample	Coagulase	Protein A	Catalase	Hemolysis
4,1B	-	+	+	a
4,2	+	-	+	ß
10,1	-	+	+	ß
14	+	-	+	a
15,2	+	-	+	a
16,1	+	+	+	a
17,2	-	+	+	a
31,1	+	-	+	a
32,1	+	+/-	+	a
35,1	-	+	+	ß
37,3	+	-	+	ß
CL1	+	-	+	ß
CL2	+	-	+	ß
CL3	+	-	+	ß
CL4	+	-	+	ß
CL5	+	-	+	ß
CL6	+	-	+	ß
CL7	+	-	+	ß
CL8	+	-	+	ß
CL9	+	-	+	ß
CL10	+	-	+	ß
CL11	+	-	+	ß
CL12	+	-	+	ß
CL13	+	-	+	ß
CL14	+	-	+	ß
CL15	+	-	+	ß
CL16	+	-	+	ß
CL17	+	-	+	ß
CL18	+	-	+	ß
CL19	+	-	+	ß
CL20	+	-	+	ß
CL21	+	-	+	ß
CL22	+	-	+	ß
CL23	+	-	+	ß
CL24	+	-	+	ß
CL25	+	-	+	ß
CL26	+	-	+	ß
CL27	+	-	+	ß
RN450	+	+/-	+	ß
RN4220	+	+/-	+	ß

Identification of *S. aureus.* Using data generated during the initial characterization, the parameters of sample collection were optimized, and, after environmental sample 11, only those strains exhibiting the colony morphology of *S. aureus* were isolated for further biochemical analysis. Additional samples were donated by the Naval Medical Clinic, Annapolis. These samples (designated CL1-CL27) were isolated from patients in the clinic from 10 Dec 2003 through 11 Mar 2004. The strains were identified by the NMCL staff as *S. aureus* and provided on blood agar plates. Due to patient privacy concerns, information concerning the patient's identity and specific infection type were not provided. All clinical strains were verified and characterized in the same manner as environmental strains. In addition, the two strains RN450 and RN4220, characterized and utilized as high-passage laboratory research strains, were included as reference points in the study. The results of the characterization of all strains are described in Table 5.

Antibiotic Susceptibility of *S. aureus* Isolates. Antibiotic susceptibility testing plates were prepared using standard microbiological methods. The diffusion of antibiotic into the media inhibits bacterial growth in a circular region known as the zone of clearance for that antibiotic. The diameter of each of the zones of clearance (Fig. 7) was recorded and interpreted by standards established by the National Committee for Clinical Laboratory Standards (NCCLS). All diameters were recorded in millimeters, and the results are interpreted in Table 6.

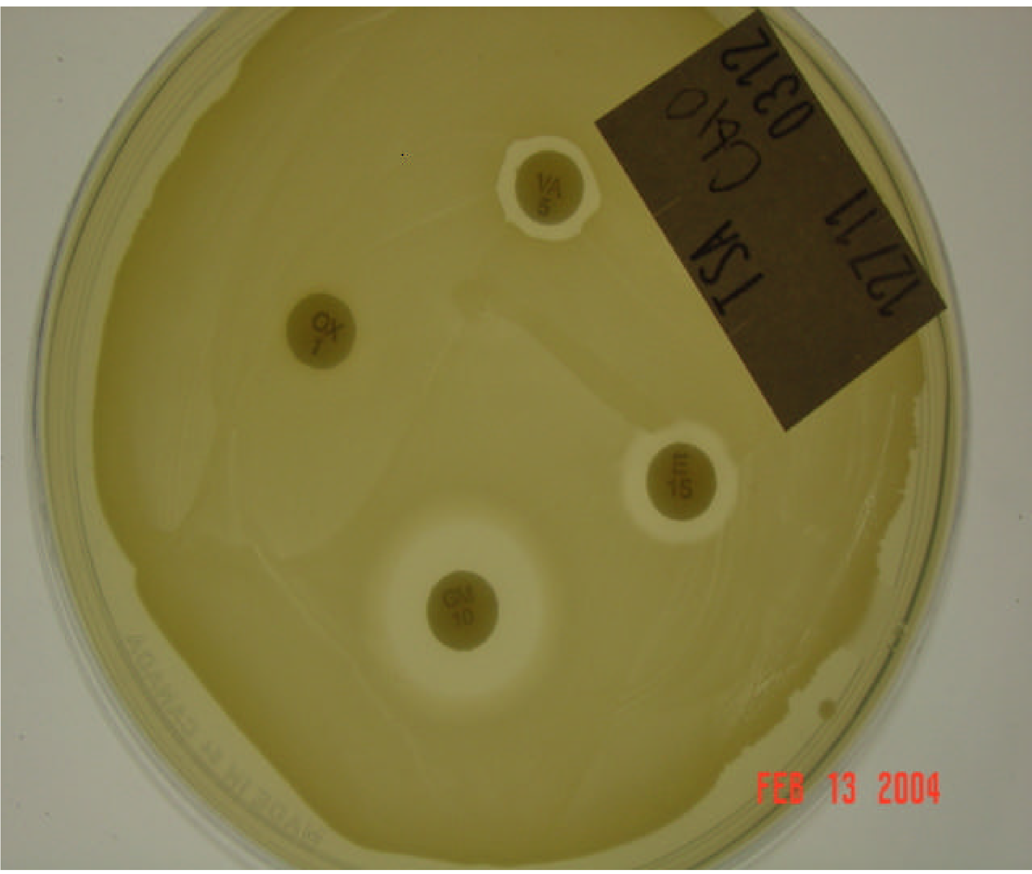


Figure 7: Zones of antibiotic clearance for colony CL10.

The four antibiotics used in this project: erythromycin (E), gentamicin (GM), oxacillin (OX), and vancomycin (VA) were chosen because they represent different classes of antibiotics and because they are used specifically in the antibiotic therapy of *S. aureus* infections.

Table 6: Antibiotic Zone of Clearance Diameter Interpretation

Sample	Zone of Clearance Diameter (mm)				Resistance Similarity Code	Resistance Type
	E	GM	OX	VA		
4,1B	14.5	31	16.0(h)	13.5	2111	1
4,2	21	13.5	19.5	9.5	2111	1
10,1	0	24	21.5	10	3111	2
14	34	22	0	12.5	1131	3
15,2	9	28	15	13.5	3111	2
16,1	11	31	0	14.5	3131	4
17,2	11.5	27.5	11.5	12	3131	4
31,1	19	14	14	9	2211	5
32,1	44	24	0	15	1131	3
35,1	23	24	16	13	2111	1
37,3	17	20	12	10	2121	6
CL1	9.5	16	14	9.5	3111	2
CL2	20.5	15	20	12	2111	1
CL3	20	14	18	9	2211	5
CL4	19	14	10	10	2231	7
CL5	9	14	14	9	3211	8
CL6	16	12	11	8	2321	9
CL7	10	13	0	8	3231	10
CL8	0	14	13	10	3211	8
CL9	19	15	12	10	2121	6
CL10	10	13	0	9	3231	10
CL11	11	15	0	9	3131	4
CL12	17	12	12	9	2321	9
CL13	18	15	13	9	2111	1
CL14	16	13	17	9	2211	5
CL15	17	14	12	9	2121	6
CL16	11	15	13	9	3111	2
CL17	15	15	10	9	2131	11
CL18	13	14	13	9	3111	2
CL19	13	14	13	8	3111	2
CL20	0	13	0	10	3231	10
CL21	15	13	12	9	2221	12
CL22	17	13	11	9	2221	12
CL23	17	13	12	9	2221	12
CL24	9	13	0	9	3231	10
CL25	0	12	0	9	3331	13
CL26	0	15	0	10	3131	4
CL27	0	20	0	9	3131	4
RN450	19	14	19	9	2211	5
RN4220	19	14	19	9	2211	5

=susceptible
=intermediate resistance
=resistant

Erythromycin is a macrolide antibiotic used to treat a wide variety of bacterial infections. Erythromycin's mode of action differs from the beta-lactam antibiotics, such as penicillin, in that the antibiotic inhibits protein synthesis rather than targeting the bacterial cell wall. Despite significant *in vitro* erythromycin resistance in *S. aureus* populations, there remains a low

frequency of clinical treatment failure. Erythromycin remains a reasonable agent in the treatment of uncomplicated superficial skin infections.

Gentamicin is a bactericidal aminoglycoside antibiotic. The cationic antibiotic molecules create fissures in the outer cell membrane, resulting in leakage of intracellular contents and enhanced antibiotic uptake. Aminoglycosides display bactericidal, concentration-dependent killing action and are active against a wide range of aerobic gram-negative bacilli. They are also active against staphylococci and certain mycobacteria. Aminoglycosides are effective even when the bacterial inoculum is large, and resistance rarely develops during the course of treatment. (Montie *et al.*, 1995)

Oxacillin, like methicillin, is a semi-synthetic penicillin derivative that blocks the formation of bacterial cell walls. Researchers developed these drugs to be resistant to penicillinase activity and combat antibiotic resistance. Oxacillin is tested instead of methicillin because it is more resistant to degradation in storage and is more likely to detect most heteroresistant strains. Accurate detection of antibiotic resistance can be difficult due to the presence of two subpopulations (one susceptible and the other resistant) that may coexist within a culture of a single strain. All cells in a culture may carry the genetic information for resistance but only a small number can express the resistance in an artificial environment, such as a plate, rather than inside a living organism. This phenomenon is termed heteroresistance and occurs frequently in staphylococci resistant to penicillinase-stable penicillins, such as oxacillin. In addition, methicillin is no longer commercially available in the United States. Antimicrobials like oxacillin and nafcillin now are used for treatment of *S. aureus* infections. Since methicillin was first used to test and treat infections by *S. aureus*, the acronym MRSA is still used by many to describe these isolates because of its historic role.

Vancomycin is a glycopeptide that acts by interfering with the construction of cell walls in bacteria. Vancomycin often is the only drug of choice for treatment of severe MRSA infections, although some strains remain susceptible to fluoroquinolones, trimethoprim, sulfamethoxazole, gentamicin, or rifampin.

For each antibiotic, different levels of susceptibility were assigned numerical values, and each strain is defined by the pattern of resistance it displayed (resistance similarity code). Thirteen different resistance types were identified using this method and the relationships between them are graphed in Figure 8.

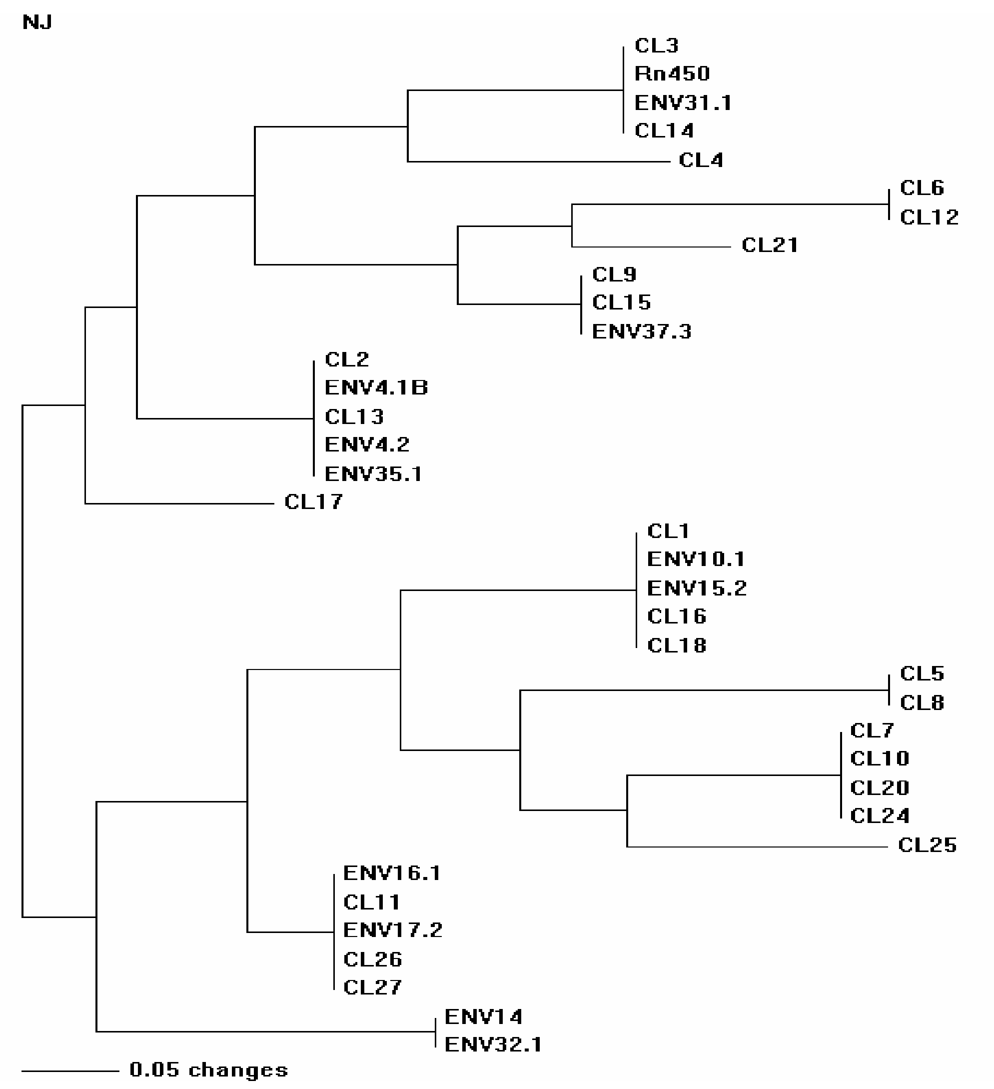


Figure 8: Strain differentiation by patterns of antibiotic susceptibility.

There are no obvious trends in the patterns of antibiotic resistance. This would indicate that there is no causal relationship between the expression of antibiotic resistance and the environment or pathogenicity of a given strain. As expected, all samples are susceptible to vancomycin. Vancomycin resistant *S. aureus* has only been reported rarely in hospital settings. Most of the strains are multiple drug resistant, with notable exceptions being environmental strains 14 and 32,1. These strains are only resistant to oxacillin. Since oxacillin is typically used to treat resistant bacteria, it is unlikely that these strains developed this resistance from clinical exposure. Most likely, they received the resistance genes via a plasmid from other bacteria.

Table 7: Final Concentrations of Chromosomal DNA

Sample	Final Concentration (ng/µL)
4,1B	191.5
4,2	1027
10,1	186
14	149
15,2	327
16,1	111
17,2	70
31,1	406
32,1	646.5
35,1	214
37,3	
CL1	381
CL2	318
CL3	454
CL4	492
CL5	607
CL6	207
CL7	373
CL8	450
CL9	123
CL10	817
CL11	170
CL12	165
CL13	293
CL14	370
CL15	271
CL16	110
CL17	133
CL18	208
CL19	286
CL20	164
CL21	411
CL22	113.5
CL23	212
CL24	111
CL25	363
CL26	119
CL27	348
RN450	308
RN4220	204

Chromosomal DNA Extraction. The final concentration of chromosomal DNA was calculated from the solution absorbance at 260 nm. Only those samples resulting in DNA concentrations above 100 ng/µL (at least 10 µg of DNA total) were considered of high enough quality to utilize in amplification reactions. Although several strains required multiple preparation attempts, all 36 samples studied resulted in sufficiently pure and concentrated DNA samples. The final concentrations of the samples analyzed are given in Table 7.

PCR Products. The sequences of the primers used in PCR reactions are given in Table 8.

Table 8: Primer Sequences for PCR and Sequencing Reactions[a]

Gene	Primer	Sequence (5'-3')
Carbamate kinase (arcC)	arcC-Up	TTGATTCACCAGCGCGTATTGTC
	arcC-Dn	AGGTATCTGCTTCAATCAGCG
Shikimate dehydrogenase (aroE)	aroE-Up	ATCGGAAATCCTATTTCACATTC
	aroE-Dn	GGTGTTGTATTAATAACGATATC
Glycerol kinase (glpF)	glpF-Up	CTAGGAACTGCAATCTTAATCC
	glpF-Dn	TGGTAAAATCGCATGTCCAATTC
Guanylate kinase (gmk)	gmk-Up	ATCGTTTTATCGGGACCATC
	gmk-Dn	TCATTAACTACAACGTAATCGTA
Phosphate acetyltransferase (pta)	pta-Up	GTTAAAATCGTATTACCTGAAGG
	pta-Dn	GACCCTTTTGTTGAAAAGCTTAA
Trisephosphate isomerase (tpi)	tpi-Up	TCGTTCATTCTGAACGTCGTGAA
	tpi-Dn	TTTGCACCTTCTAACAATTGTAC
Acetyl coenzyme A acetyltransferase (yqiL)	yqiL-Up	CAGCATACAGGACACCTATTGGC
	yqiL-Dn	CGTTGAGGAATCGATACTGGAAC

[a](Enright *et al.*, 2000)

The expected size of each fragment is shown in table 9. All PCR products were checked by gel electrophoresis for size and integrity.

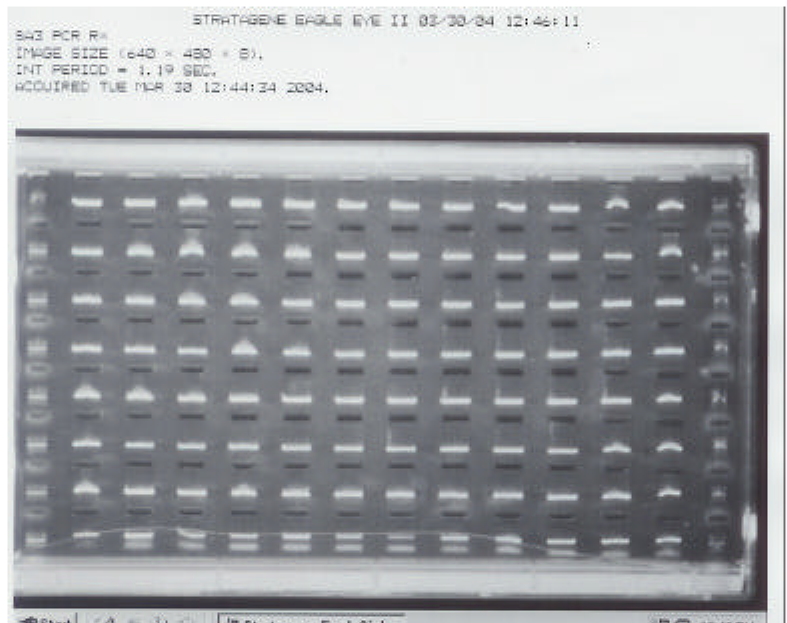
Table 9: Sequence Length of MLST Genes[a]

Gene	Sequence length (bp)	No. of alleles
arcC	456	17
aroE	456	17
glpF	465	11
gmk	429	11
pta	474	15
tpi	402	14
yqiL	516	16

[a](Enright *et al.*, 2000)

Fragments of DNA migrate by electrophoresis through agarose polymer gel according to their molecular weight, given by the number of nucleotide base pairs per fragment. Known molecular size markers (on the left and right side of Figure 9) are used to visualize the relative size of product fragments by mobility. The sequence lengths of the fragments used in this project are given in Table 9. The agarose gel of the PCR products (Figure 9) shows strong, single bands that are approximately 500 bp in length. This indicates that the PCR reaction was successful and the correct sequences were amplified.

Figure 9: PCR products on agarose gel. The first and last lanes contain markers for comparison and determination of fragment size. The remaining lanes (columns) contain PCR products from individual *S. aureus* chromosomal samples, with each row containing the product from an individual locus, and the very bottom doublets containing amplified rRNAs as positive controls.



DNA Sequencing. In the DNA Sequencer, a laser is used to excite dyes on nucleotides. The dyes emit at different wavelengths which a camera then records and sends to the computer for analysis. The output is a spectrum resembling Figure 10. The spectral output of each sequence was reviewed and ambiguities were resolved by comparing outputs of the forward and reverse reactions. The consensus sequence of the forward and reverse reactions is the sequence of the fragment.

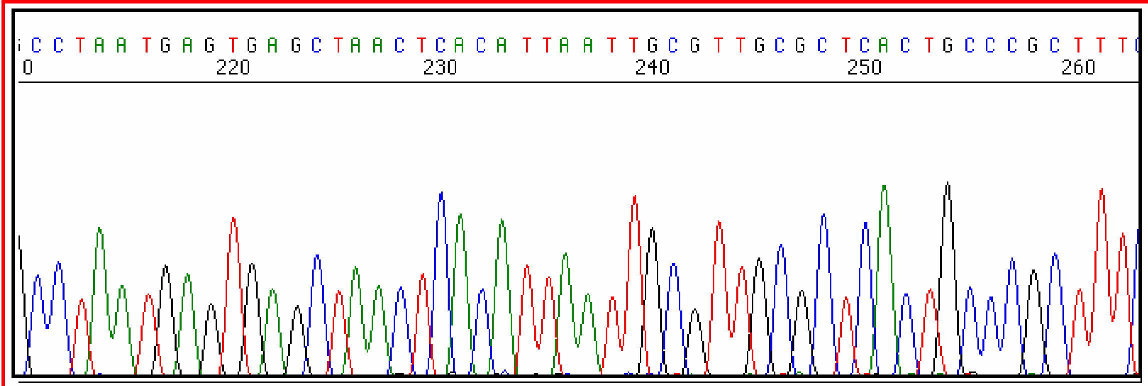


Figure 10. Sample Chromatogram

Sequential Analysis. The genome of *S. aureus* is polymorphic, meaning that there are several variants (alleles) of a particular gene that occur simultaneously in a population. A locus is the specific physical location of a gene on the chromosome. For each locus, the sequences obtained from all samples were compared to online MLST databases and the different sequences were assigned allele numbers (Table 10). For each sample, the alleles at each of the seven loci defined the allelic profile which corresponded to its strain assignment in the MLST scheme (Enright *et al.,* 2000).

Table 10: MLST Allele Numbers

Sample	arc	aro	glp	gmk	pta	tpi	yqi
CL3	13	13	1	1	12	11	13
CL1	2	2	5	2	6	3	2
CL2	2	2	2	2	6	3	2
CL4	4	3	1	1	11	4	11
CL5	3	3	1	1	1	1	10
CL6	13	13	1	1	12	11	13
CL7	3	3	1	1	4	4	3
4.1B	3	1	1	8	1	1	1
10.1	10	14	8	13	10	3	2
14	27	4	1	1	4	6	11
15.2	1	4	1	4	12	29	10
16.1	27	3	1	1	13	6	11
CL8	3	3	1	1	4	4	3
CL9	8	2	2	2	6	3	2
CL10	3	3	1	1	4	4	3
CL11	3	3	1	1	4	4	3
CL12	19	23	15	2	19	20	15
CL13	1	4	1	4	12	1	10
Rn450	3	3	1	1	4	4	3
4.2	3	1	1	8	1	1	1
17.2	13	13	1	1	12	11	13
31.1	1	4	1	4	12	1	10
32.1	1	3	1	1	4	4	11
35.1	1	1	1	1	25	23	13
CL14	1	4	1	4	12	1	10
CL15	3	3	1	1	4	4	3
CL16	3	3	1	1	4	4	3
CL17	22	1	14	23	12	4	31
CL18	1	4	1	4	12	1	10
CL20	3	3	1	1	4	4	3
CL21	10	14	8	13	10	3	2
CL24	3	3	1	1	4	4	3
CL25	3	3	1	1	4	4	3
CL26	3	3	1	1	4	4	3
CL27	3	3	1	1	4	4	3
37.3	3	3	1	1	4	4	3

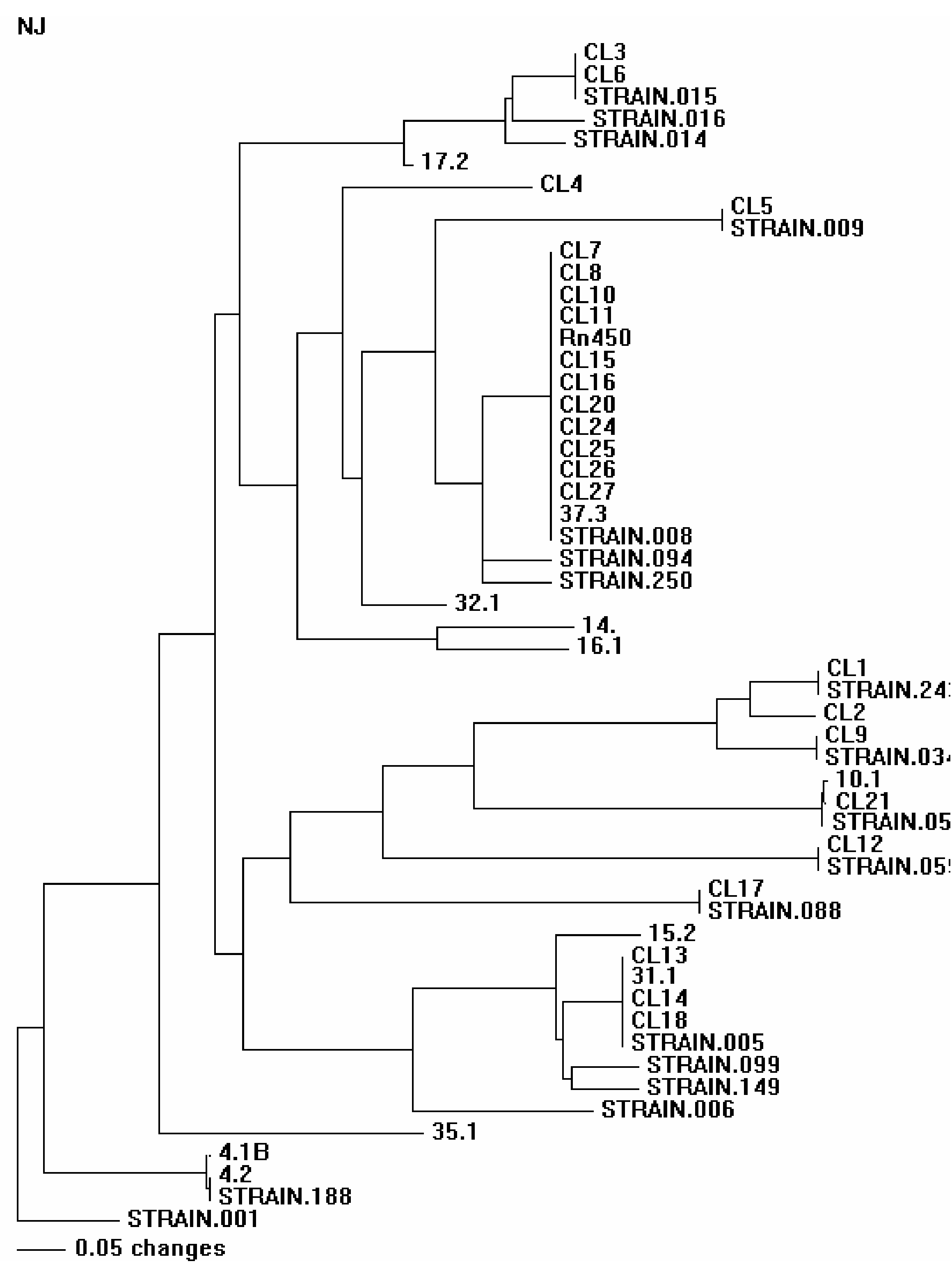


Figure 11: Strain Differentiation by Multi Locus Sequence Typing (MLST)

The samples may be grouped phylogenetically using the MLST into essentially four groups. Group I consists of environmental strains found on the yard without corresponding clinical samples. Group II is comprised of clinical isolates without corresponding environmental samples. Group III includes clinical samples that have the same strain type as environmental samples, and Group IV is made up of clinical samples that did not match any previously sequenced strains, either the environmental samples or those in the MLST database.

Group I represents strains of *S. aureus* that were isolated on the yard, but did not present in clinical cases during the time period observed during this project. This means that no one was exposed to these strains or that they were exposed, but not infected. It can be reasonably concluded that midshipmen, as well as other athletes and staff using these spaces were exposed to these strains, but there is no data as to whether or not colonization occurred. Colonization occurs when bacteria are present on or in the body without causing illness. It is unclear when and how colonized bacteria become virulent, but is most likely a function of both the virulence properties of the organism and the immune response of the host (Mandeil, 1995). Interestingly, several strains in this group produced uncharacteristic alpha hemolysis on blood agar. Most strains of *S. aureus* produce toxins that completely lyse red blood cells, producing a zone of clear beta hemolysis on agar containing blood. Non-pathogenic strains, however, sometimes do not completely lyse red blood cells, and instead convert the hemoglobin present into green methemoglobin, producing the green color that characterizes alpha hemolysis. The alpha hemolytic strains in this group may have decreased virulence properties that would begin to explain why they did not present in the clinic.

Group II includes the clinical samples without corresponding environmental samples. This means that either the infections were caused by bacteria that were present in the

environment, but not ubiquitous and therefore not represented in the relatively small sample size, or that the source of infection was not one of the studied environments. The source may have been another environment, an infected or a colonized individual, or opportunistic colonies of *S. aureus* already present on the patient. The CDC reports that approximately 25 to 30% of the population is colonized with *S. aureus* bacteria at a given time (Kluytmans *et al.*, 1997). Staphylococci are primarily transmitted between individuals by direct skin-to-skin contact. Usually the organism spreads from hands of the infected/colonized individual to the skin of another individual. It has historically been assumed, transmission of staphylococci does not occur by droplet, airborne or indirect contact with contaminated objects (fomites) (Bradley *et al.,* 1991). However, because the Naval Medical Clinic, Annapolis is not an inpatient clinic, we can reasonably assume that none of the clinical samples we received were nosocomial, or hospital acquired infections. Therefore, both the environmental and clinical samples can be considered community-associated strains of *S. aureus*. Also, the antimicrobial patterns of these strains are unique and differ from typical hospital acquired pathogens, because they are resistant to methicillin, but are not multi-drug resistant (Vandenesch, 2003). This is typically regarded as a resistance trait unique to community associated bacteria (Jessen *et al.*, 1969).

The most useful group for further study is Group III. These strains occurred both environmentally and clinically. Because the case histories of the clinical cases were confidential, no correlation can be made between individual clinical cases and any particular environmental strains, but it can be shown that the environmental strains in this group are potentially virulent. This corroborates evidence that environmental fomites may function as reservoirs for these strains, indirectly transmitting them from individual to individual and potentially causing infection. The explanation for the rapid transmission of *S. aureus* infections in hospital settings

has been the interaction of patients with each other and with health care providers (de Lencastre *et al.,* 1996). The same model can be can be used for any environment where the bacteria are ubiquitous and there is frequent skin-to-skin contact between individuals, in this case, the interaction of athletes on playing fields. But more importantly, the environment itself may play some part in the transmission of pathogenic bacteria.

The clinical strains in Group IV did not match any previously sequenced strains, either environmental or those already in the MLST database. CA-MRSA infections appear to be an emerging phenomenon worldwide, and unique strains of MRSA are increasingly responsible for community-acquired infections. The genotypes of these organisms support the recent suggestion that the dissemination of a single CA-MRSA clone did not occur around the world but rather suggests the possibility of simultaneous co-evolution of CA-MRSA organisms in different locations (Vandenesch 2003).

Conclusions and Further Study

The first and most fundamental question evoked by contemporary epidemiological studies of community associated *S. aureus* is the significance of environmental transmission in pathogenesis and the evolution of antibiotic resistance. The mechanisms of transmission of nosocomial methicillin resistant *S. aureus* (MRSA) infections are well documented and illustrate broad epidemiological trends in the general *S. aureus* population (Hiramatsu, 1995). The major route of spread is transfer from one patient to another via the contaminated hands of medical personnel. Airborne transmission is a second potential route of spread, and although airborne transmission has been described, MRSA is not commonly recovered in samples of hospital air (Sacho, 1996). Contaminated surfaces in the inanimate environment constitute a third potential reservoir from which MRSA may be transmitted, although the importance of this reservoir in clinical settings is controversial. The window for transmission via direct contact is limited by the survivability of the bacteria themselves. MRSA may survive for hours on the hands, and weeks or months in the nose, but it is unclear how long colonies may persist on contaminated surfaces creating the opportunity for indirect transmission (Sacho 1996). Some studies suggest that *S. aureus* may survive for weeks to months on inanimate surfaces, as well. General infection control procedures and antiseptic techniques may negate environmental reservoirs in hospital settings, but in non-clinical settings such as athletic fields, which are cleaned irregularly and rarely disinfected, environmental contamination may be significant.

This project clearly demonstrates that *S. aureus* can be recovered from contaminated surfaces in the community. More importantly, it can be shown at the current level of genotypic characterization that virulent strains do persist in community-associated environmental reservoirs. It was found that *S. aureus* is not ubiquitous in the athletic facilities at the Naval

Academy and the distribution patterns from the areas in which environmental cultures were obtained do not indicate a primary route of spread. In clinical settings, it has been found by quantifying the degree of nurses' contact with patients harboring MRSA that a highly significant association exists between close contact (e.g. changing wound dressings or bathing a heavily colonized patient) and the acquisition of finger and nasal carriage of MRSA by the medical personnel (Sacho 1996). If the primary route of spread in non-clinical settings was skin-to-skin contact, then similar relationships should exist between degrees of contact and bacterial colonization. However, there are no clear quantitative relationships between the individual athletic activities observed in this project and the degree or persistence of *S. aureus* contamination. The contaminated areas ranged from areas of extensive interpersonal contact such as the wrestling mats and the judo training room to areas of minimal interpersonal contact, such as the gymnastics equipment and the weight rooms. There are, however, several potentially significant environmental trends in the contaminated areas. The areas were all indoors and relatively climate-controlled. Despite substantial anecdotal evidence, no contamination was found on outdoor turf fields. No *S. aureus* strains were isolated from any of the outdoor areas sampled. This may have been due to the timing of the project. Samples were obtained during late fall and winter months when the temperature was regularly below optimum growth conditions for *S. aureus*. Outdoor sampling may benefit from more complete seasonal data. Interestingly, there was also no relationship between cleaning and contamination. Potentially virulent strains were isolated from areas, such as the wrestling mats, that are cleaned specifically to remove *S. aureus* contamination. The data suggests increased significance of environmental reservoirs in the spread of community-associated *S. aureus.*

S. aureus has a clonal population structure. Therefore, it is believed that it does not undergo extensive recombination, a process by which offspring derive a combination of genes different from that of the parent. *S. aureus* diversifies primarily by point mutations, or spontaneous changes, in individual nucleotides, and consequently demonstrates a high degree of non-random associations between genetic loci (Feil, 2003). This makes it possible to assess the relatedness of strains through measurements of genetic variation. Strain typing by genetic variation is used to characterize and respond to outbreaks of infectious disease and to describe evolutionary trends in bacterial populations.

Molecular methods have been used to differentiate strains of bacteria with varied success and efficiency. The first and most basic of these are phenotypic studies. A phenotype is defined by the observable characteristics of an organism. It is the result of the expression of an organism's genetic information in response to its environment. Therefore, the phenotype of an organism is a function of the dynamic interaction of genetic and environmental factors. As a result, organisms that are genetically similar may display different phenotypes. Conversely, organisms with the same phenotypes may have different genetic information. Consequently, it is almost impossible to use phenotypic data to model genetic variation in bacterial populations (Schmitz *et al.*, 1998).

Newer methods such as Pulsed-field Gel Electrophoresis that compare DNA fragments were adopted for their improved discriminatory ability and reproducibility. Pulsed-field Gel Electrophoresis (PFGE) is a DNA-based subtyping method that generates DNA banding patterns on an agarose gel after DNA is cut into fragments with restriction enzymes (van Belkum *et al.,* 1997). Unfortunately, these techniques are costly and difficult to use effectively. The greatest

disadvantages of PFGE and other gel methods, however, is the difficulty of comparing results between labs.

Multi-locus sequence typing (MLST) provides a highly discriminatory method that defines each strain of bacteria as a string of seven integers which produces data that can be collected in a central database on the World Wide Web. One of the greatest disadvantages of MLST is its inability to predict virulence properties and resistance patterns. It is believed that In addition, the variation within the housekeeping genes used in the MLST allelic profile accumulates relatively slowly, making it an excellent tool for studying longer-term or global pathogen epidemiology and population genetics, but poorly suited to study local epidemiology or outbreaks, which are characterized by new strain types caused by rapid accumulation of genetic variation.

There are several new typing methods that use a single locus, including the protein A encoding gene *spa* (Shopsin *et al.,* 1999; Koreen *et al.,* 2004) and the repeat region of the coagulase gene, *coa* (Shopsin *et al.,* 2000). These methods are fast, relatively inexpensive, less error prone, and they are capable of monitoring both rapid and long-term genetic variation, which makes them versatile strain typing tools (Koreen *et al.*, 2004). However, it is not well established how discriminating these methods are, and they are limited to housekeeping genes required for survival. These genes are universally present in a species, but they are often highly conserved to retain function. That is, there is little genetic variation between strains or over time.

The MLST scheme was chosen for this project for many reasons, but most importantly because it allowed comparison to the global *S. aureus* population. In addition, availability of resources and discriminatory ability made it preferable to PFGE. The most well established

method of single locus typing uses the Protein A gene, *spa*, which was not present in all of the samples used and there is no database to compare to outside strains.

Areas of further study for this project include sampling different environments or sampling one area systematically and compiling spatial statistical data. Similarly, the same areas could be sampled at different times to model the changes in local *S. aureus* population biology with respect to time. Colonization studies could be done on midshipmen and personnel that live and work in the contaminated environments, and etiological studies could be performed in the clinic to correlate the probable source of infection with empirical environmental data. With regards to strain typing, the samples could be typed using different methods and the results compared. Additional loci could also be analyzed to explain unique *in vitro* behavior of samples.

This project has laid important groundwork for further characterization of the bacterial population at the Naval Academy. The data supports the emerging significance of environmental reservoirs as a route of spread for community associated bacteria. It is critical to understand the mechanisms of colonization and infection to model the evolution and migration of bacterial populations. The unambiguous characterization of strains of clinically relevant species of bacteria helps to explain the origin and spread of antibiotic resistance, and the collection of those strain types into a common database fosters international communication and a better understanding of the global epidemiology of infection and disease.

Bibliography

Bergey, Holt, J.G., Krieg, N.R., Sneath, P.H., Bergy, D. (1994) Bergey's manual of determinative bacteriology. (9th ed.) Lippincott, Williams & Wilkins, Baltimore, MD.

Bradley S.F. *et al* (1991) Methicillin-resistant *Staphylococcus aureus*: Colonization and infection in a long-term care facility. *Annals of Internal Medicine* **115(6):** 417-422.

CDC. (1981) Community-acquired methicillin-resistant *Staphylococcus aureus* infections—Michigan. *MMWR.* **30**:185-7.

CDC. (1997) Reduced susceptibility of Staphylococcus aureus to vancomycin -- Japan, 1996. *MMWR* **46**:624-6.

Chambers, H.F. (2001) The changing epidemiology of *Staphylococcus aureus? Emerging Infectious Diseases* **7:** 178-182.

Cookson, B. D., P. Aparicio, A. Deplano, M. Streulens, R. Goering, and R. Marples. (1996) Inter-centre comparison of pulsed-field gel electrophoresis for the typing of methicillin-resistant *Staphylococcus aureus*. *J. Med. Microbiol*. **44:**179-184.

de Lencastre, H., E. P. Severina, R. B. Roberts, B. N. Kreiswirth, and A. Tomasz. (1996) Testing the efficacy of a molecular surveillance network: methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus faecium* (VREF) genotypes in six hospitals in the metropolitan New York City area. *Microb. Drug Resist.* **2:**343-351.

Enright, M. C., and B. G. Spratt**.** (1999) Multilocus sequence typing. Trends *Microbiol.* **7:**482–487.

Enright, M.C**.,** Day, N.P., Davies, C.E., Peacock, S.J. and Spratt, B.G. (2000) Multilocus sequence typing for characterization of methicillin-resistant and methicillin-susceptible clones of Staphylococcus aureus. *J. Clin. Microbiol.* **38**: 1008-1015.

Feil, E.J., Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, Peacock SJ, Smith JM, Murphy M, Spratt BG, Moore CE, Day NP. B.G. (2003) How clonal is Staphylococcus aureus? *J Bacteriol.* 185**(11)**:3307-16.

Flandrois, J.P., Fleurette, J., Eyraud, F. (1975) Detection de la proteine A de *Staphylococcus aureus* par hemagglutination conditionee. *Ann. Biol. Clin.*, **33**: 365-368.

Freundlich, L., Unified Program in Infectious Diseases, Montefiore Medical Center and Albert Einstein College of Medicine. Available online at <http://gold.aecom.yu.edu/id/micro/hemolysis.htm>

Gosbell I.B., Mercer J.L., Neville S.A., Crone S.A., Chant K.G., Jalaludin B.B., *et al.* (2001) Non-multiresistant and multiresistant methicillin-resistant *Staphylococcus aureus* in community-acquired infections. *Med J Aust* **174**:627-30.

Hiramatsu K. (1995) Molecular evolution of MRSA. *Microbiol Immunol* **39**:531-43.

Jessen O., Rosendal K., Bulow P., Faber V., Eriksen K.R. (1969) Changing staphylococci and staphylococcal infections: A ten-year study of bacteria and cases of bacteremia. *N Engl J Med* **281**:627-35.

Koreen, Larry, Srinivas V. Ramaswamy, Edward A. Graviss, Steven James M. Musser, and Barry N. Kreiswirth (2004) *spa* Typing Method for Discriminating among *Staphylococcus aureus* Isolates: Implications for Use of a Single Marker To Detect Genetic Micro- and Macrovariation. *J. Clin. Microbiol.* **42:**792-799.

Kluytmans J, Van Belkum A, Verbrugh H. (1997) Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks. *Clin Microbiol Rev* **10**:505-20.

Lally, R., Woolfrey, B. Clumping factor defective methicillin resistant *Staphylococcus aureus. Eur. J. Clin. Microbiol.,* **2**:151-152.

Levy, S.B. (1992) The Antibiotic Paradox: How Miracle Drugs are Destroying the Miracle. Plenum Press, New York.

Levy, S.B. (1998) The challenge of antibiotic resistance. *Scientific American.* **278:** 46-53.

Lowry F.D. (1998) *Staphylococcus aureus* infections. *N Engl J Med*. **339**:520-32.

Maiden, M. C. J., J. A. Bygraves, E. Feil, G. Morelli, J. E. Russell, R. Urwin, Q. Zhang, J. Zhou, K. Zurth, D. A. Caugant, I. M. Feavers, M. Achtman, and B. G. Spratt. (1998) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. USA* **95:**3140-3145.

Mandeil, G.L., Bennett, J.E., Dolin, R. (1995). Principles and practice of infectious disease. (4th ed.) Churchill Livingston, New York.

Montie T, Patamasucon P. (1995) Aminoglycosides: the complex problem of antibiotic mechanisms and clinical applications [Editorial]. *Eur J Clin Microbiol Infect Dis* **14**:85-7.

Okuma, K., K. Iwakawa, J. D. Turnidge, W. B. Grubb, J. M. Bell, F. G., O'Brien, G. W. Coombs, J. W. Pearman, F. C. Tenover, M. Kapi, C. Tiensasitorn, T. Ito, and K. Hiramatsu. (2002) Dissemination of new methicillinresistant *Staphylococcus aureus* clones in the community. *J. Clin. Microbiol.* **40:**4289–4294.

Peacock, S. J., G. D. de Silva, A. Justice, A. Cowland, C. E. Moore, C. G. Winearls, and N. P. Day. (2002) Comparison of multilocus sequence typing and pulsed-field gel electrophoresis as tools for typing *Staphylococcus aureus* isolates in a microepidemiological setting. *J. Clin. Microbiol.* **40:**3764–3770.

Ramshaw, J. A. M., J. A. Coyne, and R. C. Lewontin. (1979) The sensitivity of gel electrophoresis as a detector of genetic variation. *Genetics* **93**:1019-1037.

Sacho, Howard (1996) Methicillin Resistant *S. aureus* Infection of a CSF Shunt. *Lessons in Infectious Diseases.* **2(7):**1.

Saravolatz, L.D., Pohlod D.J., Arking L.M. (1982) Community-acquired methicillin-resistant *Staphylococcus aureus* infections: a new source for nosocomial outbreaks. *Ann Intern Med* **97**:325-9.

Schmitz, F.-J., M. Steirt, H.-V. Tichy, B. Hofmann, J. Verhoef, H.-P. Heinz, K. Köhrer, and M. E. Jones. (1998) Typing of methicillin-resistant *Staphylococcus aureus* isolates from Düsseldorf by six genotypic methods. *J. Med. Microbiol.* **47:**341-351.

Shopsin, B., Gomez, M., Montgomery, S. O., Smith, D. H., Waddington, M., Dodge, D. E., Bost, D. A., Riehman, M., Naidich, S., and Kreiswirth, B.N. (1999) Evaluation of protein A gene polymorphic region DNA sequencing for typing of *Staphylococcus aureus* strains. *J.Clin. Microbiol.* **37:** 3556-3563.

Shopsin, B., Gomez, M., Waddington, M., Riehman, M., and Kreiswirth, B.N. (2000) Use of coagulase gene (*coa*) repeat region nucleotide sequences for typing of methicillin-resistant *Staphylococcus aureus* strains. *J.Clin. Microbiol.* **38:** 3453-3456.

Spratt, B. G. (1999) Multilocus sequence typing: molecular typing of bacterial pathogens in an era of rapid DNA sequencing and the Internet. *Curr. Opin. Microbiol.* **2:**312-316

Streulens, M. J., R. Bax, A. Deplano, W. G. V. Quint, and A. van Belkum. Concordant clonal delineation of methicillin-resistant *Staphylococcus aureus* by macrorestriction analysis and polymerase chain reaction genome fingerprinting. *J. Clin. Microbiol*. **31:**1964-1970.

van Belkum, A., W. van Leewen, M. E. Kaufmann, B. Cookson, F. Forey, J. Etienne, R. Goering, F. Tenover, C. Steward, F. O'Brien, W. Grubb, P. Tassios, N. Legakis, A. Morvan, N. El Solh, R. de Ryck, M. Streulens, S. Salmenlinna, J. Vuopio-Varkila, M. Kooistra, A. Talens, W. Witte, and H. Verbrugh. (1998) Assessment of resolution and intercenter reproducibility of results of genotyping *Staphylococcus aureus* by pulsed-field gel electrophoresis of *Sma*I macrorestriction fragments: a multicenter study. *J. Clin. Microbiol.* **36:**1653-1659.

Vandenesch F, Naimi T, et al (2003) Community-Acquired Methicillin-Resistant Staphylococcus aureus Carrying Panton-Valentine Leukocidin Genes: Worldwide Emergence. *Emerg Infect Dis* **9(8)**:978-984.

Weinstein L. (1975) The pharmacologic basis of therapeutics. Macmillan, New York, NY.

Wilson, A. C., S. S. Carlson, and T. J. White. (1977) Biochemical evolution. *Annu. Rev. Biochem*. **46**:573-639.

Appendix A. Environmental Sample Locations

Table 11: Environmental Sample Locations

Sample Number	Sample Location
1	Dewey Field
2	Farragut Field
3	Ingram Track
4	Lejeune Karate Room
5	MacDonough Volleyball Courts
6	Rip Miller (Turf) Field
7	Hospital Point Intramural Fields
8	MacDonough Boxing Ring
9	Boxing Gloves/Head Gear
10	Lejeune Wrestling Mats
11	Halsey Basketball Courts
12	MacDonough Boxing Ring
13	MacDonough Weight Room
14	MacDonough Gymnastics Mats
15	Ricketts Weight Room
16	7th Wing Weight Room
17	Halsey Judo Room
18	Halsey Bubble
19	Halsey Climbing Wall
20	Halsey Indoor Track
21	Halsey Weight Room
22	MacDonough Training Pool
23	MacDonough Water Polo Pool
24	MacDonough Basketball Courts
25	MacDonough Women's Locker Room
26	MacDonough Pull-up Bars
27	MacDonough Men's Locker Room
28	MacDonough Training Room
29	7th Wing Cycling Room
30	Lejeune Training Room
31	Lejeune Men's Swimming Locker Room
32	Dahlgren Hockey Locker Room
33	Hubbard Hall Rowing Equipment
34	Hubbard Hall Men's Locker Room
35	4th Wing Squash Courts
36	4th Wing Squash Equipment
37	MacDonough Raquetball Courts
38	Lejeune Women's Locker Room
39	Halsey Women's Locker Room
40	Hubbard Hall Women's Locker Room

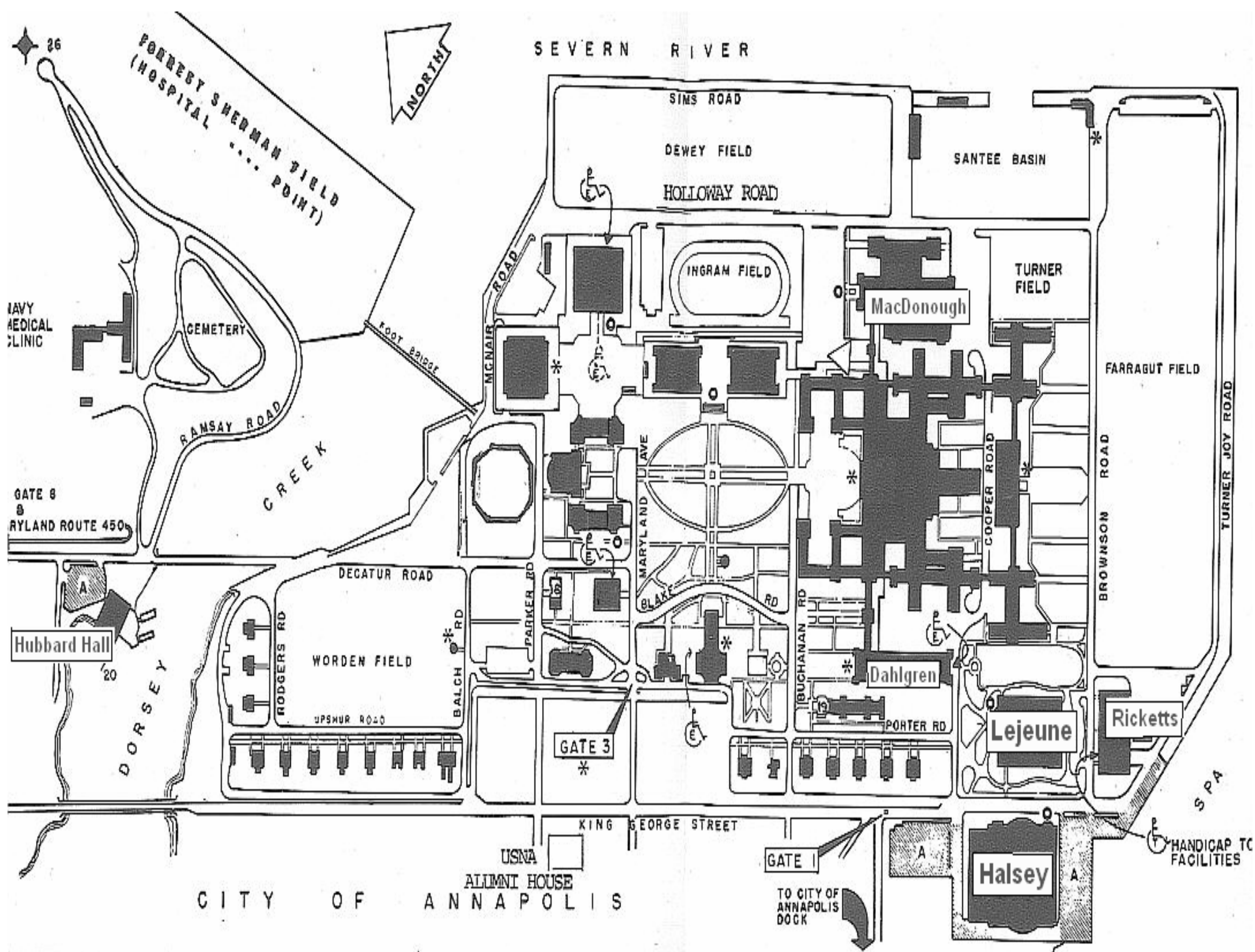


Figure 12: Map of United States Naval Academy