

THE HUMAN MICROBIOME AND SKIN AND SOFT-TISSUE INFECTIONS

by

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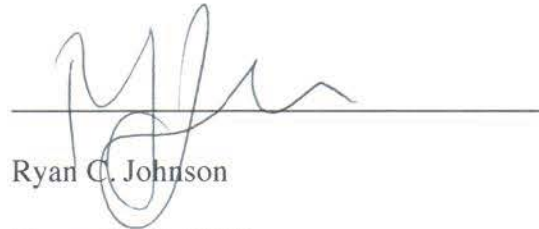
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November 3, 2015

ABSTRACT

Title of Dissertation:

The Human Microbiome and Skin and Soft-Tissue Infections

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Skin and soft tissue infections (SSTIs) are among the most prevalent and complex infections observed in both the inpatient and outpatient settings. Clinically, SSTIs can range from mild (ex. folliculitis) to severe (ex. necrotizing fasciitis) and can present as either purulent (ex. cutaneous abscess) or non-purulent (ex. cellulitis). Furthermore, SSTIs can be caused by a wide array of bacterial pathogens such as *Staphylococcus aureus* and beta-hemolytic *Streptococci* (BHS). While SSTIs are frequently reported throughout the world, certain congregate populations, such as military trainees, are at increased risk for SSTI development. Indeed, the number of SSTI-related hospital admissions exceed even those for influenza and pneumonia during the first two years of service. Although nasal colonization with *S. aureus* is an established risk factor for SSTI development, it is still unclear why some colonized individuals develop disease while others do not. Given the recent association between the human microbiome and human health, we hypothesized that fluctuations in the nasal microbiome may be associated with

SSTI development. Furthermore, given the variability in SSTI presentation, we set out to determine if specific risk factors and/or microbial profiles were unique to either cutaneous abscess or cellulitis. Using a high-throughput sequencing approach, we found that the nasal microbiomes of trainees developed SSTI had significantly less Proteobacteria compared to trainees that did not harbor a SSTI. Additionally, we found that the abundance of *S. aureus* in the nares was inversely correlated with *Corynebacterium*. With respect to bacterial etiology, we found that while most cutaneous abscesses were dominated by *S. aureus*, polymicrobial infections were frequently observed. We also characterized a unique case of cutaneous abscess caused by a strain of *S. aureus* with decreased susceptibility to chlorhexidine. While *S. aureus* was typically associated with purulent abscess, cellulitis microbiomes were mostly composed of atypical bacteria such as *Rhodanobacter*. In addition to bacterial composition, we also observed differences in risk factors associated with either cutaneous abscess or cellulitis development. Together, these data not only demonstrate a correlation between the human microbiome and SSTIs, but also highlight important microbial and epidemiological differences between purulent and non-purulent SSTIs that should be considered when treating these complex infections.

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CHAPTER ONE: Introduction

THE HUMAN MICROBIOME

Definition and Significance

The human body is a living ecosystem that contains trillions of bacteria, fungi, protozoans, and viruses. Collectively, these microbial communities make up what is known as the human microbiota. While basic culture techniques have provided a glimpse into the microbiota at specific body sites, the advent of high throughput DNA sequencing technologies has allowed researchers to characterize microbial communities with greater resolution. Indeed, the collective microbial genomes, or microbiome, at numerous anatomical locations have been described through a collaborative research consortium known as the Human Microbiome Project (HMP) (134). The HMP was established in 2008 with the primary goal to characterize the human microbiome and elucidate its role in human health and disease. Since then, the number of studies that associate disease with the human microbiome has increased dramatically (Figure 1). Fluctuations in microbiome composition have been reported for various diseases that range in severity from acne to pancreatic cancer (91; 96). As we progress through the microbiome era, the identification of microbial differences between healthy and unhealthy microbiomes will become increasingly important.

The significant contribution of the human microbiota to human health is not surprising given that the number of human-associated bacterial cells far exceeds the 37.5 trillion cells of the human body by a factor of ~ 10 (27; 266). Additionally, the human

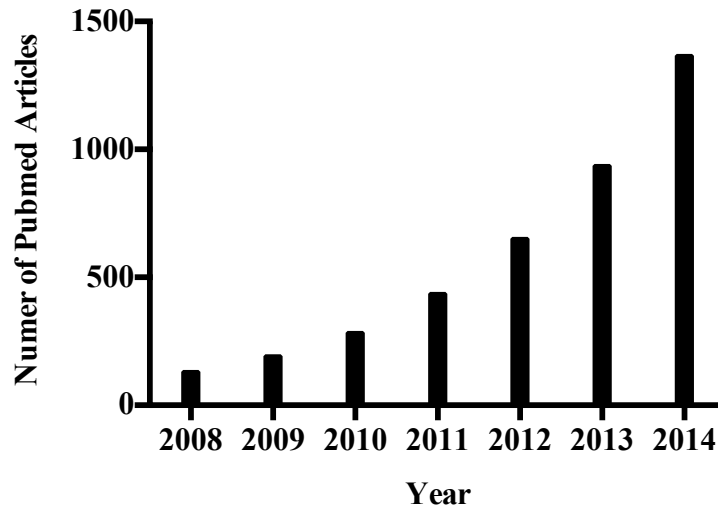


Figure 1. Microbiome and disease-related PubMed articles from 2008 to 2014. Data were downloaded from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov/pubmed>) after “microbiome disease” was entered as the search criteria.

microbiome contains hundreds of genes that have no functional homologs in the human genome (174; 245). Thus, microbes provide humans with essential biological processes that we have not acquired ourselves (116). In this regard, the human microbiota share numerous qualities similar to other organs within the body. Similar to how clinicians monitor organ function through clinical tests, scientists may potentially utilize the microbiome composition as a biomarker in the future to monitor disease progression or even assess disease susceptibilities.

Although disease presentation and subsequent microbiome dysbiosis (microbial imbalance) are important correlates, the idea of using microbiome reconstruction as a therapeutic has gained traction in the medical field as of late. The most well-documented instance of successful microbiome reconstruction to combat disease has been the implementation of fecal transplantation to replenish the gut microbiota during life-threatening *Clostridium difficile* infection (16). Since fecal transplantation is a novel and unappealing treatment option, other microbiome therapeutics such as probiotics have become increasingly popular (248). Fecal transplants and probiotics represent just two of the potentially limitless microbiome-related therapeutics. Current research is underway to determine which bacterial organism(s) can be supplemented at various body sites as a means to shift the microbiome from that of a diseased state to a healthy state.

Impact on Human Health

As early as the 1950's, it became evident that the human microbiota have a major impact on human health (293). However, the intricacies of which bacteria and other microbes influence our everyday life have only recently begun to come to light. Bacteria

are inherently given a bad reputation as agents that cause disease. However, thanks to the HMP and other studies, it is now clear that most bacterial species are nonpathogenic. In fact, some bacterial species engage in mutualistic behaviors with their human host (14). Perhaps the most well known example of bacteria-human mutualism is the intricate interaction of human metabolism with bacteria that reside within the gut. The human gut is an incredibly diverse microbial ecosystem comprised of over 1000 bacterial species (337). In return for a nutrient rich habitat provided by the human host, various gut bacteria can convert indigestible carbohydrates to digestible short-chain fatty acids (SCFAs) (253). This process is critical given that nearly 10-20% of all ingested carbohydrates are not solubilized by the intestinal tract (252). The bulk of bacteria-produced SCFAs are rapidly absorbed by the colon and serve as important energy sources for colonic-epithelial cells, promote tight-junction stability, and aid in epithelial cell repair and proliferation (253). In addition to SCFAs, the gut microbiota provide a plethora of vitamins that the human host is incapable of synthesizing *de novo*. These include vitamin K, thiamine, riboflavin, pyridoxine, pantothenic and nicotinic acids, biotin and folates (12; 167; 279). Additional studies have linked bacteria in the gut to other necessary physiological processes that include fat storage, glucose usage, and drug metabolism (13; 39; 327).

Interestingly, evidence suggests that the gut microbiota has a more global influence on human health. For example, the microbiota-gut-brain (MGB) axis is a concept that describes a bi-directional crosstalk between the central nervous system (CNS) and the gut microbiota. In support of the MGB axis hypothesis, a report in 2004 found that germfree mice exhibit an exaggerated hypothalamic-pituitary-adrenal (HPA)

stress response that is reversed when the mice are reconstituted with the gut bacterium *Bifidobacterium infantis* (285). The supplementation of gut microbiota in germfree mice has also been shown to alleviate anxiety-like behaviors (22; 215). Additionally, some gut microbiota are capable of neurotransmitter synthesis; *Bifidobacterium* and *Lactobacillus* both convert glutamate to γ -aminobutyric acid (GABA), an important inhibitory neurotransmitter required for proper CNS function (18; 128). Dysregulation of GABA has been linked to numerous neurological disorders including anxiety, epilepsy, and stiff-person syndrome (172; 181; 297). Consequently, modulation of the gut microbiota to treat neurological disorders has become an increasingly popular research field over the past decade.

There are fewer, but equally important studies and hypotheses that suggest that the CNS has the ability to alter the composition of the gut microbiome (15; 108). This is likely mediated by the direct brain-controlled secretion of signaling molecules that are detected by neurotransmitter receptors on enteric bacteria (118). Conversely, indirect CNS influence of the gut microbiota may occur through neuronal regulation of intestinal permeability, mucus secretion, and gut motility (17; 68; 249; 259). Preliminary work in our laboratory may also suggest an association between traumatic brain injury (TBI) and subsequent alteration of the gut microbiota (J. Singh, J.J. Gilbreath, M. Lagraoui, B.C. Schaefer, D.S. Merrell, unpublished results). While this facet of the MGB axis is a developing field of research, future studies will likely reveal additional CNS factors that influence the bacteria in the gut.

In addition to the MGB axis, there exists an expansive interplay between the gut microbiota and the human immune system. With respect to the innate immune response,

a healthy gut microbiota is required for proper NF- κ B suppression, antimicrobial peptide secretion, neutrophil activity, and inflammatory homeostasis of intestinal epithelial cells via TLR9 signaling (40; 51; 119; 130; 272). The adaptive immune system's interaction with bacteria in the gut is complex given that microbes in the body are inherently foreign and should invoke an immune response. Rather than simply ignoring the gut microflora, the bacteria are utilized as an immunological training ground required for proper immune system maturation. Indeed, germfree mice exhibit numerous adaptive immunological defects that include reduced B cell and CD4⁺/CD8⁺ T cell counts, insufficient Peyer's patch development, lack of Th17 cell induction, and reduced IgA secretion (122; 137; 143; 183; 240; 257). This immune system priming serves as the basis of what is known as the "microflora hypothesis." Similar to the hygiene hypothesis, the microflora hypothesis stipulates that limited microbial exposure results in suboptimal colonization of the infant gut, which in turn results in immune system deficiencies and subsequent allergy development (333). In support of the microflora hypothesis, numerous reports have associated gut dysbiosis with allergic diseases including atopic eczema, asthma, and rhinitis (2; 11; 28; 303).

Although microbial diversity in the gut is imperative for proper immune system function, studies have also linked the gut microbiota to autoimmune disorders. Inflammatory bowel disease (IBD), which includes Crohn's disease and ulcerative colitis, is an autoimmune disease characterized by chronic inflammation of the gastrointestinal tract. Interestingly, while IBD symptoms are primarily mediated by the immune system, antibiotic/probiotic therapy have proven beneficial (234; 309); a clear indication of microbial involvement. Moreover, the composition of the gut flora in IBD patients is

significantly altered compared to healthy patients (185). In addition to IBD, rheumatoid arthritis (RA) also appears to be influenced by the bacteria in the gut. In a T cell-mediated arthritis murine model (II-1 receptor negative), gut colonized mice rapidly develop arthritis while germfree mice do not (1). Reconstitution of germfree mice with the gut bacterium *Lactobacillus bifidus* restored disease progression. Other autoimmune diseases associated with the gut microbiota include diabetes, systemic lupus erythematosus, and multiple sclerosis (37; 164; 341). Given the role of gut bacteria in both immune system maturation and disease, we now appreciate the delicate balance that must exist between bacteria and the immune system in healthy individuals.

Not surprisingly, the largest organ of the body, the skin, also plays host to a diverse array of bacterial species. Unlike the gut, the role of bacteria present on the skin has not been well characterized. What we do know, however, is that the microbial composition of the skin at various body sites is extremely variable and is further complicated by environmental and host factors (115). With respect to human health, the skin commensal, *Staphylococcus epidermidis*, actively secretes anti-microbial compounds against both *Staphylococcus aureus* and Group A *Streptococcus* (54; 55; 138). Interestingly, *S. epidermidis* is also capable of immune system suppression in response to skin injury via TLR3 inhibition (160). Despite its clear role in protecting the host from pathogenic organisms, *S. epidermidis* has also emerged as an important opportunistic pathogen. In fact, indwelling medical device contamination by *S. epidermidis* represents one of the leading causes of nosocomial infections (300). Similar to *S. epidermidis*, another common skin commensal, *Propionibacterium acnes*, harbors both mutualistic and pathogenic qualities. *P. acnes* is the predominant bacterium found

within skin pilosebaceous units, or pores, and is known for its ability to produce propionic acid, which can suppress *S. aureus* growth and reduce the size of *S. aureus*-associated skin lesions in a mouse model (96; 273). On the other hand, *P. acnes* is associated with the adolescent malady acne vulgaris (175). Mechanistically, *P. acnes*' role in the pathogenesis of acne is multifactorial with both inflammatory and strain-specific components (25). Of particular interest, a recent report described a fermentation byproduct of *S. epidermidis*, succinic acid, which inhibits the growth of *P. acnes* (318). This byproduct may be a promising probiotic therapy for a condition that inflicts nearly 80% of all adolescents in the United States (35).

S. epidermidis and *P. acnes* represent two skin commensals that appear to play a role in both human health and disease. However, when the entire skin microbiota is considered, research has demonstrated that the microbial composition of the skin can change dramatically in response to human disease. In patients with atopic dermatitis (AD), a relapsing inflammatory skin disorder, disease severity is associated with a reduction in skin bacterial diversity (155). This lack of bacterial diversity is mainly driven by a stark increase in *S. aureus* abundance compared to healthy controls. Additionally, significant variations in bacterial abundance levels are also observed for *S. epidermidis*, *Streptococcus*, *Corynebacterium*, as well as *Propionibacterium*, which emphasizes the importance of comprehensive characterization of microbial communities during human disease (155).

Overall, our understanding of the human microbiome and how it influences human health has transformed the medical field. Researchers are rapidly uncovering the complex bacterial ecosystems that reside on the human body; however, the ability to

augment the microbial composition to prevent or treat specific diseases is an area of research that requires further exploration.

Defining the Human Microbiome

In 1973, Gilbert and Maxam successfully elucidated and published the sequence of the *lac* operator; the first ever published sequence of DNA (111). Four years later, the Sanger method of DNA sequencing was published and followed by the development of polymerase chain reaction (PCR) in 1983 (261; 265). In 2005, Life Sciences (a Roche company) introduced 454 high-throughput DNA sequencing technology, the first documented next-generation DNA sequencer. Since then, various other next-generation sequencing platforms have been introduced including Illumina/Solexa (2006), SOLiD (2007), Helicos (2009), Ion Torrent (2011) and PacBio Systems (2011). Together, next-generation DNA sequencing and PCR represent the two fundamental tools required for microbiome characterization. A brief overview of the steps required to analyze the microbial composition of a body site are as described below.

Whether it is a fecal sample, or a swab obtained from the axilla of patient, the first step to define a microbiome is to extract genomic DNA from the bacteria present in the sample. The genomic DNA then serves as template for PCR amplification of the 16S ribosomal RNA gene (*rrnA*). The 16S gene encodes a small subunit of the 30S prokaryotic ribosome and is not only present among all bacterial species, but is highly conserved as well. In other words, the 16S gene serves as molecular barcode. Indeed, phylogenetic mapping of the 16S gene, and the 18S eukaryotic homologue, was used to establish the 3 domains of life: Archaea, Bacteria, and Eukaryota (330). The gene itself is

approximately 1.5 kb in length and consists of 9 variable regions (V1-V9) that allow for discrimination between bacterial species. Ideally, full-length 16S gene sequences would allow for the greatest resolution between species, but given the current limitations of next-generation sequencing, researchers are limited to specific regions of the 16S gene. Fortunately, the conserved regions of the 16S gene that flank the 9 variable regions are ideal for “universal” primer design and subsequent amplification by PCR. However, because the 16S gene does not evolve evenly across its length, the region chosen for PCR amplification must be carefully considered (269). For example, it was determined that the V1 to V3 region is capable of discerning *S. aureus* from other coagulase-negative *Staphylococcus* species as well as *Streptococcus* species (42; 60). Of note, the HMP consortium also confirmed the significant resolution provided by the V1-V3 region (134).

Upon PCR amplification of the 16S gene variable region(s), the amplicons are subjected to high-throughput sequencing. Of the various sequencing platforms mentioned above, microbiome research currently most often utilizes either 454 or Illumina. 454 is advantageous given its long read length of up to 700 base pairs; however, the limited number of reads (1-2 million per sequencing reaction) may not sufficiently depict the total level of diversity represented in the sample. This is especially problematic if the sequencing reaction contains multiple samples (multiplexed). On the other hand, the Illumina platform is capable of generating over 30 million reads per run at a fraction of the price of 454. Although the read length of the Illumina platform is slightly reduced compared to 454 (~300-500 base pairs for MiSeq), the technology is rapidly advancing and will likely render 454 sequencing obsolete in the near future. Despite the advantages

and disadvantages of each platform, they each provide the researcher with millions of 16S amplicon sequences that are used to define the microbiome of the sample.

Microbiome data analysis relies primarily on the clustering of similar sequences together. As mentioned previously, because the 16S gene does not evolve uniformly along its length, a similarity threshold level must be established that will determine if two 16S reads are considered the same, even if they may not truly represent the same species. It was previously determined that species having 70% or greater DNA similarity routinely have more than 97% sequence identity between their 16S rRNA genes (278). Thus, a similarity level of 97% is frequently utilized to cluster sequences into operational taxonomic units (OTUs). Taxonomic information can be assigned for each sequence and/or OTU via alignment to a curated RNA database such as GreenGenes, Ribosomal Database Project, or SILVA (57; 191; 247). Alignment to a well-curated database is essential given that nearly five percent of all 16S rRNA sequences in GenBank may contain anomalies (9). OTU generation allows the researcher to perform various microbial diversity analyses to assess species richness and evenness. Currently, two commonly used software packages are in place that allow researchers to analyze their specific microbiome data sets: QIIME and Mothur (38; 270). Both analysis pipelines contain the molecular tools required for quality assessment, PCR chimera detection, contaminate removal, sequence clustering, taxonomic assignment, and diversity analyses of the 16S rRNA sequences.

While 16S rRNA PCR and sequencing has revolutionized the field of biology, important caveats exist that must be considered. One of the most overlooked problems in microbiome research is DNA contamination. The introduction of foreign DNA into the

PCR step that utilizes “universal” primers can result in amplification of the contaminating DNA and may dramatically influence downstream analyses. Indeed, this issue has been recognized by many groups and has been assigned the term “kitome”, or the microbiome of the kits used to process the samples (262; 321). This is especially problematic if the bacterial load of the sample is low (262). The use of negative controls is therefore strongly encouraged. Another problem with “universal” primers is that, in some unique cases, they may not bind to a particular species’ 16S gene. Furthermore, the PCR may be biased if the primers preferentially anneal to a specific bacterial species’ 16S gene (152). In either case, the region that is amplified as well as the primers used must be carefully considered. Additionally, a single bacterial species can have up to 15 copies of the 16S gene on its genome, which may inflate the actual relative abundance of an organism in the sample (147; 150). Therefore, computational manipulation to correct for 16S rRNA copy number may be required to accurately characterize a microbiome.

The Nasal Microbiome

The nasal vestibule, or anterior nares (AN), is a semi-dry environment lined with squamous epithelium that contains tactile hair (vibrissae), sweat and sebaceous glands (110). As a major external entry point to the respiratory system, the AN is exposed to upwards of a million bacterial cells for every cubic meter of atmosphere (178). While many inhaled foreign bodies are actively expelled from the respiratory tract via the “mucocillary escalator” (308), select bacterial species are retained in the AN. Indeed, the AN is a clinically relevant habitat that harbors a diverse repertoire of bacterial inhabitants, both commensal and pathogenic (101; 223; 334). For example,

approximately 20-30% of the world's population carries the Gram-positive pathogen, *Staphylococcus aureus*, in their AN (154). In addition to *S. aureus*, other bacterial species have evolved to survive within the AN including *Corynebacterium* spp, *Staphylococcus epidermidis*, and *Propionibacterium* spp (328; 334). Although host factors can largely influence the colonization of bacteria in the AN, recent data suggest that the composition of the nasal microbiota is indeed environmentally determined (180). With respect to bacterial abundance, the bulk of the AN landscape is dominated by two phyla: Firmicutes and Actinobacteria. In fact, these two phyla alone typically represent over 90% of all bacterial species present in the AN (101; 334). To a lesser extent, the Proteobacteria and Bacteroidetes phyla are also present in the AN ($\leq 10\%$ abundance) (170; 223). The contribution of these four phyla to human health is still a very active field of research.

Numerous studies have attempted to characterize the “healthy” AN, however, it has become increasingly evident that the microbial composition of the AN is incredibly variable. For example, researchers found striking differences in the nasal microbial communities between children and adults (223). Interestingly, children appear to harbor more Proteobacteria and Bacteroidetes in their AN (223). In addition to age, the composition of the nasal microbiota fluctuates over time. In a longitudinal *S. aureus* colonization study of 43 patients that were initially *S. aureus* negative in the AN, over half (55.8%) became carriers 52 weeks later (6). Similarly, another study of 12 healthy adults that were sampled 4 times over a three-week period found that half of the patients were intermittently colonized with *S. aureus* while the remaining half were persistent carriers (334). Collectively, these studies not only emphasize the variable nature of the

nasal microbiota, but also suggest that a consensus “healthy” nasal microbiome may not be definable.

The observed microbial variation in the AN has prompted researchers to investigate specific correlations that may have future clinical implications. For instance, numerous reports have shown that nasal colonization with *S. aureus* is a risk factor for subsequent *S. aureus*-related disease (79; 153; 322). As such, treatments aimed at *S. aureus* clearance would prove beneficial to human health. A recurring phenomenon observed in the nares is the apparent inverse correlation between species of the *Corynebacterium* genus and *S. aureus* (170; 334). Indeed, 71% of *S. aureus* carriers became non-carriers after artificial implantation of *Corynebacterium* in the nares (302). While the mechanism behind this bacterial interference remains unknown, it represents yet another potential microbiome-altering probiotic therapy to prevent disease. Additional interactions of *S. aureus* with other bacterial inhabitants of the nose are shown in Figure 2 (31; 125; 149; 179; 228; 255).

Unlike the gut and skin, our understanding of how the microbial composition of the AN is altered in response to human disease is limited. This is largely due to the inability to discern between a diseased state and interpersonal microbiome variation. In a recent study that investigated fluctuations of the nasal microbiota associated with chronic rhinosinusitis (CRS), while the researchers observed a reduction in bacterial diversity in patients suffering from CRS compared to healthy controls, it was determined that the observed variation between individuals trumped variations associated with disease status (29). Therefore, further work is required in order to tease out how the nasal microbiome fluctuates at an intra as well as interpersonal level.

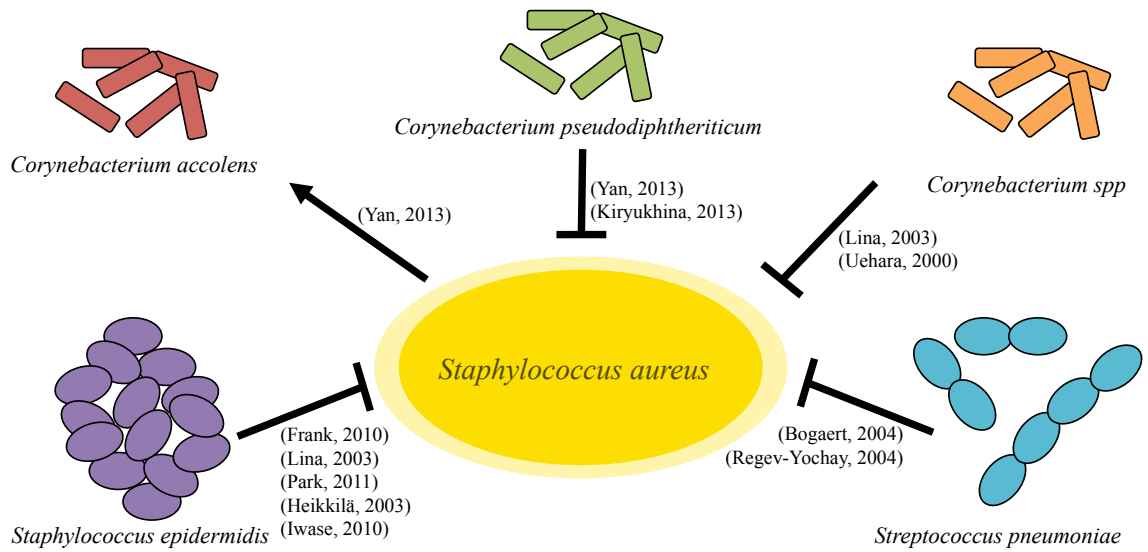


Figure 2. Interactions of *Staphylococcus aureus* with other common bacteria found within the anterior nares and nasal cavity.

Arrows indicate the promotion of growth while blunt ended lines indicate growth inhibition. Studies listed in parentheses support the interaction of the two organisms. spp = species.

SKIN AND SOFT-TISSUE INFECTIONS

Clinical Presentation and Etiology

Skin and soft-tissue infections (SSTIs) can be broadly defined as any microbial invasion of the skin or underlying soft tissue that invokes an immune response. These infections can range in clinical severity from mild and self-limiting to severe and life-threatening. As such, a classification system was implemented in 1998 by the US Food and Drug Administration that identifies SSTIs as either uncomplicated or complicated. Uncomplicated SSTIs, which include cellulitis, simple abscesses, erysipelas, and folliculitis, are superficial in nature and respond rapidly to antibiotic treatment, drainage, and debridement (281). A spectrum of uncomplicated SSTIs is depicted in Figure 3. Alternatively, complicated SSTIs, which include major abscesses, burns, infected ulcers and necrotizing fasciitis, involve deeper tissues that typically require surgical interventions (187). These severe infections are often accompanied by systemic maladies such as sepsis and neutropenia (70). SSTIs present on individuals with an underlying disease (*e.g.* immunocompromised, diabetes mellitus, obesity) that compromises treatment options may also be considered complicated (106).

The vast majority of SSTIs, both complicated and uncomplicated, are typically caused by the bacterial pathogen *S. aureus*. Indeed, the rate of *S. aureus*-associated SSTIs has risen with the emergence of methicillin-resistant *S. aureus* (MRSA) (284). In addition to beta-lactam antibiotics, *S. aureus* has evolved and/or acquired resistance to multiple classes of antibiotics and biocides such as quinolones, glycopeptides, aminoglycosides,

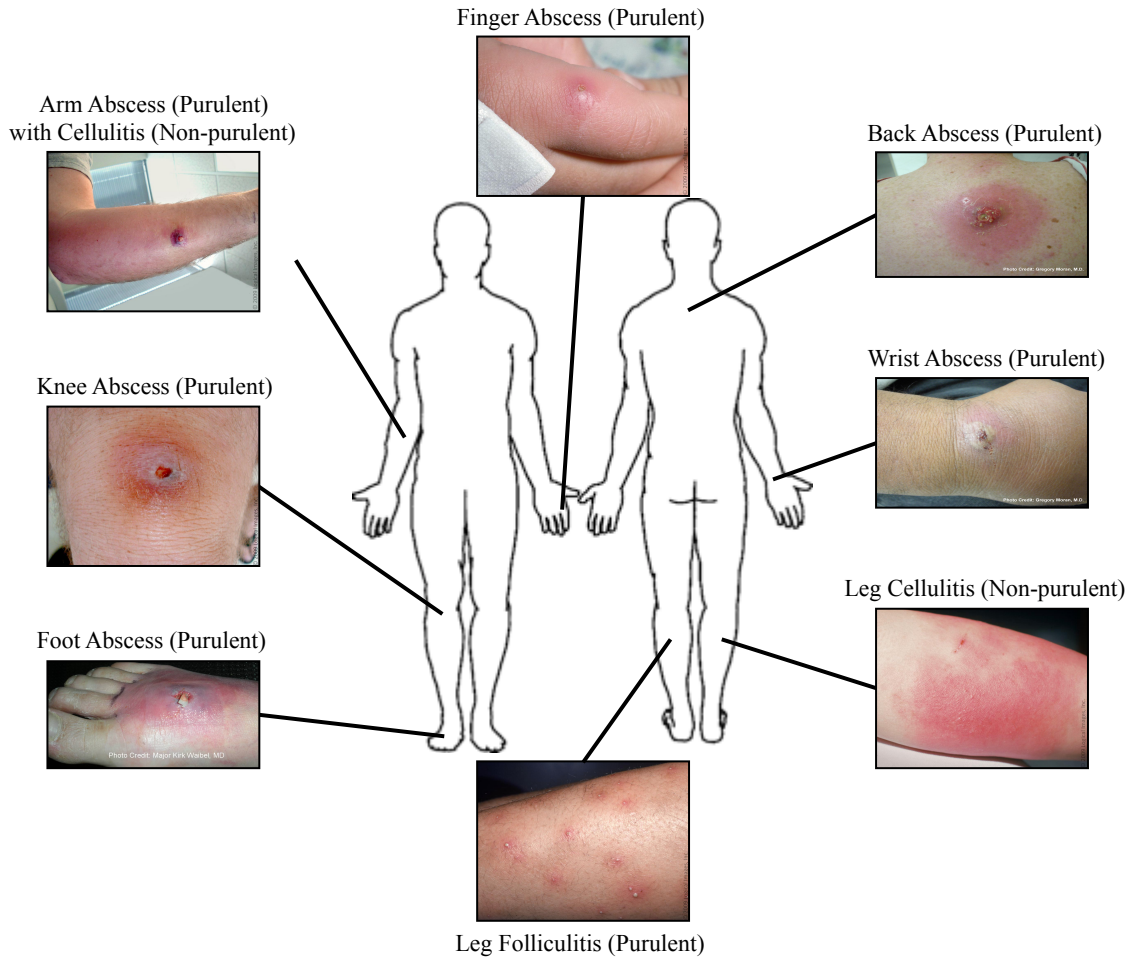


Figure 3. Clinical photographs of common skin and soft-tissue infections (SSTIs). SSTIs are noted as either purulent or non-purulent. All images are credited to Dr. Gregory Moran or Logical Images Incorporated. Body outline image courtesy of imgbuddy.com.

oxazolidinones, and quaternary ammonium compounds (30; 44; 109; 189; 256; 275; 290; 296; 298). While the vast majority of *S. aureus* resistance mechanisms have been molecularly elucidated, novel mechanisms are frequently identified. This rapid evolution of *S. aureus* continues to nullify even the newest of antimicrobial compounds and emphasizes the need for future antibiotic design.

Although *S. aureus* is associated with most SSTIs, other Gram-positive, as well as Gram-negative bacterial species, are also routinely isolated. These include beta-hemolytic streptococci (Groups A, B, C, and G), *Pseudomonas aeruginosa*, *Enterococcus* species, and *Escherichia coli* (201). Data suggest that the etiology of SSTIs is also influenced by any preexisting disease as evidenced by the increased prevalence of Group B streptococci SSTIs in diabetics and the elderly (86; 90). Furthermore, culture data can be difficult to obtain for SSTIs that are non-purulent, specifically for cellulitis infections. While serological data, syringe aspirates, and punch biopsies suggest that some cellulitis infections are caused by *S. aureus* and beta-hemolytic streptococci, in nearly 80% of all cases, no bacterium is isolated (71; 142; 171; 216; 235; 286). While culture remains the gold standard for SSTI etiology, researchers and clinicians would greatly benefit from increased sensitivity detection methods such as DNA sequencing.

Treatment

Given the recent spread of MRSA and sharp rise in SSTI cases, the Infectious Diseases Society of America (IDSA) was forced to update their guidelines regarding SSTI treatment (281). Identification and targeted treatment of the infecting pathogen is the preferred practice, however, most clinicians are forced to treat empirically given the

difficulty associated with discerning bacterial etiology. This is especially worrisome given the rise in bacterial antibiotic resistance. Therefore, as emphasized by the IDSA, further research into the rapid identification of the causes of SSTIs, particularly cellulitis, is needed (281).

Per the IDSA's recommendations, the algorithm for SSTI management requires that the SSTI be initially identified as either purulent or non-purulent (281). For all purulent SSTIs, incision and drainage followed by culture and sensitivity testing should be performed. If forced to treat empirically, the recommended antibiotics for severe purulent SSTIs include vancomycin, daptomycin, linezolid, televancin, or ceftaroline. For moderate SSTIs, empiric antibiotic treatment should include trimethoprim/sulfamethoxazole (TMP/SMX) or doxycycline. Non-purulent SSTIs, such as cellulitis and erysipelas, can be treated with penicillin, cephalosporin, dicloxacillin, or clindamycin. Severe cases of non-purulent SSTI such as necrotizing fasciitis may require surgical debridement followed by empiric vancomycin plus piperacillin/tazobactam. These treatment options should be tailored accordingly if MRSA or another antibiotic resistant pathogen is identified (281).

While the IDSA recommendations were recently updated, there are additional complicating factors that make SSTI treatment exceptionally difficult. For instance, it has been shown that SSTIs can in fact be polymicrobial in nature (230; 320). Importantly, data suggest that mixed infections that involve *S. aureus* demonstrate enhanced pathogenesis (211). Also, the responsiveness of *S. aureus* to therapeutics in a polymicrobial setting has yet to be evaluated. Secondly, our basic understanding of cellulitis is limited. While most clinicians treat cellulitis with antibiotics, the use of anti-

inflammatories has also proven efficacious and suggests an immunological component to these infections that is currently unknown (65). These common oversights need to be considered in the future for the successful treatment of SSTIs.

Epidemiology and Military Impact

The ubiquity of *S. aureus* and the susceptibility of both healthy and non-healthy individuals makes SSTIs among the most prevalent infections in both community and hospital settings. In 2005 alone, there were over 14 million SSTI related hospitalizations in the United States, and the numbers continue to rise (127; 284). It is predicted that for every 1,000 physician, outpatient, or emergency room visits in the United States, over 48 of them are due to SSTI; a substantial increase from 32 visits per 1,000 people in 1997 (127). This surge in SSTI rates, as mentioned prior, is directly associated with the emergence of MRSA, particularly community-associated MRSA (CA-MRSA) (284). Indeed, the number of *S. aureus* associated SSTIs increased by a staggering 123% from 2001 to 2009 (284). SSTIs are not only a significant epidemiological issue, but also have a profound financial impact. From 2001 to 2008, the total cost of *S. aureus*-associated SSTI hospitalizations in the United States increased by 44%, which equated to over \$4.8 billion (284). Thus, SSTIs represent a pressing public health issue that requires immediate attention.

While no individual is known to be immune to SSTI, there are established risk factors that increase one's susceptibility to future infection. Intuitively, repeated exposure and/or colonization with *S. aureus* increases the risk for SSTI development (156). This is especially true in hospitals and other health-care settings that foster an environment with

elevated *S. aureus* abundance levels. In addition to *S. aureus* exposure, individuals with underlying diseases such as diabetes and HIV are more likely to develop SSTIs than their non-diseased counterparts (64; 254). Other SSTI risk factors include obesity, prior SSTI, injection drug use, and poor hygiene (277; 288). Risk factors associated with specific SSTIs (*e.g.* abscess and cellulitis) have yet to be determined.

Given *S. aureus*' ability to survive on both biotic and abiotic surfaces, certain congregate settings where *S. aureus* can be efficiently transferred from one host to the next are routinely the site of localized SSTI outbreaks. Examples of such outbreaks include a French prison in 2010, a collegiate football team from 2002 to 2004, and a hospital nursery in 2010 (32; 258; 264). Furthermore, trainees in the military are disproportionately affected by SSTIs. Astonishingly, SSTI-related hospital admissions outnumber those for pneumonia and influenza within the first two years of military service (5). The increased prevalence of SSTIs in the military is also reflected by the numerous outbreaks reported at various military training facilities (36; 342). Indeed, it is predicted that at Fort Benning, one of the nation's largest Army training facilities, approximately 1 in 10 trainees will go on to develop an SSTI during their basic training (3). With about 20,000 new trainees per year, Fort Benning is a hotbed for SSTIs.

Although they are rarely fatal, SSTIs can cause significant morbidity. In the context of the military, this can greatly impair training participation and completion. In 2005, of the 4% of recruits at Fort Benning that were infected with CA-MRSA, 39% were placed on limited duty for approximately 5.3 days (206). Moreover, 10% of the CA-MRSA infected trainees required hospitalization. Because of the immense burden that SSTIs impose on military trainees at Fort Benning, researchers have attempted to reduce

SSTI rates via prophylactic hygiene strategies. In a recent cluster-randomized hygiene study at Fort Benning, researchers determined that chlorhexidine body wash did not reduce SSTI rates among military trainees (82). Given the potent anti-*S. aureus* effect of chlorhexidine and low prevalence of chlorhexidine resistance among *S. aureus* isolates in the U.S., the data from this clinical trial suggest that additional factors that will be key for reduction of SSTI rates have yet to be uncovered (52; 87; 190; 268).

Association of the Nasal Microbiome with SSTI

Currently, about 30% of the human population is either transiently or permanently colonized by *S. aureus* (85; 154). While this pathogen can inhabit various body sites, *S. aureus* preferentially colonizes the anterior nares (322). As mentioned previously, nasal colonization is a known risk factor for SSTI development (79; 105). It is hypothesized that the nares-colonizing strain of *S. aureus* can inoculate open cuts and wounds located at remote areas of the human body and cause SSTI. Indeed, in nearly 80% of cases, the infecting strain of *S. aureus* is identical to the individual's colonizing strain (67; 153; 236; 322). While nasal colonization with *S. aureus* increases SSTI risk, it does not guarantee subsequent infection (66). Furthermore, the anterior nares serve as a reservoir for other bacterial species that are routinely isolated from SSTIs such as coagulase-negative *Staphylococcus* and beta-hemolytic *Streptococcus* (251). Together, these data suggest a potential association of the bacteria in the nose with SSTIs. Characterization of this association could be useful for identification of individuals that are at increased risk for future SSTI development.

STAPHYLOCOCCUS AUREUS

SSTI Associated Virulence Factors

S. aureus is not only a leading cause of SSTIs, but is also routinely associated with other diseases such as osteomyelitis, bacteremia, endocarditis, and toxic shock syndrome (93; 140; 209; 224). In order to establish infection, *S. aureus* is equipped with an impressive number of virulence factors that contribute to increased pathogenesis in the host as well as aid in immune system evasion. In the context of SSTIs, specific virulence factors are essential for disease progression and will be discussed below.

Of the most well characterized virulence factors, the prophage-encoded Pantone-Valentine Leukocidin (PVL) protein is a beta-pore forming cytotoxin that has been shown to contribute to SSTI pathogenesis via keratinocyte apoptosis and leukocyte destruction (48; 112). The importance of PVL to *S. aureus* virulence with respect to SSTIs is exemplified by the low prevalence of PVL-positive strains isolated from healthy individuals (< 2%) as opposed to SSTI-associated isolates (> 30%) (19; 196; 243; 311). In addition to PVL, *S. aureus* produces other cytolytic toxins, which include delta toxin, alpha hemolysin, phenol soluble modulins (PSMs), leukocidins, and gamma hemolysin (26; 145; 207; 237). While the exact contribution of these various factors to SSTI pathogenesis is still an evolving area of research, these toxins are highly immunogenic and may be utilized as targets for vaccine development and immunotherapy (7). In fact, antagonism of alpha hemolysin with antibodies was shown to reduce SSTI severity in a mouse model (263). Although data suggest a contribution of the other cytotoxins to SSTI pathogenesis, the exact mechanisms have yet to be determined (24; 177).

In addition to cytotoxins, *S. aureus* has evolved additional mechanisms to avoid killing by the host immune system. Immune clearance of foreign cells is largely dependent on the ability to create antibodies that are specific to antigens on the surface of the cell. To overcome this defense, *S. aureus* is coated in a potent antiphagocytic protein known as capsular polysaccharide (CP) (214; 292). Similar to other bacterial capsules, CP obstructs the interaction between bound antibodies or complement factor C3b and the receptors on the phagocytic cell (222). *S. aureus* also inhibits phagocytosis via protein A (SpA) synthesis. SpA binds to antibodies, specifically IgG, and effectively inhibits the opsonization process (10; 239). Other immune modulators produced by *S. aureus* include the chemotaxis inhibitory protein of *S. aureus* (CHIPS) and extracellular adherence protein (Eap) (241; 329). Furthermore, *S. aureus* is notorious for its ability to create barricades that prevent immune cells from reaching the bacterium. For example, two clotting factors produced by *S. aureus*, coagulase and von Willebrand factor binding protein (vWbp), are necessary for kidney abscess formation in a mouse model (47). These two proteins activate host prothrombin, which catalyzes the conversion of fibrinogen to fibrin (227). This reaction results in the clotting of blood and plasma around the bacterium and provides protection from invading immune cells (47). Additionally, *S. aureus* is capable of biofilm formation, which not only protects the bacterium from phagocytosis, but also confers greater resistance to antibiotics (8; 294). Of interest, data suggest that *S. aureus* isolates from SSTIs are capable of biofilm formation, regardless of disease type (158). Whether SSTI pathogenesis is influenced by coagulase/vWbp expression or biofilm formation is still a debated topic and requires further elucidation.

Molecular Determinants of Methicillin Resistance

Of the numerous virulence factors associated with *S. aureus*, the ability to rapidly acquire resistance to antibiotics is indisputably the greatest threat to human health. Indeed, the Center for Disease Control and Prevention (CDC) lists both vancomycin resistant *S. aureus* (VRSA) and MRSA as two of the top 18 drug-resistant threats in the United States (276). Methicillin resistance, in particular, has resulted in a global pandemic of *S. aureus* infections that is ongoing to this day (195). At the molecular level, methicillin resistance is conferred by the *mecA* gene, which encodes the low-affinity penicillin-binding protein PBP2A that is carried on a mobile genetic element known as the staphylococcal chromosome cassette *mec* (SCC*mec*) (21). This cassette is variable from one strain to the next and can range in size from 21 to 60 kb in size (326). This variation has been exploited as a molecular typing technique that is used to characterize isolates of *S. aureus* (335). To date, there are 11 types of identified SCC*mec* cassettes (136). Despite the variability, all SCC*mec* cassettes have 4 unifying features: the *mecA* gene, the cassette chromosomal recombinase gene(s) (*ccr*), inverted and direct DNA repeats flanking each end, and integration into the *S. aureus* chromosome at the 3' end of a specific gene of unknown function designated *orfX* (136). Of note, the most prevalent clone of MRSA in the United States, USA300, typically carries the SCC*mec* type IV cassette (291).

Hospital-Acquired versus Community-Acquired MRSA

With increased use of methicillin in the hospital setting, MRSA was initially considered a strict nosocomial pathogen. However, in the early 1990's, isolates of MRSA

began to emerge in the community. These isolates infected healthy individuals with no prior association with health-care facilities (301). It was initially presumed that hospital-acquired MRSA (HA-MRSA) simply jumped from the health-care setting into the community. However, community-acquired MRSA (CA-MRSA) was deemed genetically distinct from HA-MRSA (301). Although it is hypothesized that CA-MRSA is the result of circulating MSSA strains that acquired the *SCCmec* cassette, why/how methicillin resistance emerged in a niche with little to no selective pressure remains a mystery.

At the genotypic level, CA-MRSA and HA-MRSA can be differentiated by two key features: *SCCmec* type and presence of genes encoding PVL. While CA-MRSA typically encodes a type IV *SCCmec* cassette, HA-MRSA strains harbor the larger type I, II, or III *SCCmec* element (94). Furthermore, PVL is highly prevalent in CA-MRSA isolates and is only rarely observed among strains of HA-MRSA (66). These genetic differences are also accompanied by epidemiological and clinical variations as well. Whereas HA-MRSA is routinely associated with multidrug resistance, CA-MRSA is susceptible to most non β -lactam antibiotics (210; 307). Moreover, while both MRSA types are capable of causing disease in both healthy and non-healthy people, CA-MRSA infections tend to present as SSTIs in younger, otherwise healthy individuals (66). Conversely, HA-MRSA infections afflict individuals with exposure to health-care facilities and typically manifest as pneumonia, bacteremia, and indwelling medical device infections (66). Although CA-MRSA has gained considerable attention due to its recent emergence and rapid spread, both MRSA types are capable of causing significant morbidity and mortality throughout the world and thus, should be monitored closely.

GOALS AND SPECIFIC AIMS

We now appreciate that colonization with *S. aureus*, particularly in the anterior nares, is a *bona fide* risk factor for SSTI development. However, it is still unclear why some carriers develop SSTI while others do not. This ambiguity is largely due to an incomplete understanding of host genetics, strain differences, immune response, and risk factors associated with SSTIs. Given the apparent link between the nasal microbiota and SSTI formation, we believe that the nose may harbor valuable clues regarding SSTI susceptibility that have yet to be discovered. To this end, the first goal of this dissertation was to correlate variations in the nasal microbiota with purulent abscess formation. This goal was divided into two aims: in Aim 1 we compared the nasal microbiomes of military trainees that either did or did not develop SSTI (described in Chapter 2). For Aim 2, we assessed the impact of *S. aureus*, both methicillin-resistant and methicillin-sensitive, on nasal and abscess microbiome composition. These studies not only highlight the complexity of the nasal and abscess microbiomes, but also indicate key SSTI-associated variations in the nasal microbiome that should prove useful for both clinicians and researchers alike.

Despite the medical and financial burden of SSTIs, remarkably little is known regarding the microbial etiology of SSTIs. While *S. aureus* is the main organism isolated from purulent SSTIs, other organisms are also frequently isolated. Furthermore, since bacteria are rarely isolated from cellulitis, their microbial composition remains largely unknown. Given the differences in clinical presentation between purulent and non-purulent SSTIs, we hypothesize that the microbial composition of each SSTI will vary.

Therefore, the second goal of this dissertation was to elucidate the microbial composition of both purulent and non-purulent (cellulitis) SSTIs. To address this goal, our first aim was to utilize high-throughput sequencing to analyze and compare the various SSTI microbiomes (described in Chapter 3). For aim 2, we characterized an SSTI caused by a clinically unique strain of *S. aureus* that was resistant to chlorhexidine (described in Chapter 4). Together, this dissertation provides insight into the nasal and SSTI microbiomes and will hopefully contribute to the treatment, as well as prevention, of SSTIs in the future.

CHAPTER TWO: Correlation Between Nasal Microbiome Composition and Remote Purulent Skin and Soft-Tissue Infections

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The work presented in this chapter is the sole work of R.C. Johnson with the following exceptions: abscess cultures were processed by Martin Army Community Hospital microbiology laboratory; study oversight at Fort Benning provided M.W. Ellis and C.D. Schlett; sample acquisition provided by J.B. Lanier; patient data collected by T. Cui.

ABSTRACT

The incidence of skin and soft-tissue infections (SSTIs) has dramatically increased over the past decade, resulting in significant morbidity in millions of otherwise healthy individuals worldwide. Certain groups, like military personnel, are at increased risk for SSTI development. Although nasal colonization with *Staphylococcus aureus* is an important risk factor for the development of SSTIs, it is not clear why some colonized individuals develop disease while others do not. Recent studies have revealed the importance of microbial diversity in human health. Therefore, we hypothesized that the nasal microbiome may provide valuable insight into SSTI development.

To examine this hypothesis, we obtained anterior nares samples from military trainees with cutaneous abscesses and from asymptomatic (Non-SSTI) participants. We also obtained samples from within abscess cavities. Specimens were analyzed by culture and the microbial community within each sample was characterized using a 16S sequencing-based approach.

We collected specimens from 46 Non-SSTI participants and from 40 participants with abscesses. We observed a significantly higher percent abundance of Proteobacteria in the anterior nares in Non-SSTI participants ($p < 0.0001$) compared to participants with abscesses. Additionally, we noted a significant inverse correlation between *Corynebacterium* spp. and *S. aureus* ($p = 0.0001$). The sensitivity of standard microbiological culture for abscesses was 71.4%. These data expand our knowledge of the complexity of the nasal and abscess microbiomes, and potentially pave the way for novel therapeutic and prophylactic countermeasures against SSTI.

INTRODUCTION

Staphylococcus aureus is a leading pathogen in both community and hospital settings. Infections with *S. aureus* range from invasive disease such as bacteremia and pneumonia to generally less severe skin and soft-tissue infections (SSTIs) (100; 202; 310). SSTIs, especially those caused by USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) have emerged as a common, burdensome, and costly disease (127; 284; 289). Individuals in congregate settings (e.g., athletic teams, prison inmates, military trainees) are at increased risk for SSTI (151; 162; 166; 342).

Given that approximately one third of people may exhibit *S. aureus* carriage, it is unclear why some develop SSTI while others do not (154). While it is clear that host genetics, immune response, and strain differences contribute (33; 75; 132; 233; 260; 305), there are likely other important factors. As the anterior nares appears to be a critical *S. aureus* reservoir (310; 322; 323) and because antecedent nasal carriage increases the risk for infection (79; 102; 310) a better understanding of nasal microbial ecology may harbor valuable clues regarding SSTI susceptibility.

The anterior nares are a dynamic microbial battleground between pathogens and commensals (101; 223). The concept that changes in the host microbiome may influence human health is well documented (50); however, few studies have investigated how the nasal microbiome is altered in response to human disease (41; 92). Importantly, no study has investigated whether the microbial composition in the anterior nares can influence susceptibility to infection. Therefore, we hypothesized that variations in the nasal microbiome might be associated with purulent SSTIs and *S. aureus* colonization. The purpose of this study was to describe the microbial composition of the anterior nares in persons with and without cutaneous abscesses, as well as the microbial composition within cutaneous abscesses.

MATERIALS AND METHODS

Study participants and study design

This observational investigation was conducted from May 2010 to January 2012 in the setting of an ongoing prospective, field-based, cluster-randomized trial aimed at

preventing SSTI (83). Study participants were all male US Army soldiers age 17-39 who were undergoing Infantry training at Fort Benning, Georgia. The investigation was approved by the Uniformed Services University Infectious Diseases Institutional Review Board.

Enrollment and data collection

We enrolled two groups of participants: trainees with abscesses and asymptomatic (Non-SSTI) controls. Trainees who presented to the Troop Medical Clinic (TMC) with cutaneous abscesses that required incision and drainage were eligible to participate as abscess subjects. Trainees who presented with non-infectious complaints (e.g. ankle sprain) were eligible to participate as Non-SSTI subjects. After written informed consent, all participants underwent anterior nares sampling with two swabs (BD BBL CultureSwabs® (BD Diagnostic, Sparks MD)). One swab was used for microbiological culture and the other for microbiome analysis. Each swab was inserted about one centimeter into one nostril, rubbed in a circular/twirling fashion at least three times along the nasal septum and the superior, lateral, and inferior surfaces of the nare. The same swab was then used to sample the other nostril following the same procedure. For cutaneous abscesses, after skin was prepared with chlorhexidine, incision and drainage was performed in a standard fashion by TMC healthcare personnel. Two swabs were obtained from within the abscess cavity. One specimen was sent for standard microbiological culture, and the other for microbiome analysis. Within five minutes of collection, microbiome specimens were frozen at -20°C at the TMC. These samples were subsequently transported on dry ice and maintained at -80°C until molecular analysis.

Microbiological and molecular analysis

Abscess cultures were processed by the Martin Army Community Hospital microbiology laboratory according to standard protocols. Anterior nares specimens were processed as previously described (268). *S. aureus* isolates underwent PFGE to assess relatedness and PCR to assess for resistance and virulence determinants as previously described (83).

DNA extraction

Total genomic DNA was extracted from the samples using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich). Briefly, the swab heads were submerged in 500 μ L of Gram-Positive Lysis Solution consisting of lysozyme (45mg/mL), mutanolysin (125U/mL) and lysostaphin (0.16mg/mL) for 30 minutes at 37°C, followed by the addition of Proteinase K (0.95mg/mL) and 500 μ L of Lysis Solution C for 10 minutes at 55°C. Column purification of genomic DNA was conducted according to the manufacturer's recommendations.

DNA amplification and sequencing

Post DNA extraction, the V1-V3 region of the 16S rRNA gene was amplified from each sample using the universal 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-ATTACCGCGGCTGCTGG-3') primers (163). Briefly, 0.15 μ L of AccuPrime High Fidelity Taq DNA polymerase (Invitrogen) was added to a mixture containing 1X

AccuPrime PCR Buffer II, 0.2 μ M of each primer, and 15 μ L of genomic DNA to a final volume of 20 μ L. All reactions were performed minimally in triplicate and combined after amplicon verification on a 1% agarose gel. The combined reactions were cleaned using the Qiagen MinElute Reaction Cleanup Kit (per manufacturer's recommendation), quantified using a Nanodrop (NanoDrop 8000, Thermo Scientific), and added in a 1:1 ratio to a single 1.5mL eppendorf vial. 126 samples were multiplexed in two separate pyrosequencing runs (63 samples per run) using the Roche GS FLX Titanium 454 sequencer at the Tufts University Genomics Core Facility. Nasal and abscess samples were randomized between the two sequencing runs to reduce sequencing bias.

The reverse PCR primer contained a 6-nucleotide 'barcode' sequence that was unique to each sample, which allowed us to multiplex multiple reactions per pyrosequencing run. PCR reaction conditions were in accordance with the 16S 454 Sequencing Protocol of the Human Microbiome Consortium (http://www.hmpdacc.org/doc/16S_Sequencing_SOP_4.2.2.pdf).

Sequence processing

Sequences were processed according to the 454 standard operating procedure (SOP; http://www.mothur.org/wiki/454_SOP) using the open-source software program, mothur (v.1.31.2)(270). Sequences greater than 200 base pairs were de-noised using the mothur adapted PyroNoise algorithm (250) and aligned using the SILVA reference alignment (244). Any sequence containing more than 1 mismatch to the barcode and/or 2 mismatches to the primer sequence were discarded. Sequences within 2 base pairs of a more abundant sequence were pre-clustered together. PCR chimeras were identified and

removed using the mothur implementation of UCHIME (73). After de-nosing, trimming, and pre-clustering of raw sequences, all reads were classified using the Ribosomal Database Project (RDP) Bayesian classifier (315) using an 80% bootstrap confidence level over 100 iterations. Contaminant sequences, including mitochondria, chloroplasts, archaea, eukaryotes, and sequences classified as “unknown” at the Kingdom level, were removed from the study. All remaining sequences were clustered into operational taxonomic units (OTUs) defined by a 97% similarity level according to the average-neighbor algorithm. To avoid the effects of varying sequencing depths, all samples were rarefied to 2,245 reads per sample. Lastly, sequences were classified to the species level using the GreenGenes 16S rRNA gene database (May 2013 release) (69).

Diversity analysis and statistics

All alpha and beta-diversity analyses were computed using mothur. The inverse Simpson diversity estimator (invsimpson) was used for the alpha-diversity analyses since it can accurately assess diversity levels without being dramatically affected by sampling effort. Phylogenetic diversity was assessed using Faith’s index (88). Statistical testing for percent abundance, invsimpson, Faith’s index and observed Operational Taxonomic Unit (OTU) data were based on the Mann-Whitney test using GraphPad Prism. Linear regression analyses were also performed using GraphPad Prism. To determine if two populations (example: *S. aureus* carriers versus non-carriers) were significantly different based on bacterial composition, we used the analysis of molecular variance (AMOVA) test for the mothur generated Yue & Clayton (Θ_{YC}) and Jaccard distance matrices (139; 338). AMOVA was also used to test phylogenetic differences between communities

using the unweighted and weighted-Unifrac distances generated from a neighbor-joining tree containing all sequences. The neighbor-joining tree was created in mothur using the clearcut command and was also used when assessing phylogenetic diversity using Faith's index. The interrelationships between populations were spatially visualized by non-metric multidimensional scaling (NMDS) using R. When determining differentially represented OTUs between the communities, we utilized the mothur adapted Metastats software (324). OTUs that differed in abundance had a p and q value below 0.05, and were required to have a mean percent abundance greater than 0.1 in at least one of the communities.

RESULTS

Baseline participant characteristics

Overall, the study population consisted of 30,209 trainees, and 137 were enrolled in the microbiome portion of the study. Of these 137 subjects, 46 Non-SSTI, and 40 abscess participant samples were sequenced. Of the 40 abscess participants, 2 had received antimicrobials approximately 18-26 days prior to sampling and were subsequently removed from the analysis. We categorized Non-SSTI participants according to anterior nares culture results (15-MRSA, 15-methicillin-susceptible *S. aureus* (MSSA), 16-No *S. aureus* (NoSA)) and abscess participants based on anterior nares (11-MRSA, 16-MSSA, 11-NoSA) as well as abscess culture results (16-MRSA, 15-MSSA, 7-NoSA). Of the 31 *S. aureus* abscess participants, 15 (48%; 7-MRSA, 8-MSSA) had nasal-abscess concordant isolates as determined by PFGE. 15 of the 16 MRSA abscess isolates were

USA300. Study participant characteristics are outlined in Table 1. Age was shown to not have an influence on the nasal microbiome composition (Table 2). Additionally, the abscess microbiomes appeared similar in structure regardless of broadly defined anatomic location (Table 2).

Sequencing results

Sequencing of 86 participants' samples (46 Non-SSTI nasal; 40 abscess nasal, and 40 abscess cavities) were conducted in two separate pyrosequencing reactions yielding a total of 3,252,212 raw sequences; 66% of these reads remained after quality processing and contaminant removal. In addition to the two patient samples removed due to recent antimicrobial treatment, one abscess sample, which contained only 987 associated reads, was excluded from the analysis. On average, each remaining sample contained 16,538 (range 2,245-44,189) reads with an average read length of 194 (range 169-219) nucleotides. To obviate bias based on sequencing depth, 2,245 reads were randomly sub-sampled for each specimen. Good's coverage values ($\geq 97\%$) as well as rarefaction curves (data not shown) suggested that this sampling size adequately represented the total observed biodiversity for each specimen. A table showing the percent abundance of each taxon for each sample is provided (Table 5 (S1)).

Phyla-level anterior nares microbiome

Analysis of the relative abundance of bacteria present within the 84 anterior nares samples revealed that the microbiome was largely composed of four phyla (Actinobacteria, Firmicutes, Proteobacteria, Bacteroidetes), and was predominantly composed of bacteria from the Actinobacteria and Firmicutes phyla (average percent abundance 56.8% and 39.7%, respectively; Figure 4A). The presence of Actinobacteria

Table 1. Study participant characteristics

| | Abscess (n=38) | Non-SSTI (n=46) |
|---|----------------|-----------------|
| Age | | |
| Median years (range) | 20 (17-33) | 20.5 (18-39) |
| Race / Ethnicity^a | | |
| White, non-Hispanic | 28 (74) | - ^b |
| Hispanic | 5 (13.2) | - |
| Black, non-Hispanic | 3 (7.9) | - |
| Other, non-Hispanic | 1 (2.6) | - |
| Nasal Colonization | | |
| MRSA | 11 (28.9) | 15 (32.6) |
| MSSA | 16 (42.1) | 15 (32.6) |
| NoSA | 11 (28.9) | 16 (34.8) |
| Abscess Colonization | | |
| MRSA | 16 (42.1) | NA |
| MSSA | 15 (39.5) | NA |
| NoSA | 7 (18.4) | NA |
| Site of Infection^c | | |
| Lower extremity | 19 (50) | NA |
| Ankle | 1 (2.6) | |
| Buttock | 5 (13.2) | |
| Foot | 1 (2.6) | |
| Knee | 7 (18.4) | |
| Thigh | 5 (13.2) | |
| Upper extremity | 11 (28.9) | NA |
| Arm | 2 (5.3) | |
| Elbow | 5 (13.2) | |
| Finger | 1 (2.6) | |
| Forearm | 3 (7.9) | |
| Thorax | 4 (10.5) | NA |
| Axilla | 3 (7.9) | |
| Back | 1 (2.6) | |
| Head and neck | 3 (7.9) | NA |
| Neck | 2 (5.3) | |
| Scalp | 1 (2.6) | |
| Hygiene Study Group^d | | |
| Standard | 8 (21) | 20 (43.5) |
| Enhanced Standard | 11 (28.9) | 20 (43.5) |
| Chlorhexidine | 19 (50) | 6 (13) |
| Known/Suspected SSTI/MRSA Infection in the Past Year^e | | |
| | 2 (5.3) | |

Unless specified, numbers in parenthesis correspond to percentage of total individuals in either Abscess or Non-SSTI groups.

Abbreviations: Abscess, individuals that developed SSTI; Non-SSTI, individuals that did not develop SSTI; MRSA, Methicillin-Resistant *Staphylococcus aureus*; MSSA, Methicillin-Sensitive *Staphylococcus aureus*; NoSA, No *Staphylococcus aureus*; NA, Not Applicable

^aRace / Ethnicity data for Non-SSTI individuals as well as one abscess individual was not obtained.

^bDashes correspond to data not available

^cSite of Infection data for one individual was not obtained

^dMilitary trainees were enrolled in an ongoing hygiene trial aimed at preventing SSTI prior to this study.

^eData for 2 individuals was not obtained.

Table 2. Microbiome composition differences

| Community-1 | Community-2 | Jaccard p-value^a | Φ_{vc} p-value^a |
|---|---|------------------------------------|---|
| Nasal microbiomes- <20 years old | Nasal microbiomes- Ages 20-29 | 0.180 | 0.490 |
| Nasal microbiomes- Ages 20-29 | Nasal microbiomes- >29 years old | 0.612 | 0.731 |
| Nasal microbiomes- <20 years old | Nasal microbiomes- >29 years old | 0.276 | 0.675 |
| Abscess microbiomes- Upper extremities | Abscess microbiomes- Lower extremities | 0.378 | 0.257 |
| Abscess microbiomes- Upper extremities | Abscess microbiomes- Thorax | 0.486 | 0.515 |
| Abscess microbiomes- Upper extremities | Abscess microbiomes- Head/Neck | 0.536 | 0.725 |
| Abscess microbiomes- Lower extremities | Abscess microbiomes- Thorax | 0.305 | 0.379 |
| Abscess microbiomes- Lower extremities | Abscess microbiomes- Head/Neck | 0.519 | 0.512 |
| Abscess microbiomes- Head/Neck | Abscess microbiomes- Thorax | 0.435 | 0.063 |

^aP-values are the result of performing analysis of molecular variance (AMOVA) on the Jaccard and Φ_{vc} distance matrices.

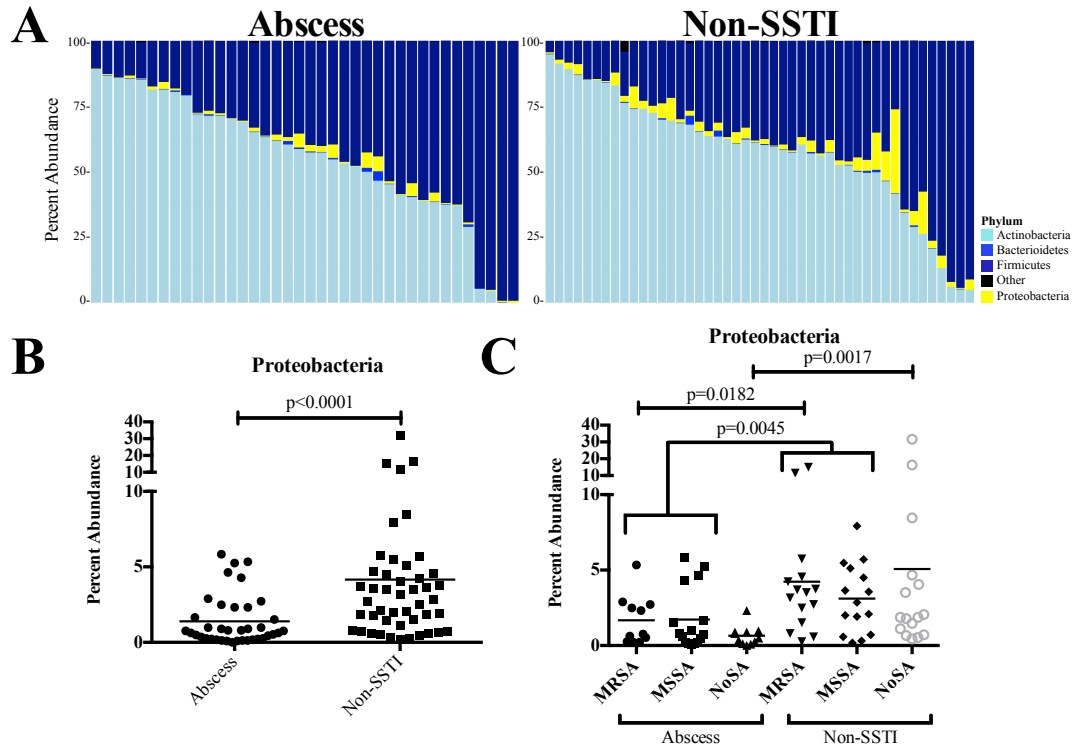


Figure 4. Characterization of the nasal microbiomes for those that did (Abscess) and did not develop SSTI (Non-SSTI).

A, Breakdown of each nasal specimen by phyla. Each column represents one swab. **B**, Percent abundance of Proteobacteria in the nose for the Abscess and Non-SSTI groups. **C**, Proteobacteria abundance broken down by nasal culture results (MRSA, MSSA, and NoSA) for the Abscess and Non-SSTI groups. Horizontal lines represent the average percent abundance. Significant comparisons were calculated using the Mann-Whitney statistical test.

and Firmicutes was inversely correlated in the anterior nares (Figure 5A). There was a significantly higher percent abundance of Proteobacteria in the anterior nares of the Non-SSTI group compared to the abscess participants (average percent abundance 1.4% vs 4.1%; $p < 0.0001$; Figure 4B). This increase in Proteobacteria was not attributed to any single bacterium, but was due to an overall increase in multiple genera including *Neisseria* and *Haemophilus* (Table 3). When analyzed based on *S. aureus* nasal carriage status (MRSA, MSSA, or NoSA), we found that the Non-SSTI participants that did not carry *S. aureus* (NoSA) showed a significantly higher abundance of Proteobacteria compared to the NoSA abscess participants ($p = 0.0017$; Figure 4C). The NoSA abscess participants showed exceptionally low levels of nasal Proteobacteria. A significant difference was not observed when the MSSA groups were compared between the Non-SSTI and abscess groups ($p > 0.05$); however, MRSA carriers showed a significantly higher percent abundance of Proteobacteria in the Non-SSTI group ($p = 0.0182$; Figure 4C). Additionally, *S. aureus* carriers (MRSA + MSSA) had a significantly greater percent abundance of Proteobacteria in the Non-SSTI group ($p = 0.0045$; Figure 4C).

Genus and species-level anterior nares microbiome

Visualization of the predominant phyla, genera, and species across nasal samples suggested significant differences between the abscess and Non-SSTI samples (Figure 6). Indeed, we observed a significantly higher abundance of *Propionibacteria* ($p = 0.0292$) and *S. epidermidis* ($p = 0.0221$) in the Non-SSTI NoSA group as compared to the NoSA abscess group (Figure 7A, B). Additionally, among the abscess participants, we found that the NoSA group contained a significantly higher percentage of *Corynebacterium* as

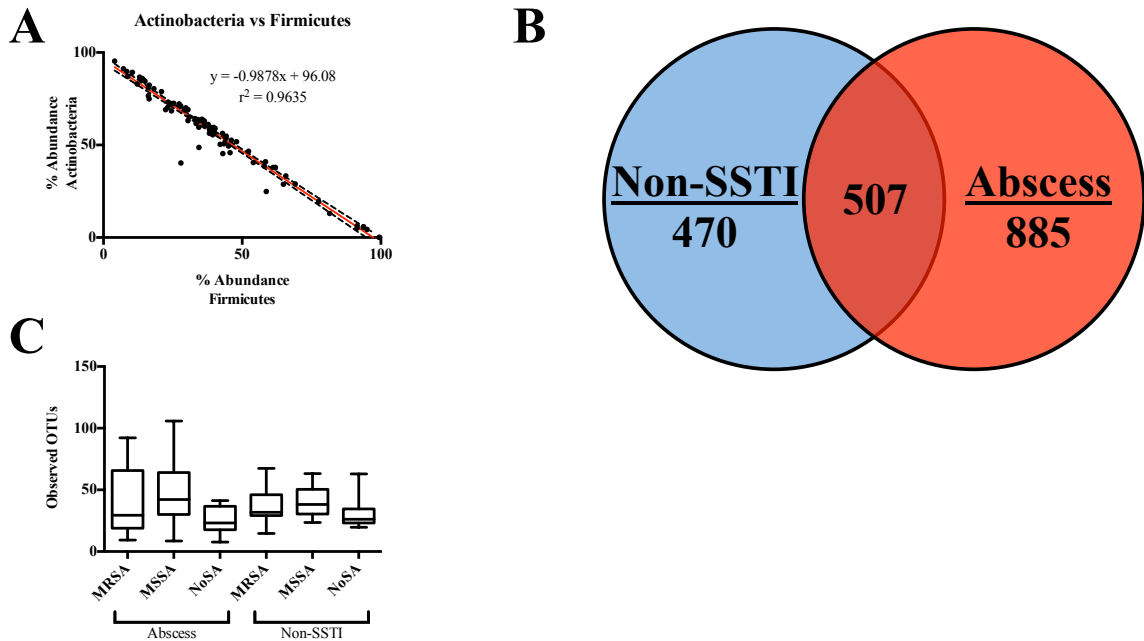


Figure 5. Characterization of the nasal microbiomes.

A, Inverse correlation between Actinobacteria and Firmicutes for all nasal samples (slope (m) = -0.9878). Red line and inset equation represent the best fit line ($y = \text{slope}(x) + y\text{-intercept}$). Dashed lines show the 95% confidence interval. r^2 = Coefficient of determination. **B**, Venn-diagram representing the number of Operational Taxonomic Units (OTUs) that were unique as well as shared between the Non-SSTI and abscess nasal groups. **C**, The number of OTUs for individuals colonized (MRSA and MSSA) or not colonized (NoSA) with *S. aureus* that either did (Abscess) or did not develop SSTI (Non-SSTI). Each box represents the interquartile range with the mean shown within. The whiskers spread from the minimum to maximum values.

Table 3. Abundance of Proteobacteria in the nares

| Genus^a | Average Percent Abundance (% ± SD) | |
|--------------------------|---|-----------------------------|
| | Abscess^b | Non-SSTI^c |
| <i>Dyella</i> | 0.41 ± 0.88 | 1.08 ± 2.63 |
| <i>Rhodanobacter</i> | 0.28 ± 0.49 | 0.99 ± 1.04 |
| <i>Haemophilus</i> | 0.01 ± 0.05 | 0.46 ± 2.09 |
| <i>Vitreoscilla</i> | 0.16 ± 0.43 | 0.79 ± 2.42 |
| <i>Neisseria</i> | 0.06 ± 0.15 | 0.24 ± 0.54 |
| <i>Moraxella</i> | 0.02 ± 0.11 | 0.15 ± 0.36 |
| <i>Burkholderia</i> | 0.03 ± 0.05 | 0.10 ± 0.14 |

SD = Standard Deviation.

^aAs determined by GreenGenes database.

^bIndividuals that developed skin and soft-tissue infections.

^cIndividuals that did not develop skin and soft-tissue infections.

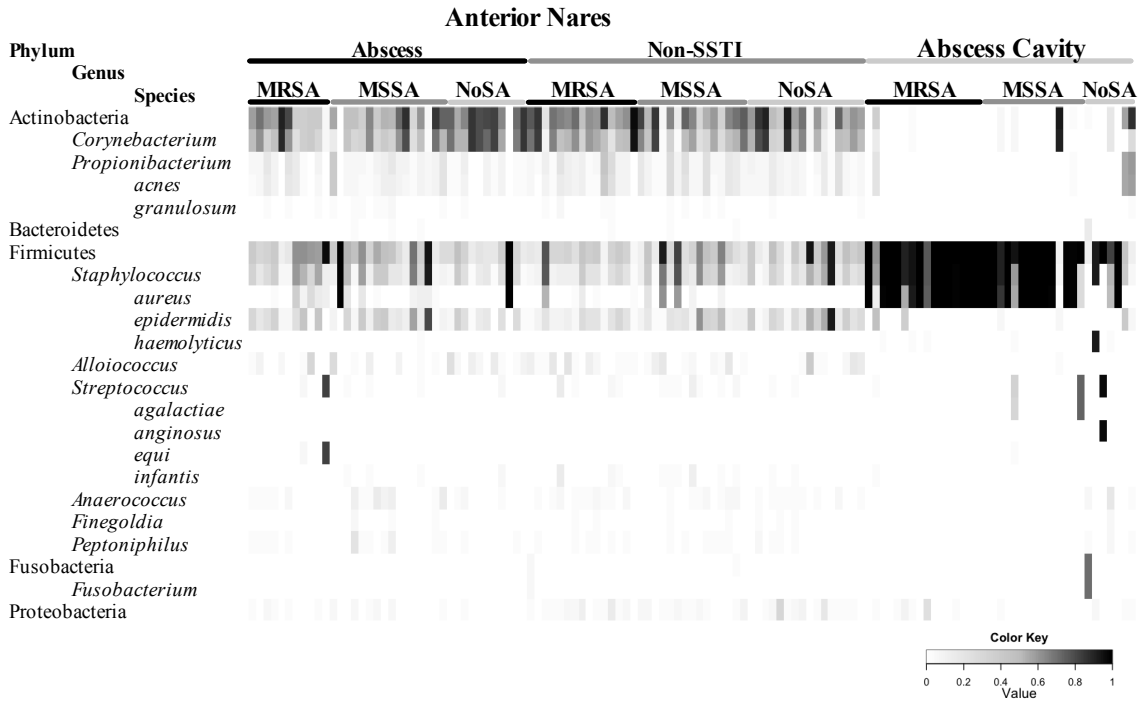


Figure 6. Heatmap showing the percent abundance for the predominant phyla, genera and species for all nasal and abscess cavity samples. The anterior nares specimens are separated by those that did (Abscess) and did not develop SSTI (Non-SSTI). Additional sub-groupings are based on nasal culture results (MRSA, MSSA, or no *S. aureus* (NoSA)). The darker the shading, the more abundant the taxa. Each column represents a single sample.

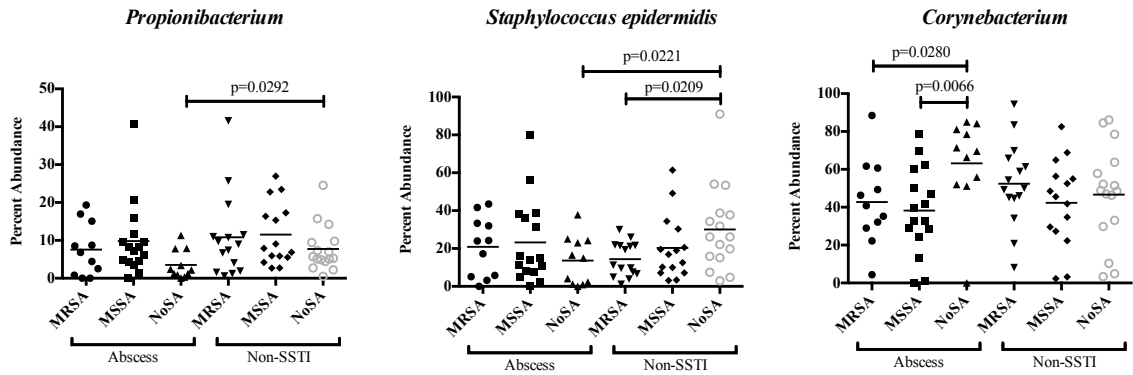


Figure 7. Percent abundance in the anterior nares for *Propionibacterium*, *Staphylococcus epidermidis*, and *Corynebacterium* (A, B, and C, respectively). Samples are categorized based on SSTI status (Abscess or Non-SSTI) as well as *S. aureus* nasal culture results (MRSA, MSSA, NoSA). Horizontal lines represent the average percent abundance. Significant comparisons were calculated using the Mann-Whitney statistical test.

compared to the MRSA and MSSA groups (Figure 7C). However, this correlation was not observed for the Non-SSTI groups.

Nasal microbiome composition analysis

We next investigated the overall variation in bacterial composition between the Non-SSTI and abscess participants' nasal microbiomes. Among the Non-SSTI and abscess participants, we observed a total of 977 and 1392 OTUs, respectively. Only 507 OTUs were shared across the groups (Figure 5B). In keeping with this, we observed a significant difference in the Jaccard distance matrix (AMOVA, $p < 0.001$), which suggests a difference in OTU membership between the Non-SSTI and abscess participants' nasal microbiomes. This OTU membership difference was present between the Non-SSTI and abscess MRSA groups (Jaccard AMOVA, $p = 0.023$; Θ_{YC} AMOVA, $p = 0.152$), MSSA groups (Jaccard AMOVA, $p = 0.01$; Θ_{YC} AMOVA, $p = 0.928$), and NoSA groups (Jaccard AMOVA, $p = 0.014$; Θ_{YC} AMOVA, $p = 0.225$), suggesting that there is a bacterial membership difference between the abscess and Non-SSTI groups' nasal microbiomes, regardless of *S. aureus* colonization status. Metastats analysis of the differentially represented OTUs between the abscess and Non-SSTI nasal microbiomes showed that many were very low in abundance. However, a substantial proportion of them were from the Proteobacteria phylum (Table 4). Phylogenetic analysis using the unweighted-UniFrac distances supported the bacterial membership difference between the abscess and Non-SSTI nasal microbiomes (unweighted-Unifrac AMOVA, $p < 0.001$). However, when the relative abundance of OTUs was considered, we did not detect differences

Table 4. Differential abundance of OTUs using Metastats

| OTU ^a | Taxonomy ^b | Community-1 (Mean Percent Abundance) | Community-2 (Mean Percent Abundance) | p-value | q-value |
|------------------|----------------------------|---|---|---------|---------|
| 0001 | <i>Staphylococcus</i> (g) | <i>S. aureus</i> positive nares (34.78) | <i>S. aureus</i> negative nares (20.63) | 0.004 | 0.018 |
| 0003 | <i>Corynebacterium</i> (g) | <i>S. aureus</i> positive nares (2.19) | <i>S. aureus</i> negative nares (9.15) | 0.002 | 0.009 |
| 0014 | Proteobacteria (p) | Abscess nares (0.29) | Non-SSTI nares (0.99) | 0.002 | 0.005 |
| 0022 | Proteobacteria (p) | Abscess nares (0.005) | Non-SSTI nares (0.45) | 0.021 | 0.041 |
| 0029 | Proteobacteria (p) | Abscess nares (0.02) | Non-SSTI nares (0.17) | 0.005 | 0.011 |

^aOperational Taxonomic Unit (OTU) designation as assigned by mothur

^b(g), Genus; (p), Phylum

between the abscess and Non-SSTI nasal communities (Θ_{yc} AMOVA, $p=0.825$; weighted-Unifrac, $p=0.603$).

Although we did not detect any differences in bacterial diversity between the abscess and Non-SSTI nasal microbiomes using the inverse Simpson calculator (data not shown), at the phylogenetic level, we found that the abscess group was significantly more diverse than the Non-SSTI nasal communities (Figure 8A). However, we did not detect any significant differences when the MRSA, MSSA and NoSA groups were compared individually between the abscess and Non-SSTI groups (Figure 8B).

***S. aureus* impact on anterior nares bacterial composition**

Given that our data suggested that *S. aureus* colonization affects the nasal architecture (Figures 6, 7), we next assessed the overall impact of *S. aureus* carriage on the nasal microbiome. As determined by the Jaccard and Θ_{yc} distances, we did not observe significant differences in anterior nares microbiome structure between MRSA and MSSA carriers among the abscess and Non-SSTI participants (data not shown). Therefore, we grouped all participants together (abscess and Non-SSTI) and subsequently reorganized them into *S. aureus*-positive and *S. aureus*-negative based on the sequencing results ($> 0\%$ abundance = *S. aureus* positive). Upon visual observation of the most abundant phyla, genera, and species, there appeared to be a higher abundance of bacteria from the Actinobacteria phylum, particularly from the genus *Corynebacterium* present in the anterior nares of *S. aureus*-negative participants (Figure 9A, B). This inverse correlation was confirmed by regression analysis (coefficient of determination (r^2) = 0.2859, slope (m) = -0.6791, $p < 0.0001$; Figure 9C). Of note, the inverse correlation was also present between *S. epidermidis* and *Corynebacterium* ($r^2 = 0.2373$, $m = -0.6524$, p

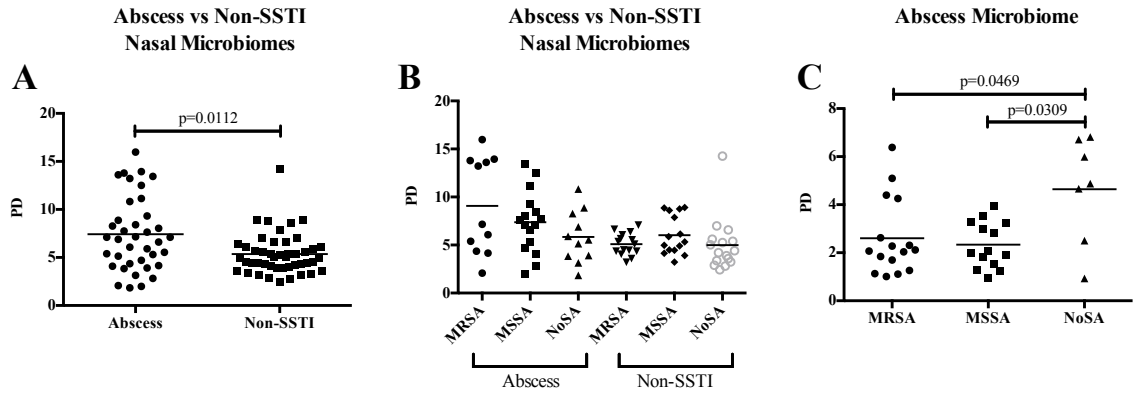


Figure 8. Phylogenetic diversity (PD) as determined using Faith's index between the **A**, abscess and Non-SSTI nasal microbiomes and **B**, various nasal microbiome subgroupings as determined by *S. aureus* colonization status (MRSA, MSSA, NoSA). **C**, PD was also computed for the abscess cavity microbiomes. The various subgrouping are based on infecting *S. aureus* strain (MRSA, MSSA, NoSA).

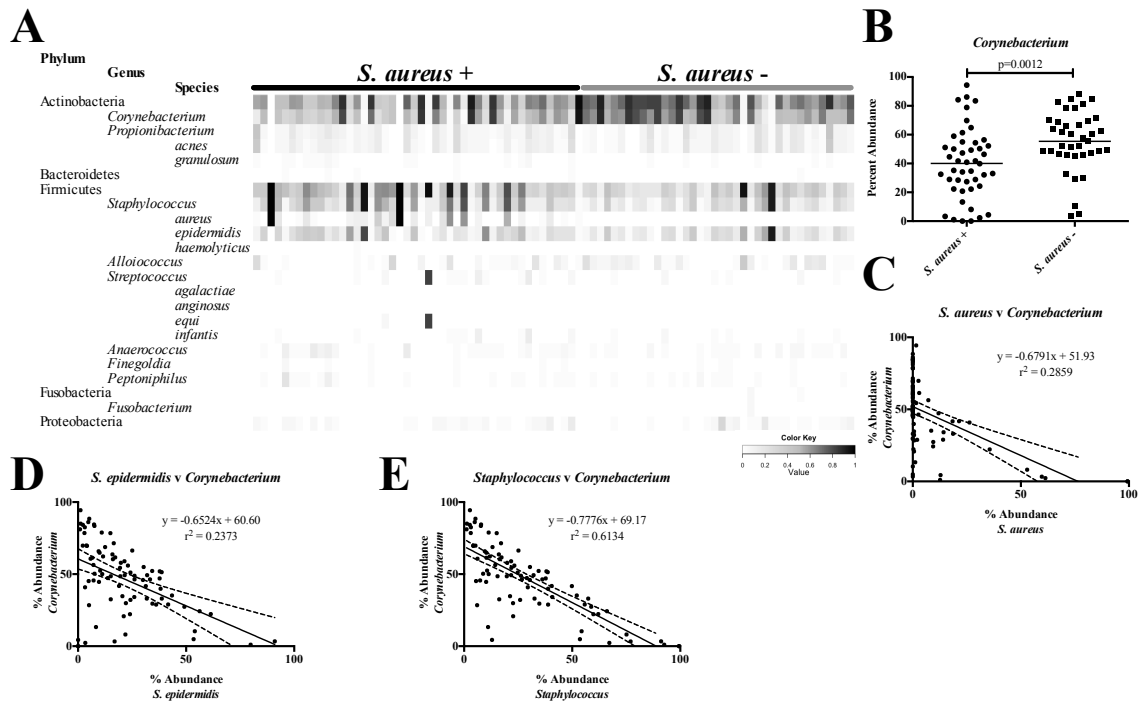


Figure 9. Impact of *Staphylococcus* on the nasal microbiome.

A, Heatmap showing the percent abundance for the predominant phyla, genera and species for nasal samples separated by *S. aureus* colonization status (based on sequencing results). The darker the shading, the more abundant the taxa. Each column represents a single sample. **B**, Percent abundance of *Corynebacterium* in the nose for individuals that were sequence positive or negative for *S. aureus*. Horizontal lines represent average percent abundance. **C**, Inverse correlation between *Corynebacterium* and *S. aureus*, **D**, *S. epidermidis*, **E**, and *Staphylococcus*. Solid lines and inset equations represent the best fit line ($y = \text{slope}(x) + y\text{-intercept}$). Dashed lines represent the 95% confidence interval. $r^2 = \text{Coefficient of determination}$.

<0.0001), but was strongest when all species of *Staphylococcus* were considered ($r^2 = 0.6134$, $m = -0.7776$, $p < 0.0001$; Figure 9D, E). Thus, *Staphylococcus* and *Corynebacterium* appear to compete for nasal landscape.

When we analyzed the overall bacterial composition between *S. aureus*-negative and positive nasal microbiomes using the Θ_{YC} and weighted-Unifrac distance matrices, we observed a significant difference in OTU abundance levels between *S. aureus*-positive and *S. aureus*-negative groups (Θ_{YC} AMOVA, $p = 0.003$; weighted-Unifrac AMOVA, $p < 0.001$). This difference could be visualized by non-metric multidimensional scaling (NMDS; Figure 10), and was shown by Metastats analysis to be largely driven by differential representation of Staphylococcal and Corynebacterial OTUs (Table 4). These data support our finding that *Staphylococcus* and *Corynebacterium* presence are inversely correlated (Figure 9) and suggests that these two genera alone can greatly impact the overall diversity of the anterior nares.

Abscess microbiome

We next characterized the 37 abscess swabs obtained from abscess participants. As shown in Figure 11A, we found that the majority of bacteria present within the abscess belonged to the Firmicutes phylum (87.6% average abundance). Of note, many of the abscesses were polymicrobial; 10 of the 37 abscesses had no single bacterial species reach more than 90% abundance (Figures 6 and 11A). At the species level, *S. aureus* dominated the majority of abscesses (73.7% average abundance). Other common bacteria found within the abscesses included: *Staphylococcus haemolyticus*, *Streptococcus agalactiae*, *Streptococcus anginosus*, *Propionibacterium acnes*, and *Corynebacterium*. We observed one sample highly rich in *Fusobacterium* (Figure 6). For the seven NoSA

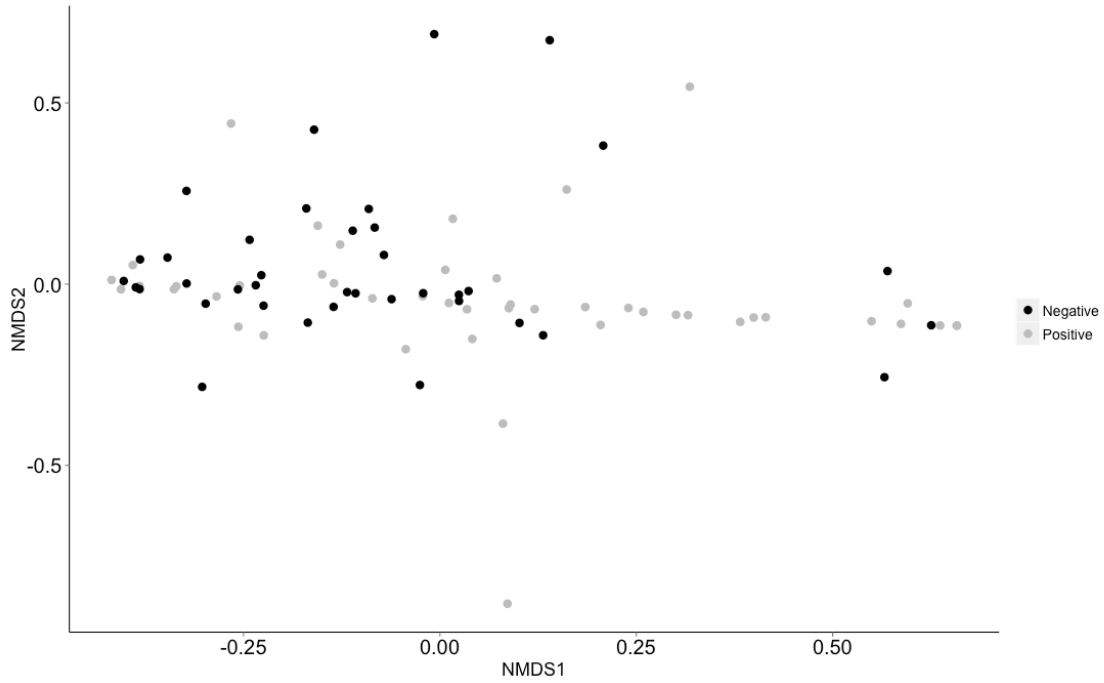


Figure 10. Non-metric multidimensional scaling (NMDS) plot showing the spatial variation between *S. aureus* positive and negative nasal communities using the Θ_{YC} distances, indicating that these communities are compositionally distinct. The spatial separation between the positive and negative groups is statistically significant (AMOVA, $p < 0.001$).

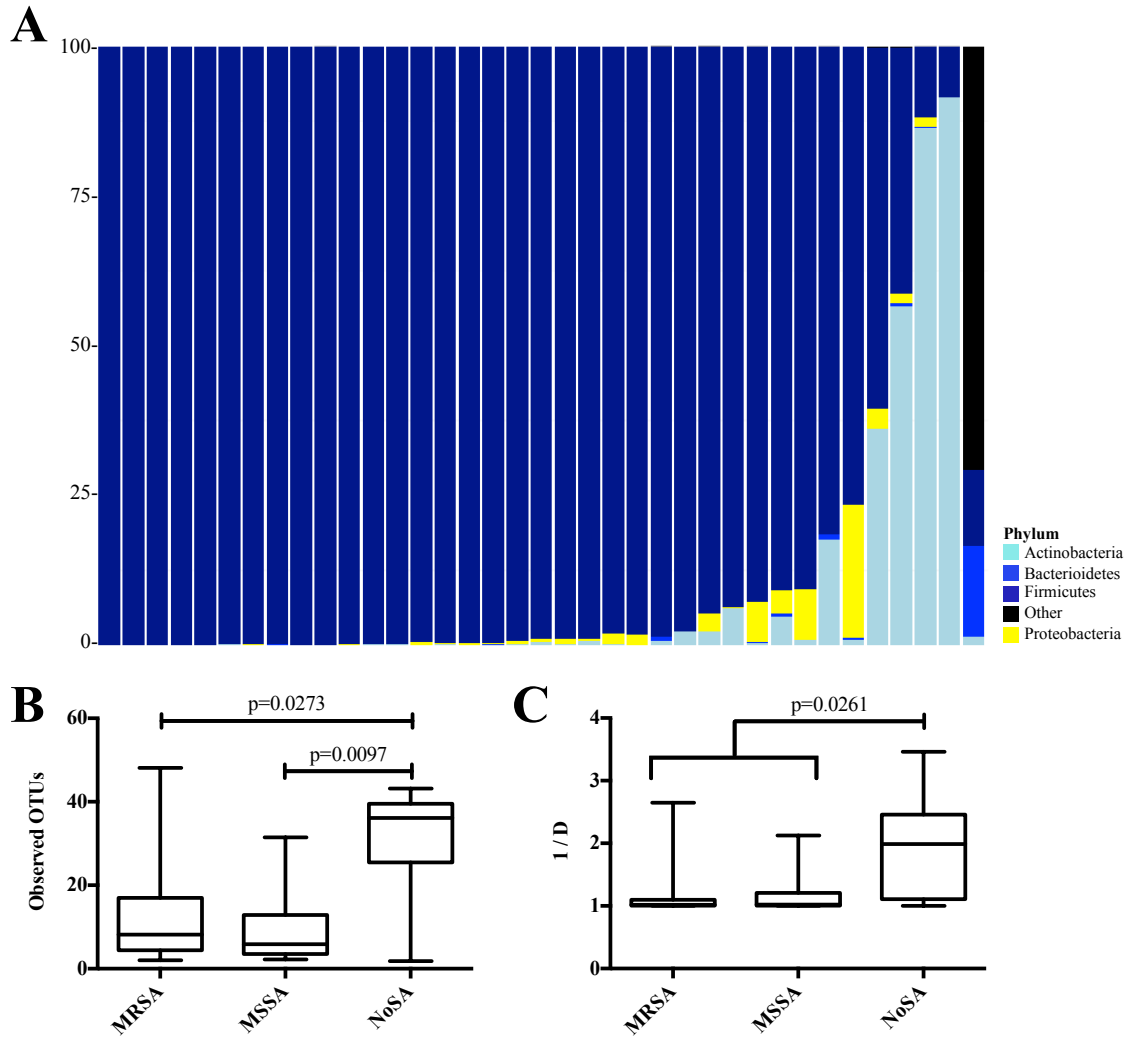


Figure 11. Characterization of the abscess cavity microbiomes.

A, Breakdown of each abscess cavity specimen by phyla. Each column represents one abscess swab. **B**, The number of observed OTUs for each abscess is shown as well as the **C**, inverse simpson index (1/D). Each box represents the interquartile range with the mean shown within. The whiskers spread from the minimum to maximum values. Significant comparisons were calculated using the Mann-Whitney statistical test.

abscesses, we collected basic microbiological information for the organisms that were cultured from the abscess cavity. These included coagulase-negative *Staphylococcus*, *Escherichia coli*, Group A and G *Streptococcus*, and *Enterobacter*. Although it is difficult to draw any correlations between nasal and abscess colonization, we note that for the individual in which Group A *Streptococcus* was cultured from the abscess, we observed an exceptionally high level of *Streptococcus* in the nares (>80 percent abundance).

Our study design allowed us to also directly compare the microbiomes of MRSA, MSSA, and no *S. aureus* (NoSA) abscesses. This analysis revealed that NoSA abscesses showed a significantly greater number of observed OTUs compared to the MRSA ($p=0.0273$) and MSSA abscesses ($p=0.0097$; Figure 11B). In addition, the NoSA group was significantly more diverse than the *S. aureus* positive abscesses (MRSA + MSSA; $p=0.0261$; Figure 11C). Using Faith's index, we also found that at the phylogenetic level, the NoSA groups were more diverse than the MRSA ($p=0.0469$) and MSSA ($p=0.0309$) abscesses (Figure 8C). Comparison of the Θ_{YC} and weighted-Unifrac distances among groups revealed that the NoSA abscesses were significantly different from the MRSA (Θ_{YC} AMOVA, $p<0.001$; weighted-Unifrac AMOVA, $p=0.002$) and MSSA (Θ_{YC} AMOVA, $p=0.004$; weighted-Unifrac AMOVA, $p=0.035$) abscesses. This suggests that bacteria found among the *S. aureus*-positive and negative abscess communities were different in abundance levels. There were no OTU abundance differences between the MRSA and MSSA abscesses. Membership-based Jaccard and unweighted-Unifrac distance analysis revealed that the MRSA and MSSA abscesses were significantly different from that of the NoSA abscesses in regards to which OTUs were present

(Jaccard AMOVA, $p=0.005$ and $p=0.021$, respectively; unweighted-Unifrac AMOVA, $p=0.002$ and $p=0.001$, respectively).

Sensitivity of culture for *S. aureus* detection

For individuals that were considered *S. aureus*-negative (NoSA) by anterior nares culture, we observed a high level of concordance with sequencing data. Only one out of the 27 culture-negative anterior nares specimens demonstrated a *S. aureus* abundance level greater than 0.1% in the sequencing results. On the other hand, abscess culture appeared significantly less reliable; 2 of the 7 culture negative abscesses were sequence positive and showed a relatively high abundance level of *S. aureus* (>49% abundance). Thus, this culture sensitivity of 71.4% for abscess samples may indicate that standard microbiology culture alone has limitations for determining abscess etiology.

DISCUSSION

In this investigation involving a large community-based population at high risk for SSTI, we made several significant observations that help describe a healthy nasal microbial composition and may help inform future prevention strategies. We observed a greater abundance of Proteobacteria in the anterior nares of the Non-SSTI participants compared to those who had developed cutaneous abscesses. Additionally, with regard to anterior nares carriage, we noted an inverse correlation between *Corynebacterium* spp and *S. aureus*.

S. aureus nasal colonization is a risk factor for the development of SSTIs (67; 322; 323); however, as demonstrated in our investigation, it is not always associated with

SSTI. Therefore, we set out to classify the nasal microbiomes of people who did or did not develop cutaneous abscess in order to identify a “marker” microbiome associated with abscess development. In agreement with other nasal microbiome studies that have primarily looked at healthy individuals (101; 170; 223; 334), we found that Actinobacteria and Firmicutes dominated the nares of all patients (Figure 4A). In particular, *Corynebacterium* and *Staphylococcus* were the most abundant genera present (Figure 5). Intriguingly, we found that participants without abscesses carried a higher abundance of Proteobacteria in their anterior nares (Figure 4B). This finding suggests that a loss of Proteobacteria in the nares may either be associated with development of SSTI or may be a “marker” of the SSTI state. While the most significant reduction of Proteobacteria was seen in NoSA abscess individuals, lower abundance was also seen in *S. aureus* colonized abscess participants (Figure 4C). Thus, even in the presence of *S. aureus* carriage, low levels of Proteobacteria appear to correlate with SSTI. In a recent study by Lemon, *et al.*, an inverse correlation between Firmicutes and Proteobacteria abundance was observed in the oropharynx (170). Their finding suggests that Proteobacteria may inhibit Firmicutes colonization in that niche. However, when we compared abundance of Proteobacteria to Firmicutes in our patients, we did not find any significant correlations (data not shown). Thus, SSTI incidence is not solely due to increased abundance of *S. aureus* (Figure 4C). For those individuals who are not *S. aureus* carriers, it is intriguing to speculate that Proteobacteria occupy the nasal niche in such a way as to prevent colonization by other pathogenic microbes. Therefore, it would be interesting to determine if Proteobacteria in the nares may in fact be protective against SSTI and could be used prophylactically to prevent SSTI.

We observed a significant inverse correlation between *S. aureus* and *Corynebacterium* abundance in the nares (Figure 9C). A similar finding has been documented by other studies (170; 302; 334). Indeed, *Corynebacterium* has been shown to directly inhibit *S. aureus* growth (170), as well as to foster clearance of *S. aureus* from the anterior nares (302). The molecular mechanism by which this occurs is not clear. Perhaps *Corynebacterium* produces a product that is toxic to *S. aureus*. Alternatively, *Corynebacterium* may successfully compete for limited nutrients required for *S. aureus* growth. If the former is the case, and the toxic molecule can be isolated and purified, it could potentially serve as a novel therapeutic agent to prevent *S. aureus* colonization.

Our data indicate that *S. aureus* colonization has a major impact on the overall composition of the nasal microbiota in our study population (Figure 10). However, our cross-sectional sampling limits our ability to discern between persistently or non-persistently colonized individuals. In a recent study that monitored the nasal microbiome of 12 healthy individuals over a course of three weeks, it was determined that while *S. aureus* carriers and non-carriers had similar nasal microbiomes, non-persistent carriers displayed a significant drop in bacterial diversity (334). Proteobacteria levels in the nares were also shown to significantly vary among persistent and non-persistent carriers (334). Additionally, microbiota changes at other body locations (axilla, groin, perianal), as well as the site of infection prior to abscess formation, may also contribute to SSTI susceptibility (336).

Although vaccine studies have been largely ineffective at preventing *S. aureus* carriage in humans (33; 62; 267), a greater understanding of the nature of the apparent competition between *S. aureus* and other taxa may hold promise for future therapeutic

design to prevent *S. aureus* carriage and SSTI development. Although bacterial antagonism against *S. aureus* has previously been investigated (138; 228; 302; 334), our data suggests that there are additional variables to consider when designing prophylactic countermeasures; in order to lower the risk of development of SSTI, one must lower *S. aureus* colonization levels, but must be cognizant about other nasal bacterial inhabitants, especially from the Proteobacteria phylum. Additionally, a reduction of specific bacteria in non-carriers, including *Propionibacterium* and *S. epidermidis*, correlates with SSTI development (Figure 7). Given these data, nasal administration of a single bacterium may not be sufficient to protect against SSTIs; a medley of bacteria may be needed.

Given that culture has been the primary method used to identify the etiological agent of abscesses (282), our study provides the first in-depth characterization of abscesses from individuals infected with MRSA, MSSA and NoSA. Not surprisingly, we found that the vast majority of MRSA and MSSA culture-positive abscesses were dominated by *S. aureus* (Figure 6). Interestingly, we identified a high number of polymicrobial infections, especially in the NoSA abscess group (Figures 6 and 11). It is possible that bacteria within a polymicrobial infection may possess a distinct transcriptional profile compared to a monomicrobial abscess. Indeed, *S. aureus* in the context of a polymicrobial infection displays enhanced pathogenesis when compared to a *S. aureus* mono-infection (212).

There are limitations to our investigation. First, this study was conducted in the context of a prospective cluster-randomized trial that involved hygiene measures. Although we did not detect any differences in the nasal microbiomes of the participants in different study groups (data not shown), it is possible that the hygiene interventions

(287), especially chlorhexidine use, may have impacted the anterior nares microbiome. However, given that some of our findings are congruent with other studies (the inverse correlation between *S. aureus* and *Corynebacterium* in the nares has also been observed elsewhere (170; 302; 334)), this suggests that chlorhexidine use did not have a major impact on the nasal microbiome. Nevertheless, our large sample provides a firm hypothesis-generating foundation upon which to build. Second, because all abscesses were drained based on clinical criteria, these culture swabs were processed in the microbiology lab using standard techniques, which is different from the enriched technique employed for the anterior nares specimens. This may in part explain the discrepancy between culture-negative and sequence-positive abscesses.

In summary, we have characterized the nasal microbiome of over 80 individuals and have described significant differences in microbial composition in the anterior nares between those with and without abscesses, and between *S. aureus* carriers and non-carriers. We also used high-throughput sequencing techniques to unveil the microbiome of abscesses. These observations expand our understanding of the complexity of the nasal and abscess microbiomes, and will potentially be useful in the design of future therapeutic and prophylactic countermeasures against *S. aureus* carriage and subsequent disease.

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| Sample ID | Sample Location | STY/S aureus colonization status | 1852R | 1859R | 1863R | 1899R | 1977R | 1993R | 2000R | 2051R | 1674R | 1766R | 1858R | 1867R | 1920R | 1939R | 1990R | 2010R | 2050R | 2139R | 2168R | |
|-------------------------|-----------------|----------------------------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|-------------|-------------|-------------|-----------|-------------|-------------|------------|------------|-------------|-----------|-----------|-----------|------------|
| | | | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose |
| | | | MSSA Absc | MSSA Absc | MSSA Absc | MSSA Absc | MSSA Absc | MSSA Absc | MSSA Absc | MSSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc | NoSA Absc |
| Taxon | | | | | | | | | | | | | | | | | | | | | | |
| p_Acidobacteria | | | 0.04454343 | | 0.13363029 | | 0 | | 0.04454343 | 0.04454343 | 0.04454343 | 0.08908686 | | | | | 0.04454343 | | | | | 0.04454343 |
| c_Acidobacteria-5 | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_Acidobacteria-6 | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_11-15 | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_Acidobacteria | | | 0.04454343 | | 0.08908686 | | 0 | | 0 | 0 | 0.04454343 | 0.08908686 | | | | | 0 | | | | | 0.04454343 |
| o_Acidobacteriales | | | 0.04454343 | | 0.08908686 | | 0 | | 0 | 0 | 0.04454343 | 0.08908686 | | | | | 0 | | | | | 0.04454343 |
| f_Acidobacteriaceae | | | 0.04454343 | | 0.08908686 | | 0 | | 0 | 0 | 0.04454343 | 0.08908686 | | | | | 0 | | | | | 0.04454343 |
| e_Elphobacter | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0.08908686 | | | | | 0 | | | | | 0 |
| t_moderum | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0.08908686 | | | | | 0 | | | | | 0 |
| e_Terriglobus | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0.08908686 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0.08908686 | | | | | 0 | | | | | 0 |
| unclassified | | | 0.04454343 | | 0.08908686 | | 0 | | 0 | 0 | 0.04454343 | 0 | | | | | 0 | | | | | 0.04454343 |
| unclassified | | | 0.04454343 | | 0.08908686 | | 0 | | 0 | 0 | 0.04454343 | 0 | | | | | 0 | | | | | 0.04454343 |
| f_Koribacteraceae | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| k_Candidatus_Koribacter | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_Chloroacidobacteria | | | 0 | | 0 | 0.04454343 | | | 0.04454343 | 0.04454343 | | | | | | | 0.04454343 | | | | | 0 |
| o_05-100 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_PK29 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_184 | | | 0 | | 0.04454343 | | 0 | | 0.04454343 | 0.04454343 | | | | | | | 0.04454343 | | | | | 0.04454343 |
| f_E186075 | | | 0 | | 0 | 0 | | | 0.04454343 | 0.04454343 | | | | | | | 0.04454343 | | | | | 0.04454343 |
| unclassified | | | 0 | | 0 | 0 | | | 0.04454343 | 0.04454343 | | | | | | | 0.04454343 | | | | | 0.04454343 |
| unclassified | | | 0 | | 0 | 0 | | | 0.04454343 | 0.04454343 | | | | | | | 0.04454343 | | | | | 0.04454343 |
| unclassified | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_04052 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_E116513 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_3825 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_Solibacteres | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_Solibacteres | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| f_MV5-65 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| f_PK34 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_5va0725 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| c_11-8 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_05-18 | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | 0 | | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| p_Actinobacteria | 50.33407572 | 71.09111403 | 86.68151448 | 28.99772787 | 60.62360802 | 5.87972739 | 84.85232385 | 72.24944321 | 54.78841871 | 63.07349666 | 85.96881196 | 78.88641425 | 80.454343 | 82.36080178 | 63.91982183 | 0.04454343 | 70.155902 | 85.61247216 | | | | |
| c_Actinomirabilia | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_Actinomirabiales | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| f_111 | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| f_181017 | | | 0 | | 0.04454343 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| unclassified | | | 0 | | 0 | | 0 | | 0 | 0 | 0 | 0 | | | | | 0 | | | | | 0 |
| o_Actinobacteria | 50.33407572 | 71.0467706 | 86.68151448 | 28.99808597 | 60.53452116 | 5.87972739 | 84.85232385 | 72.24944321 | 54.78841871 | 63.07349666 | 85.96881196 | 78.88641425 | 80.454343 | 82.36080178 | 63.91982183 | 0.04454343 | 70.155902 | 85.61247216 | | | | |
| o_Actinomycetales | 50.33407572 | 71.0467706 | 86.68151448 | 28.99808597 | 60.53452116 | 5.87972739 | 84.85232385 | 72.24944321 | 54.78841871 | 63.07349666 | 85.96881196 | 78.88641425 | 80.454343 | 82.36080178 | 63.91982183 | 0.04454343 | 70.155902 | 85.61247216 | | | | |
| o_Actinomycetales | 0.08908686 | 0.04454343 | 0.04454343 | 0.13363029 | 0 | 0.04454343 | | 0. | | | | | | | | | | | | | | |

| | | | | | | | | | | | | | | | | | | |
|---------------------|------------|------------|------------|---|-------------|-------------|-------------|-------------|-------------|-------------|------------|-------------|-------------|-------------|---|---|---|------------|
| F_Burkholderiaceae | 0 | 0 | 0 | 0 | 0.356347439 | 0 | 0.534521158 | 0.400890866 | 0.04454343 | 0 | 0.13363029 | 0.26720579 | 0.04454343 | 0.178173719 | 0 | 0 | 0 | 0 |
| E_Burkholderia | 0 | 0 | 0 | 0 | 0.356347439 | 0 | 0.534521158 | 0.400890866 | 0.04454343 | 0 | 0.13363029 | 0.26720579 | 0.04454343 | 0.178173719 | 0 | 0 | 0 | 0 |
| E_Andropogonis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Stryphilia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Graminis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Suberum | 0 | 0 | 0 | 0 | 0 | 0.178173719 | 0 | 0.178173719 | 0.08908686 | 0 | 0 | 0.13363029 | 0 | 0.13363029 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0.178173719 | 0 | 0.356347439 | 0.311804009 | 0.04454343 | 0 | 0.13363029 | 0.13363029 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 |
| E_Laetipora | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Salinipora | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Tropica | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F_Comanadaceae | 0.08908686 | 0 | 0.04454343 | 0 | 0.178173719 | 0 | 0 | 0.13363029 | 0 | 0 | 0.13363029 | 0 | 0.04454343 | 0.178173719 | 0 | 0 | 0 | 0 |
| E_Acidovorax | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Sclerotids | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Alicyclophus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Aquabacterium | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Azohydromonas | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Comanonas | 0.04454343 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Terrigena | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0.04454343 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Delta | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.08908686 | 0 | 0 | 0.178173719 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.08908686 | 0 | 0 | 0 | 0.178173719 | 0 | 0 | 0 | 0 |
| E_Diaphobacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Giesbergia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Hyemonella | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| E_Leptotrix | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Methylalum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Petrophilum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Faustbacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Pelonomas | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Purpureae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Kambacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Rubrivivax | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Gelatinosus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Schlegelia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Tepidimonas | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Variovorax | 0 | 0 | 0 | 0 | 0 | 0 | 0.13363029 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Panatolus | 0 | 0 | 0 | 0 | 0 | 0 | 0.13363029 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F_Ohalobacteraceae | 0.04454343 | 0 | 0.08908686 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.04454343 | 0.04454343 | 0.13363029 | 0.311804009 | 0.04454343 | 0 | 0 | 0 | 0.08908686 |
| E_Collimonas | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0.08908686 | 0.22271749 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0.08908686 | 0.22271749 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Cuprivivax | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Gladii | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Herbaspillum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 |
| E_Merminimonas | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 |
| E_Janthinobacterium | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Lividum | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Ohalobacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| S_Formigenes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| E_Polydubacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| S_Cosmopolitatus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0.04454343 |
| Unclassified | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0.04454343 |
| O_Ellis607 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| O_Neisseria | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.356347439 | 0.04454343 | 1.69250334 | 7.26579065 | 1.959910913 | 0.04454343 | 0.75723807 | 1.514476615 | 0 | 0 | 0 | 0 | 0 | 0.48997728 |
| F_Neisseriaceae | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.356347439 | 0.04454343 | 1.69250334 | 7.26579065 | 1.959910913 | 0.04454343 | 0.75723807 | 1.514476615 | 0 | 0 | 0 | 0 | 0 | 0.48997728 |
| E_Aquasala | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F_Magnosella | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Eikenella | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Kingella | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.08908686 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 |
| Unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.08908686 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Microvirgula | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| E_Neisseriiformis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| F_Neisseria | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0.356347439 | 0.04454343 | 1.718173719 | 0 | 0.04454343 | 0.712694878 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.26720579 |
| S_Cinerea | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 1.718173719 | 0 | 0 | 0.402808669 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| S_Sulfoloba | 0 | 0 | 0 | 0 | 0.111804009 | 0 | 0 | 0 | 0.04454343 | 0.311804009 | 0 | 0 | 0 | 0 | 0 | | | |

| Sample ID | 5937R | 5938R | 5941R | 5942R | 5943R | 5944R | 5946R | 5947R | 5981R | 5982R | 5992R | 5997R | 1674A | 1715A | 1716A | 1717A | 1742A | 1749A | |
|-----------------------------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|---------------|------------|-------------|------------|--------|------------|-------------|---|
| Sample Location | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Nose | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | |
| STY/S. aureus colonization status | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | NoSA Non-SST1 | MRSA | MRSA | MRSA | MRSA | MRSA | MRSA | |
| Taxon | | | | | | | | | | | | | | | | | | | |
| p__Acidobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.08908686 | 0.13363029 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Acidobacteria-5 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Acidobacteria-6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__B1-15 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Acidobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__Acidobacteriales | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__Acidobacteriaceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Edaphobacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| s__modestum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Terriglobus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__Noribacteriaceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Candidatus_noribacter | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Chloracidobacteria | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.08908686 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__DS-100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__PK29 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__R841 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.08908686 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__Ellin075 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__DMS02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__Ellin0513 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__R825 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Solibacteres | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__Solibacteres | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__MVS-65 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__PS4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Suo0725 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__Suo0725 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__B1-8 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__DS-18 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| p__Actinobacteria | 40.31180401 | 91.22494432 | 56.39198238 | 69.08885969 | 5.523385301 | 59.42091541 | 24.85523835 | 6.369710468 | 73.1401118 | 52.65033408 | 60.75723811 | 56.30289532 | 0.13363029 | 36.08017817 | 0.17817719 | 0 | 0.04454343 | 0.801781737 | 0 |
| c__Actinomycetia | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| o__Actinimicrobiales | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__C111 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__B1017 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| c__Actinobacteria | 40.31180401 | 91.22494432 | 56.39198238 | 69.08885969 | 5.523385301 | 59.3763198 | 24.81069042 | 6.369710468 | 73.1401118 | 52.65033408 | 60.53452116 | 56.25831589 | 0.13363029 | 36.08017817 | 0.17817719 | 0 | 0.04454343 | 0.801781737 | 0 |
| o__Actinomycetia | 40.31180401 | 91.22494432 | 56.39198238 | 69.08885969 | 5.523385301 | 59.3763198 | 24.81069042 | 6.369710468 | 73.1401118 | 52.65033408 | 60.53452116 | 56.25831589 | 0.13363029 | 36.08017817 | 0.17817719 | 0 | 0.04454343 | 0.801781737 | 0 |
| f__Actinomycetaceae | 0 | 0 | 0.13363029 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.48997728 | 0 | 0.48997728 | 0 | 0 | 0 | 0 | 0 |
| g__Actinobaculum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Actinomycetes | 0 | 0 | 0.08908686 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.48997728 | 0 | 0.48997728 | 0 | 0 | 0 | 0 |
| s__purpureus | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| s__pyovaginalis | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0.48997728 | 0 | 0 | 0 | 0 |
| g__Moliblunus | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Varibaculum | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| unclassified | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| f__Actinomycetaceae | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.04454343 | 0 | 0 | 0 | 0 | 0 | 0 |
| g__Actinoalotrichus | 0 | 0 | 0 | | | | | | | | | | | | | | | | |

| Sample ID | 1751A | 1766A | 1767A | 1858A | 1863A | 1867A | 1978A | 1990A | 2012A | 2123A | 1683A | 1737A | 1781A | 1790A | 1859A | 1920A | 1933A | 1977A | 1993A | |
|------------------------------------|-------------|-------------|-------------|------------|------------|-------------|------------|--------|------------|--------|-------------|-------------|------------|-------------|-------------|--------|-------------|------------|--------|--|
| Sample Location | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | Abcess | |
| STTV/S. aureus colonization status | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | MSSA | |
| Taxon | | | | | | | | | | | | | | | | | | | | |
| p__Acidobacteria | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| c__Acidobacteria-S | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| c__Acidobacteria-6 | | | | | | | | | | | | | | | | | | | | |
| o__ll1-15 | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| c__Acidobacteria | | | | | | | | | | | | | | | | | | | | |
| o__Acidobacteriales | | | | | | | | | | | | | | | | | | | | |
| f__Acidobacteriaceae | | | | | | | | | | | | | | | | | | | | |
| g__Edaphobacter | | | | | | | | | | | | | | | | | | | | |
| s__modestum | | | | | | | | | | | | | | | | | | | | |
| g__Terriglobus | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| f__Vorbacteriaceae | | | | | | | | | | | | | | | | | | | | |
| g__Candidatus_koribacter | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| c__Chloracidobacteria | | | | | | | | | | | | | | | | | | | | |
| o__OS-100 | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| o__PK29 | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| o__PB41 | | | | | | | | | | | | | | | | | | | | |
| f__Ellin075 | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
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| c__D4012 | | | | | | | | | | | | | | | | | | | | |
| o__Ellin0513 | | | | | | | | | | | | | | | | | | | | |
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| c__8B25 | | | | | | | | | | | | | | | | | | | | |
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| c__Solibacteres | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| o__Solibacteriales | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| f__MAY65 | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| unclassified | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| unclassified | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| f__PK54 | | | | | | | | | | | | | | | | | | | | |
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| c__Sva0725 | | | | | | | | | | | | | | | | | | | | |
| o__Sva0725 | | | | | | | | | | | | | | | | | | | | |
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| c__ll3-4 | | | | | | | | | | | | | | | | | | | | |
| o__OS-18 | | | | | | | | | | | | | | | | | | | | |
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| p__Actinobacteria | 2.273714922 | 0.935412027 | 0.178173719 | 0.04454343 | 0.04454343 | 0.178173719 | 0.13363029 | | 0.04454343 | | 0.489977228 | 4.810690423 | 0.08908686 | 6.319536748 | 0.712694878 | | 0.178173719 | 0.04454343 | | |
| o__Actinomicrobiales | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| o__Actinomicrobiales | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| f__C111 | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
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| f__EB1017 | 0.04454343 | | | | | | | | | | | | | | | | | | | |
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| unclassified | 0.04454343 | | | | | | | | | | | | | | | | | | | |
| c__Actinobacteria | 2.182628062 | 0.935412027 | 0.178173719 | 0.04454343 | 0.04454343 | 0.178173719 | 0.13363029 | | 0.04454343 | | 0.489977228 | 4.810690423 | 0.08908686 | 6.346993318 | 0.712694878 | | 0.178173719 | 0.04454343 | | |
| o__Actinomycetales | 2.182628062 | 0.935412027 | 0.178173719 | 0.04454343 | 0.04454343 | 0.178173719 | 0.13363029 | | 0.04454343 | | 0.489977228 | 4.810690423 | 0.08908686 | 6.346993318 | 0.712694878 | | 0.178173719 | 0.04454343 | | |
| f__Actinomycetales | | | | | | | | | | | | | | | | | | | | |
| g__Actinobaculum | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| g__Actinomycetes | | | 0.04454343 | | | | | | | | | | | | | | | | | |
| s__europaeus | | | | | | | | | | | | | | | | | | | | |
| s__pyvagnalis | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | 0.04454343 | | | | | | | | | | | | | | | | | |
| g__Molibaculum | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| g__Varibaculum | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| f__Actinomycetaceae | | | 0.04454343 | | | | | | | | | | | | | | | | | |
| g__Actinoalloteichus | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| g__Actinera | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| g__Lentzea | | | | | | | | | | | | | | | | | | | | |
| s__violacea | | | 0.04454343 | | | | | | | | | | | | | | | | | |
| g__Saccharothrix | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| f__Neutrobergiaceae | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| f__Rogorielaceae | | | | | | | | | | | | | | | | | | | | |
| g__Georgenia | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| f__Brevibacteriaceae | | | 0.04454343 | | | 0.04454343 | | | | | | | | | | | | | | |
| g__Brevibacterium | | | 0.04454343 | | | 0.04454343 | | | | | | | | | | | | | | |
| s__caeseum | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | 0.04454343 | | | 0.04454343 | | | | | | | | | | | | | | |
| s__paucivorans | | | | | | | | | | | | | | | | | | | | |
| f__Cellulomonadaceae | | | | | | | | | | | | | | | | | | | | |
| g__Actinotalea | | | | | | | | | | | | | | | | | | | | |
| unclassified | | | | | | | | | | | | | | | | | | | | |
| g__Cellulomonas | | | | | | | | | | | | | | | | | | | | |
| g__Lida | | | | | | | | | | | | | | | | | | | | |
| f__Corynebacteriaceae | 1.514476615 | 0.400890869 | 0.178173719 | 0.04454343 | | 0.04454343 | 0.04454343 | | 0.04454343 | | | 4.231625835 | 0.04454343 | 5.968819599 | 0.668151448 | | 0.13363029 | | | |
| g__Corynebacterium | 1.514476615 | 0.400890869 | 0.178173719 | 0.04454343 | | 0.04454343 | | | | | | | | | | | | | | |

CHAPTER THREE: Bacterial Etiology and Risk Factors Associated with Cellulitis and Purulent Skin Abscesses in Military Trainees

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The work presented in this chapter is the sole work of R.C. Johnson with the following exceptions: abscess/cellulitis cultures were processed by Martin Army Community Hospital microbiology laboratory; study oversight at Fort Benning provided M.W. Ellis, E.V. Millar, and C.D. Schlett; sample acquisition provided by J.B. Lanier; risk factor assessment performed by C.D. Schlett, E.V. Millar, D. Mor; pulsed-field gel electrophoresis and antibiotic susceptibility testing performed by E. Elassal; ASO and DNase B titers performed by D.K. Bishop; full-length 16S rRNA sequencing performed by P.T. LaBreck; 454 sequencing performed by C.L. Redden and K.A. Bishop-Lilly.

ABSTRACT

Skin and soft-tissue infections (SSTIs) remain one of the most predominant bacterial infections seen in the hospital today. Certain populations, such as military personnel, are disproportionately affected by SSTIs. A recent investigation in our laboratory revealed that while purulent SSTIs in military infantry were primarily dominated by the Gram-positive pathogen *Staphylococcus aureus*, there were also a large

number of polymicrobial infections. Although *S. aureus* is associated with purulent SSTI, it is unclear to what degree this pathogen causes cellulitis. Furthermore, while broad risk factors associated with SSTI have been established, specific risk factors associated with either purulent (abscesses) or non-purulent (cellulitis) infections have not been thoroughly elucidated. To address these knowledge gaps, we enrolled 200 military trainees at Fort Benning who developed either cutaneous abscesses or cellulitis and assessed exposure to known SSTI risk factors. This analysis revealed that while most risk factors were not significantly different between the disease types, nasal colonization with MRSA was associated with abscess formation, while cellulitis development was statistically linked to prior blister formation and ethnicity. To investigate differences in microbial composition between the two SSTIs, we collected 18 swabs and 22 leading edge aspirates from individuals suffering from cutaneous abscess and cellulitis, respectively. Genomic DNA was extracted from all samples and the V1 to V3 region of the 16S rRNA gene was PCR amplified and sequenced using 454 high-throughput sequencing technology. Additionally, we collected relevant clinical information from the military trainees including anti-streptolysin O (ASO) and anti-DNase B titers. In agreement with our previous study, we found that while most of the purulent abscesses were primarily composed of *S. aureus*, mixed infections were common. Interestingly, the bacterial compositions of the cellulitis microbiomes were dramatically different from the purulent abscesses; bacteria from the Proteobacteria phylum predominated. These data emphasize important epidemiological and microbial differences between purulent and non-purulent SSTIs that should be considered when treating these complicated infections.

INTRODUCTION

Skin and soft-tissue infections (SSTIs) are among the most frequently observed infections in the ambulatory and hospital settings (72; 127; 226). In 2005 alone, approximately 14.2 million SSTI cases were reported in the United States; a 65% increase from 1997 (127). While severe SSTIs such as necrotizing fasciitis are routinely reported, the bulk of disease manifests as abscesses and cellulitis. In fact, cutaneous abscesses and cellulitis accounted for 95% of the observed SSTI increase from 1997 to 2005 (127). While numerous SSTI risk factors have been established in response to this increased prevalence, whether certain risk factors are specifically associated with either cutaneous abscess or cellulitis formation have yet to be determined.

Although SSTIs afflict millions of individuals world-wide, certain congregate populations, such as military service members, are at increased risk (36; 342). Indeed, there were roughly 62,000 ambulatory visits and 1,500 hospitalizations attributed to SSTIs in the military in 2010 (4). The alarming increase in SSTI prevalence in the military and general public is largely attributed to the emergence of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) (148). In an era of drug resistant bacterial pathogens, such as CA-MRSA, bacterial culture and antibiotic susceptibility testing is imperative for proper treatment of SSTI. While CA-MRSA has been identified as the leading cause of most purulent SSTIs (203; 283), to what extent this pathogen causes cellulitis infections remains unknown.

Without a site to culture, bacterial etiology for non-purulent cellulitis has been historically unsuccessful. However, dogma suggests that cellulitis is caused by both *S.*

aureus and beta-hemolytic streptococci (BHS); the actual degree each pathogen contributes is unclear (117; 251; 295). Additionally, despite diverse sample collection methods (punch biopsy, needle aspirate and blood sample), a pathogen is isolated less than 30% of the time (71; 216; 235; 286). In other words, microbial diagnosis is undefined in more than 70% of all cellulitis cases. To further complicate matters, recent studies have shown that SSTIs, particularly purulent abscesses, can be polymicrobial in nature (144). The changing epidemiology and uncertainty of microbial etiology have exposed important SSTI knowledge gaps. Herein we report our findings from an epidemiological analysis to discern SSTI-specific risk factors as well as detailed characterization of the microbiomes of both purulent and non-purulent SSTI using a high-throughput sequencing strategy. Together, our data indicate that while abscess and cellulitis are both considered SSTIs, they represent clinically unique infections that may require individualized treatments.

MATERIALS AND METHODS

Study design and participants

We enrolled a total of 240 U.S. Army infantry trainees stationed at Fort Benning, GA; 40 were part of the microbiome portion of the study and 200 were utilized for SSTI risk factor assessment. The study population was all male, ages 17-39, and in good physical condition. Patient demographics for the 40 microbiome study participants are shown in Table 6 and Table 7. All military trainees enrolled were part of an ongoing research study aimed at elucidation of the epidemiology, etiology, immunology, and

economic burden of SSTIs in military trainees. The study was approved by the Uniformed Services University Infectious Disease Institutional Review Board.

Enrollment and data collection

Military trainees that presented to the Troop Medical Clinic (TMC) with an SSTI were enrolled in the study and placed into one of two groups contingent on infection type: cutaneous abscess or cellulitis group. While the cutaneous abscess group presented with an infection that required incision and drainage, the cellulitis group presented with diffuse inflammation of the skin with no purulent exudate. All participants were assessed for nasal colonization by *S. aureus* as previously described (144). Exclusion criteria included prior antibiotic administration within two weeks before initial presentation, infection on the face or associated with a surgical site, animal or human bite wound cellulitis, evidence or suspected bacteremia/sepsis/deep soft tissue infection, or any underlying condition such as neutropenia, vascular insufficiency or diabetes.

For the 18 microbiome participants with cutaneous abscess, the skin at the site of infection was sterilized with chlorhexidine prior to incision. Two swabs were then obtained from within the abscess cavity: one was sent for microbiological culture, and the other was used for microbiome analysis. For the 22 cellulitis infections, we obtained samples via needle aspiration using a 22-gauge needle attached to a 10 ml plastic syringe loaded with sterile phosphate buffered saline (PBS). The needle was inserted into the subcutaneous tissue of the leading edge of inflammation and 1.5 ml of PBS was slowly injected and then immediately aspirated. One half of the recovered PBS (approximately

Table 6. Purulent abscess patient information

| Patient ID | Age / Race | Site of Infection | Abscess Culture | Area Erythema (initial/days 2-3/days 21-28) ^a | Body Site Colonization | | | |
|------------|------------|-------------------|--------------------|--|------------------------|------------|----------|----------|
| | | | | | Nose | Oropharynx | Perianal | Inguinal |
| 1782 | 22/White | Hand | ND | ND | CNS | CNS | CNS | MSSA |
| 1868 | 17/White | Elbow | MSSA | ND | MSSA | CNS | MSSA | CNS |
| 1871 | 21/White | Elbow | Other ^b | ND | MRSA | NG | CNS | CNS |
| 1876 | 18/White | Buttock | MRSA | 8.5/-/0 | CNS | CNS | MRSA | ND |
| 1914 | 18/White | Foot | ND | ND | CNS | CNS | NC | CNS |
| 1955 | 17/White | Lower Leg | MRSA | ND | CNS | NC | NC | NC |
| 1957 | 17/Asian | Forearm | MRSA | ND | MSSA | MSSA | NC | NC |
| 1983 | 18/White | Chest | ND | ND | CNS | CNS | NC | NC |
| 2018 | 18/White | Foot | MRSA | ND | MSSA | MSSA | CNS | CNS |
| 2058 | 26/White | Axilla | ND | ND | MSSA | CNS | NC | CNS |
| 2067 | 22/White | Lower Leg | ND | 42.5/-/0 | MSSA | MSSA | CNS | CNS |
| 2086 | 19/Asian | Forearm | ND | ND | CNS | CNS | NC | NC |
| 2094 | 24/White | Knee | ND | ND | CNS | MSSA | NC | MSSA |
| 2120 | 19/White | Axilla | ND | ND | MSSA | MSSA | NC | CNS |
| 2152 | 19/Other | Axilla | ND | ND | MSSA | ND | NC | NC |
| 2157 | 19/White | Hand | Other ^b | ND | NC | MSSA | NC | NC |
| 2160 | 18/White | Foot | Other ^b | ND | MSSA | CNS | NC | NC |
| 2202 | 18/White | Axilla | MRSA | ND | CNS | CNS | NC | CNS |

Abbreviations and symbols: ND, not determined; NG, no growth; CNS, Coagulase-negative *Staphylococcus*; NC, not consented; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*

^a Area measured in centimeters. A dash indicates no measurement taken.

^b Indicates non-*S. aureus* bacterial growth.

Table 7. Cellulitis patient information

| Patient ID | Age / Race | Site of Infection | ASO Titers ^a (Acute/Conv) | Anti-DNase B Titers ^a (Acute/Conv) | Aspirate Culture | Area Erythema (initial/days 2-3/days 21-28) ^b | Body Site Colonization | | | | Subsequent abscess formation |
|------------|------------|-------------------|--------------------------------------|---|------------------|--|------------------------|------------|----------|----------|------------------------------|
| | | | | | | | Nose | Oropharynx | Perianal | Inguinal | |
| 1680 | 19/White | Foot | ND | ND | ND | 203/144/0 | MSSA | MSSA | NC | CNS | N |
| 1739 | 17/White | Lower Leg | 9/7 | <95 /<95 | NG | 216/212.5/0 | CNS | CNS | CNS | MSSA | N |
| 1773 | 21/White | Forearm | 499/483 | <95/<95 | NG | 228/205/0 | CNS | NG | MSSA | CNS | N |
| 2055 | 18/White | Foot | 437/423 | <95/102 | NG | 405/240/0 | CNS | CNS | NC | NC | N |
| 2080 | 23/White | Foot | 92/324 | <95/<95 | ND | - | MSSA | CNS | MSSA | MSSA | N |
| 2083 | 18/White | Foot | 448/NA | <95/NA | CNS | 48/25/- | MSSA | CNS | MSSA | MSSA | ND |
| 2124 | 20/White | Foot | 220/206 | <95/<95 | NG | 115/110/0 | MSSA | MSSA | MSSA | MSSA | N |
| 2128 | 22/White | Elbow | 52/47 | <95/109 | NG | 228/144/0 | MSSA | CNS | CNS | CNS | N |
| 2129 | 22/White | Forearm | 100/100 | 109/<95 | NG | 225/0/0 | CNS | CNS | CNS | CNS | N |
| 2171 | 24/White | Thigh | 439/129 | 207/124 | NG | 238/84/0 | MSSA | CNS | MSSA | MSSA | Y |
| 2172 | 19/White | Foot | 119/113 | <95/<95 | NG | 115.5/116/0 | MSSA | MSSA | CNS | CNS | N |
| 2203 | 21/White | Foot | 41/37 | 97/111 | NG | - | MSSA | NG | NC | MSSA | N |
| 2213 | 18/White | Knee | 85/87 | <95/<95 | NG | 289/-/0 | MSSA | MSSA | MSSA | CNS | N |
| 2250 | 24/White | Foot | 58/62 | <95/<95 | NG | 72.25/36/0 | MSSA | CNS | NC | MSSA | N |
| 2306 | 22/White | Foot | 25/25 | <95/<95 | NG | 38.25/0/0 | CNS | CNS | NC | CNS | N |
| 2326 | 23/White | Foot | 226/229 | 100/289 | NG | 135/24/0 | MSSA | MSSA | CNS | CNS | N |
| 2367 | 20/White | Knee | 247/259 | <95/<95 | NG | 532/49/0 | MSSA | MSSA | MSSA | MSSA | N |
| 2423 | 21/White | Foot | 92/86 | 103/<95 | ND | 36/30/0 | NC | NC | NC | NC | N |
| 2637 | 24/White | Elbow | 10/12 | <95/<95 | NG | 144/0/0 | MSSA | MSSA | CNS | MSSA | N |
| 2758 | 24/White | Arm, Elbow | 13/<6 | <95/<95 | ND | - | MRSA | MRSA | MRSA | MRSA | N |
| 2867 | 18/White | Thigh | 147/138 | <95/<95 | NG | 100/36/0 | MSSA | MSSA | NC | NC | N |
| 2913 | 18/White | Thigh | 19/16 | <95/<95 | NG | 90/0/0 | CNS | CNS | CNS | CNS | N |

Abbreviations and symbols: ND, not determined; NG, no growth; N, no; Y, yes; CNS, Coagulase-negative *Staphylococcus*; NC, not consented; MSSA, methicillin-sensitive *S. aureus*; MRSA, methicillin-resistant *S. aureus*

^a ASO and DNase B titers are represented as IU/ml and U/ml, respectively. Normal ranges: ASO <200 IU/ml, DNase B <301 U/ml. Titers were measured upon initial admission to the TMC (acute) and at 12-14 days post admission (conv). Elevated titer levels are depicted in bold.

^b Area measured in centimeters. A dash indicates no measurement taken.

0.5 ml) was immediately frozen at -80°C and sent for microbiome analysis. The remaining half was promptly sent for microbiological culture. Post aspiration, the patient underwent phlebotomy to obtain serum samples for immunological surveillance (acute samples) and was monitored for 21-28 days at which time a second phlebotomy was performed (convalescent samples).

In addition to infection culture analysis, additional swabs were simultaneously taken and used to assess carriage of *S. aureus* at various body sites including the oropharynx, inguinal, and perianal regions. Anatomical site colonization samples were cultured in enriched broth as previously described (77). Follow-up assessments at days 2-3 and 21-28 for all patients were conducted to monitor SSTI resolution.

For the 200 SSTI risk factor assessment participants (103 abscess, 97 cellulitis), pertinent medical and demographic information was collected. Additionally, the patients were issued a questionnaire to assess prior exposure to SSTI risk factors. Data from the questionnaire is shown in Table 8.

Microbiological and molecular analysis

Culture analyses for both cellulitis and abscess samples were conducted according to standard procedures at the Martin Army Community Hospital microbiology laboratory (78). *S. aureus* isolates were typed using pulsed-field gel electrophoresis (PFGE) and resistance/virulence determinants were assessed using PCR as previously described (82).

Serological analysis

Acute and convalescent blood samples were allowed to clot for a minimum of 30 minutes and serum was isolated by centrifugation at 2500 rpm for 15 minutes. Anti-

Table 8. Abscess and cellulitis risk factor assessment

| | SSTI Cases (n=200) | | P-Value ^b |
|--|----------------------|------------------------|----------------------|
| | Abscess Only (n=103) | Cellulitis Only (n=97) | |
| Median Age, years (Range) | 19 (18-21) | 20 (18-22) | 0.08 |
| Ethnicity: White | 74 (71.8) | 83 (85.6) | 0.02 |
| Median (Range) No. of Weeks in Training at Enrollment | 5 (3-8) | 4 (2-7) | 0.2 |
| Season of Enrollment | | | 0.59 |
| Spring | 21 (20.4) | 21 (21.6) | |
| Summer | 49 (47.6) | 39 (40.2) | |
| Fall | 22 (21.4) | 21 (21.6) | |
| Winter | 11 (10.7) | 16 (16.5) | |
| Nasal Colonization with <i>S. aureus</i> | 54 (52.4) | 49 (50.5) | 0.79 |
| Nasal Colonization with MRSA^c | 24 (44.4) | 24 (14.3) | 0.002 |
| Risk Factor: Past Year | | | |
| Admitted to a Hospital | 5 (4.9) | 4 (4.1) | 0.79 |
| Worked at a Hospital | 4 (3.9) | 2 (2.1) | 0.68 |
| Known or Suspected SSTI/MSSA Infection | 7 (6.8) | 6 (6.2) | 0.85 |
| Taken an Antibiotic in Past 6 mos. | 18 (17.5) | 10 (10.3) | 0.14 |
| Risk Factor: 3 mos. Prior to Fort Benning | | | |
| Contact with a Person with SSTI | 4 (3.9) | 2 (2.1) | 0.68 |
| Risk Factor: While at Fort Benning | | | |
| History of SSTI | 1 (1) | 3 (3.1) | 0.36 |
| Contact with a Person with SSTI | 30 (29.1) | 25 (25.8) | 0.57 |
| Abrasions/Cuts | 57 (55.3) | 50 (51.5) | 0.59 |
| Blister | 44 (42.7) | 59 (60.8) | 0.01 |
| Insect Bite | 29 (28.2) | 29 (29.9) | 0.79 |
| Risk Factor: Trainee Survey | | | |
| Do Not Wash Hands or Use Sanitizer | 0 (0) | 1 (1) | 0.79 |
| Do Not Shower Daily | 0 (0) | 0 (0) | 0.79 |
| Share Towels and Clothing | 2 (1.9) | 6 (6.2) | 0.16 |
| Share or Borrow Razors | 1 (1) | 1 (1) | 1 |
| Do Not Wash Towels | 1 (1) | 4 (4.1) | 0.14 |
| Do Not Wash PT Uniform | 0 (0) | 2 (2.1) | 0.14 |
| Do Not Wash ACU | 0 (0) | 2 (2.1) | 0.14 |
| Shave Body Parts Other than Face | 17 (16.5) | 18 (18.6) | 0.73 |
| Bunkmate/Battle Buddy had Skin/MRSA Infection | 13 (12.6) | 7 (7.2) | 0.2 |
| Sexual Contact in Past 6 mos. with Someone with SSTI | 2 (1.9) | 0 (0) | 0.5 |

^a Unless specified, the numbers in parenthesis correspond to the percentage of total individuals with either abscess or cellulitis.

^b P-values calculated with chi square test. For counts less than 5, Fisher's exact test was used. P-values for median data were generated using the Wilcoxon rank-sum test.

^c Numbers in parenthesis correspond to the percentage of total individuals that were nasal carriers of *S. aureus*

streptococcal DNase B, and Streptolysin O (ASO) antibody titer determinations were performed at the reference laboratory QuestDiagnostics (Chantilly, VA).

DNA extraction, amplification, and sequencing

For the purulent abscess swabs, bacterial genomic DNA extraction and PCR amplification was performed in an identical fashion as previously described (144). For the cellulitis aspirates, 0.5 mls of the recovered PBS was mixed with an equal volume of lysis solution (GenElute bacterial genomic DNA kit (Sigma-Aldrich)) containing lysozyme (45 mg/ml), mutanolysin (125 U/ml), and lysostaphin (0.16 mg/ml) and allowed to incubate for 30 min at 37°C. Proteinase K (0.95 mg/ml) and lysis solution C (1 ml) were subsequently added to the sample and incubated for 10 minutes at 55°C. The remaining column purification steps were performed to the manufacturer's specifications. Amplification of the V1-V3 region of the 16S rRNA gene from the purulent abscess and cellulitis specimens was performed as previously described (144). All 40 samples were multiplexed and sequenced in a single 454 pyrosequencing run using the Roche GS FLX Titanium 454 sequencer at the Naval Medical Research Center in Fredrick, Maryland. Raw DNA sequences were quality processed and assigned taxonomy information using Mothur (v.1.34.4) as previously described (144). To control for possible reagent contaminants, we also prepared 0.5 mls of sterile PBS for 16S amplification and sequencing using the same reagents and 454 sequencer as described above. The PBS sample was sequenced in a separate pyrosequencing reaction in combination with an ongoing fecal microbiome study in our laboratory and yielded 3 usable reads; all of which were assigned to the alphaproteobacteria class.

Full-length 16S rRNA gene sequencing

Total genomic DNA from four cellulitis samples were subjected to PCR amplification of the 16S rRNA gene using the 8F and 1492R primers (299). The PCRs were performed in 50 µl reactions containing nuclease-free water, 125 U FidelityTaq DNA polymerase (Affymetrix), and 0.6 µM of the forward and reverse primers. PCR mixtures were incubated at 95°C for 90 seconds, 34 cycles of 95°C for 30 seconds, 50°C for 30 seconds and 68°C for 2 minutes followed by a final extension of 68°C for 5 minutes. Five technical replicates of each PCR reaction were combined and purified using the QIAquick PCR Purification Kit (Qiagen). The purified PCR products were then ligated into the pGEM-T easy vector (Promega) and transformed into *E. coli* TOP10 cells. Transformants were selected on LB agar supplemented with Ampicillin (Amp) (100 µg/ml), X-gal (40 µg/ml) and IPTG (1 µM). At least 10 white colonies per transformation were selected and grown overnight in LB-Amp broth with shaking at 200 rpm. Plasmids were isolated using the QIAprep Spin Miniprep Kit (Qiagen).

Seven sequencing reactions were performed for each sample to ensure complete coverage of both strands of the 16S rRNA gene. The insert within the pGEM-T easy vector was sequenced using the following primers: T7, SP6, 8F, 1492R, 515F, 806R, and 919F (Promega) (299; 319). PCR and sequencing were performed as previously described (80). DNA chromatograms were visualized using Chromas Lite (Technelysium). Full-length 16S sequences were manually assembled and taxonomy information was assigned after alignment to the RDP database (57).

Statistical analyses

For the risk factor assessment analysis, p-values for the “median age, years (range)” and “median (range) no. of weeks in training at enrollment” were calculated with the Wilcoxon rank-sum test. For all other comparisons, the chi square test was used to generate p-values. When variable counts were less than 5, the Fisher’s exact p-value was used.

RESULTS

Cellulitis and abscess risk-factor assessment

While numerous established risk factors for SSTI development have been identified, no group has assessed the contribution of these risk factors to either abscess or cellulitis formation within the same population. Thus, we enrolled 103 military trainees that developed a purulent abscess and 97 that presented with cellulitis and assessed for known SSTI risk factors (Table 8). For the majority of the risk factors assessed, the percentages of trainees in the abscess and cellulitis groups were not significantly different. However, we found that white ethnicity and blister formation were significantly associated with cellulitis development. Conversely, for patients that were *S. aureus* carriers, MRSA nasal colonization was linked to abscess formation.

Participant infection and body site culture

Of the 18 microbiome participants that developed cutaneous abscess, 9 had bacterial growth upon abscess culture. Of these 9, 5 were positive for MRSA (55.6%), 1

for MSSA (11.1%), and 3 grew non-*S. aureus* bacteria (33.3%) (Table 5). Unlike the cutaneous abscesses, only one of the 18 cellulitis aspirates analyzed (5.6%) grew bacteria (Coagulase-negative *Staphylococcus* (CNS)) (Table 7). Body site culture analysis for all 40 microbiome participants revealed that *S. aureus* (MRSA and MSSA) was routinely found throughout the body (abscess group: nose-52.9%, oropharynx-37.5%, perianal-33.3%, inguinal-20%) (cellulitis group: nose-71.4%, oropharynx-42.9%, perianal-53.3%, inguinal-47.4%) (Tables 6 & 7). While MSSA and CNS were frequently isolated from all body sites, MRSA was only detected 6 times total: twice in the nose, once in the oropharynx, twice in the perianal region, and once at the inguinal body site. Of note, one cellulitis patient (ID# 2758) had MRSA cultured from all 4 body sites tested.

ASO and anti-DNase B titer analysis

Previous studies have linked *Streptococcus* with cellulitis etiology (129; 142; 165; 216). We therefore assessed *Streptococcus* exposure using a serological approach. Upon admission to the clinic, 7 of the 22 cellulitis patients (31.8%) had elevated ASO titers (Table 7). Of those 7 patients, 5 maintained elevated ASO titers up to 21-28 days post SSTI diagnosis. We observed one patient that presented with normal ASO levels that eventually increased to above normal levels 21-28 days later, as well as a separate patient with the opposite ASO titer profile: elevated acute ASO titers and normal convalescent ASO titers. Anti-DNase B titers were unremarkable for all cellulitis patients at both acute and convalescent time points. Of note, linear regression analysis revealed no correlation between ASO titer levels and *Streptococcus* abundance levels (data not shown).

Sequencing results

While risk factor assessment revealed minimal differences between cellulitis and purulent abscesses, we also wanted to discern any differences in bacterial composition between the two SSTIs using a high-throughput sequencing approach. In total, we sequenced 40 SSTI samples (18 purulent abscess and 22 cellulitis) in a single 454 pyrosequencing run and yielded 1,515,532 raw sequences. 953,505 (63%) reads were removed from the analysis post quality filtering and contaminate/chimera detection. Overall, our sample set contained 1,007 unique reads with an average read length of 257 (range, 230 to 283) nucleotides. On average, there were 30,744 reads associated with each purulent abscess sample (range, 6,663 to 55,430). However, we observed a dramatic reduction in associated reads for the cellulitis samples. In fact, 4 of the 22 cellulitis samples had 0 associated reads. For the remaining 18, there were on average 480 associated reads per cellulitis sample (range, 149 to 956). This initial observation suggests that the bacterial load within the cellulitis samples is dramatically reduced compared to the purulent samples. Good's coverage values suggests that the number of reads associated with each sample accurately depict the total level of biodiversity (abscess samples: >99.4%; cellulitis samples: >95.9%). Subsampling was not performed for our data set given that no diversity comparisons were utilized. The percent abundance of each observed taxon for both infection types and the negative control is included in Table 10 (S2).

Purulent abscess microbiome

Analysis of the 18 purulent abscess samples revealed that the most abundant bacterial phylum present was Firmicutes (98.2% average abundance) (Figure 12A). At the species level, the most abundant bacterium present was *Staphylococcus aureus* (92.9% average abundance) (Figure 12C). Similar to a previous study (144), we observed a significant number of mixed infections; approximately 22% of the samples (4 of 18) had no single bacterial species reach an abundance level greater than 90%. While all purulent samples were sequence positive for *S. aureus*, additional abscess cohabitants included *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Staphylococcus haemolyticus*, and *Staphylococcus lugdunensis*.

Cellulitis microbiome

We next assigned taxonomy information to the 18 needle-aspirate samples that yielded usable sequences. In contrast to the purulent abscess samples, the most abundant bacterial phylum observed was Proteobacteria (89.8% average abundance) with Firmicutes representing the next most abundant phylum (5.9% average abundance) (Figure 12B). Further taxonomic investigation revealed that the most abundant bacterial species present was the gammaproteobacterium *Rhodanobacter terrae* (66.8% average abundance). While *Rhodanobacter* dominated the vast majority of cellulitis samples, other clinically relevant genera present included *Streptococcus*, *Staphylococcus*, *Propionibacterium*, and *Burkholderia* (Figure 12D).

To confirm and extend the taxonomy observation obtained by looking at the V1-V3 portion of the 16S rRNA, we next amplified, cloned and sequenced at least 10 full-length 16S genes from four separate cellulitis samples: 2055, 2080, 2203, and 2250. In

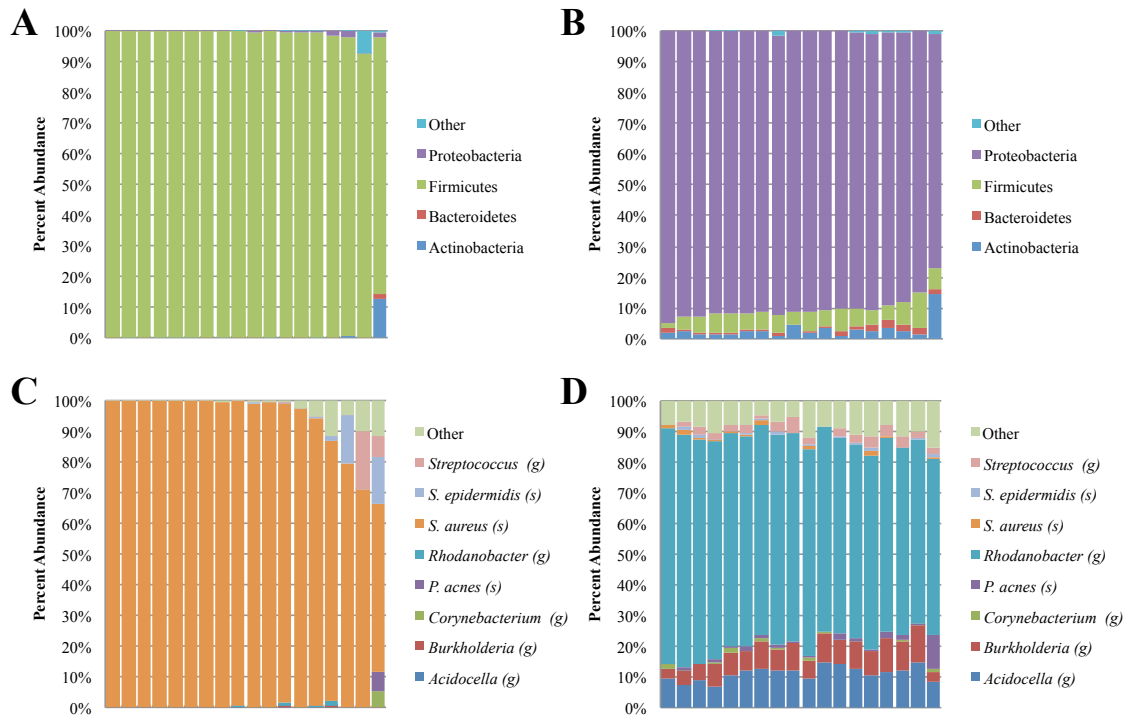


Figure 12. Microbial composition within purulent abscesses (A and C) and cellulitis (B and D) samples.

The microbiomes were characterized at the bacterial phylum level (A and B) as well as the genus (g) and species (s) levels (C and D). Each column corresponds to a single SSTI microbiome.

Table 9. Full-Length 16S rRNA taxonomy from Cellulitis Samples

| | 2055 | 2080 | 2203 | 2250 |
|----------------|---|---|--|---|
| Clone 1 | <i>Rhodanobacter terrae</i> ; SPg; FJ405366 100% | uncultured bacterium; nbu286c01c1; KF063132 100% | uncultured bacterium; nbu286c01c1; KF063132 98.8% | uncultured bacterium; nbu286c01c1; KF063132 100% |
| | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 99.8% | <i>Dyella</i> sp. K4; FR874237 99.1% | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 99.1% |
| | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 99.8% | <i>Rhodanobacter</i> sp. A2-60; KF441591 98.4% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.3% | <i>Rhodanobacter</i> sp. A2-60; KF441591 98.4% |
| Clone 2 | <i>Rhodanobacter terrae</i> ; SPg; FJ405366 100% | uncultured bacterium; nbu286c01c1; KF063132 99.0% | uncultured bacterium; nbu286c01c1; KF063132 99.0% | uncultured bacterium; nbu286c01c1; KF063132 99.8% |
| | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 99.8% | <i>Dyella</i> sp. K4; FR874237 99.0% | <i>Dyella</i> sp. K4; FR874237 0.939 | <i>Dyella</i> sp. K4; FR874237 0.989 |
| | <i>Rhodanobacter terrae</i> str. SPg 96.42% | <i>Rhodanobacter</i> sp. A2-60; KF441591 98.3% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 98.3% |
| Clone 3 | uncultured bacterium; nbu286c01c1; KF063132 99.2% | uncultured bacterium; nbu286c01c1; KF063132 99.4% | uncultured bacterium; nbu286c01c1; KF063132 99.4% | uncultured bacterium; nbu286c01c1; KF063132 99.3% |
| | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 94.3% | <i>Dyella</i> sp. K4; FR874237 94.4% |
| | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.8% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.9% | <i>Rhodanobacter</i> sp. A2-60; KF441591 89.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.1% |
| Clone 4 | uncultured bacterium; nbu286c01c1; KF063132 99.9% | uncultured bacterium; nbu286c01c1; KF063132 99.9% | uncultured bacterium; nbu286c01c1; KF063132 98.5% | uncultured bacterium; nbu286c01c1; KF063132 99.3% |
| | <i>Dyella</i> sp. K4; FR874237 0.943 | <i>Dyella</i> sp. K4; FR874237 94.8% | <i>Dyella</i> sp. K4; FR874237 93.7% | <i>Dyella</i> sp. K4; FR874237 94.2% |
| | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.6% | <i>Rhodanobacter</i> sp. A2-60; KF441591 91.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.0% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.1% |
| Clone 5 | uncultured bacterium; nbu286c01c1; KF063132 99.9% | uncultured bacterium; nbu286c01c1; KF063132 99.1% | uncultured bacterium; nbu286c01c1; KF063132 99.4% | uncultured bacterium; nbu286c01c1; KF063132 99.1% |
| | <i>Dyella</i> sp. K4; FR874237 0.948 | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 94.0% |
| | <i>Rhodanobacter</i> sp. A2-60; KF441591 91.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.2% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.6% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.5% |
| Clone 6 | uncultured bacterium; nbu286c01c1; KF063132 99.2% | uncultured bacterium; nbu286c01c1; KF063132 99.0% | bacterium BM0247; JQ680693 100% | uncultured bacterium; nbu286c01c1; KF063132 99.5% |
| | <i>Dyella</i> sp. K4; FR874237 94.1% | <i>Dyella</i> sp. K4; FR874237 0.933 | <i>Escherichia coli</i> ; r47; JQ661027 99.8% | <i>Dyella</i> sp. K4; FR874237 94.3% |
| | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 89.5% | <i>Escherichia coli</i> ; H16; JN129456 99.8% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.6% |
| Clone 7 | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 100% | uncultured bacterium; nbu286c01c1; KF063132 99.5% | <i>Rhodanobacter terrae</i> ; SPg; FJ405366 100% | uncultured bacterium; nbu286c01c1; KF063132 99.3% |
| | <i>Rhodanobacter</i> sp. B64; EU194895 100% | <i>Dyella</i> sp. K4; FR874237 94.4% | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 99.8% | <i>Dyella</i> sp. K4; FR874237 94.2% |
| | <i>Rhodanobacter terrae</i> ; SPg; FJ405366 99.8% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.7% | <i>Rhodanobacter</i> sp. B64; EU194895 99.5% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.3% |
| Clone 8 | <i>Escherichia coli</i> ; r47; JQ661027 99.1% | uncultured bacterium; nbu286c01c1; KF063132 99.4% | uncultured bacterium; nbu286c01c1; KF063132 98.3% | Uncultured bacterium JF204832 100% |
| | uncultured organism; ELU0124-T310-S-NI_000496; HQ791715 99.1% | <i>Dyella</i> sp. K4; FR874237 94.2% | <i>Dyella</i> sp. K4; FR874237 93.3% | uncultured bacterium; ncd2350h08c1; JF205603 100% |
| | <i>Escherichia coli</i> ; NBRC 12062; AB680228 99.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.6% | <i>Rhodanobacter</i> sp. A2-60; KF441591 89.9% | <i>Streptococcus oralis</i> ATCC 700233 99.6% |
| Clone 9 | uncultured bacterium GQ096144 99.6% | uncultured bacterium; nbu286c01c1; KF063132 99.1% | uncultured bacterium; nbu286c01c1; KF063132 99.6% | uncultured bacterium; nbu286c01c1; KF063132 99.4% |
| | <i>Acinetobacter parvus</i> KJ806336 99.6% | <i>Dyella</i> sp. K4; FR874237 93.9% | <i>Dyella</i> sp. K4; FR874237 94.5% | <i>Dyella</i> sp. K4; FR874237 94.2% |
| | uncultured bacterium; nbw109h05c1; GQ007804 99.3% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.4% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.9% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.6% |

| | | | | |
|-----------------|--|---|---|---|
| Clone 10 | <i>Rhodanobacter glycinis</i> ; MO64; EU912469 98.7% | uncultured bacterium; nbu286c01c1; KF063132 99.9% | uncultured bacterium; nbu286c01c1; KF063132 99.1% | uncultured bacterium; nbu286c01c1; KF063132 98.0% |
| | <i>Rhodanobacter</i> sp. B64; EU194895 98.7% | <i>Dyella</i> sp. K4; FR874237 94.8% | <i>Dyella</i> sp. K4; FR874237 93.9% | <i>Dyella</i> sp. K4; FR874237 93.2% |
| | <i>Rhodanobacter terrae</i> ; SPg; FJ405366 98.0% | <i>Rhodanobacter</i> sp. A2-60; KF441591 91.1% | <i>Rhodanobacter</i> sp. A2-60; KF441591 88.8% | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.0% |
| Clone 11 | uncultured bacterium; nbu286c01c1; KF063132 99.9% | | uncultured bacterium; nbu286c01c1; KF063132 99.5% | |
| | <i>Dyella</i> sp. K4; FR874237 94.8% | | <i>Dyella</i> sp. K4; FR874237 94.6% | |
| | <i>Rhodanobacter</i> sp. A2-60; KF441591 91.1% | | <i>Rhodanobacter</i> sp. A2-60; KF441591 90.9% | |

Taxonomy information according to the RDP database. GenBank accession listed after species name. Top three hits per clone are given. Percentages represent percent identity.

support of the 454 sequencing data, a large proportion of the full-length 16S sequences were classified as *Rhodanobacter* (Table 9). In addition, many of the sequences were assigned to the closely related *Dyella* genus. Besides *Rhodanobacter* and *Dyella*, we also detected *Acinetobacter* and *Streptococcus*. Additionally, two of the samples were identified as *E. coli*. While this may indicate infection with *E. coli*, it may also be due to purification of contaminate *E. coli* DNA from the *E. coli* TOP 10 cells that carried the cloned 16S sequences.

DISCUSSION

Given the dramatic differences in physical presentation between cutaneous abscess and cellulitis, it stands to reason that there may be specific risk factors and/or microbial profiles associated with each SSTI type. In our military specific study population, we found that the bulk of the risk factors assessed were not significantly different between cutaneous abscess and cellulitis patients. However, we did find that white ethnicity and prior blister formation were strongly associated with cellulitis while cutaneous abscess formation was linked to nasal colonization with MRSA. These apparent risk factor differences prompted a subsequent investigation into the bacterial composition of SSTIs. Our high-throughput sequencing and culture data confirmed that *S. aureus* is typically associated with cutaneous abscess. For cellulitis aspirates, however, we observed a dramatic difference in microbiota as compared to abscesses; *Rhodanobacter/Dyella* were identified as the predominant genera. To our knowledge, this is the first clinical investigation that utilizes an epidemiological approach in addition to

high-throughput sequencing to characterize the two most commonly diagnosed SSTIs, cellulitis and cutaneous abscess.

To date, there are numerous risk factors associated with SSTI development. These include, but are not limited to injectional drug use, diabetes, and antecedent *S. aureus* carriage (79; 251; 280). Unfortunately, most of these studies failed to differentiate between the various SSTI types. By separating military trainees with SSTI into either an “abscess only” or “cellulitis only” group, we were able to discern any SSTI-specific risk factors (Table 8) Our findings are in agreement with a UK-based prospective study of 150 patients with lower limb cellulitis and 300 healthy controls that also observed an association between cellulitis development and white ethnicity (120). Of note, that study also suggested that preceding blister formation may be a risk factor of cellulitis but the findings failed to reach statistical significance (120). With respect to abscess formation, we found that trainees colonized with MRSA were more likely to harbor an abscess rather than cellulitis. This is not entirely surprising given the high prevalence of MRSA-associated abscesses in the military and general population (89; 206; 226). Although these data suggest a clear association between MRSA and abscess formation, it may also infer that MRSA is less likely to be the cause of cellulitis infections. This is further supported by the low abundance of *Staphylococcus aureus* detected in the skin of cellulitis infection (< 2% percent abundance) (Figure 12). While the exact contribution of *S. aureus* to cellulitis infections remains unclear, our data suggests that other bacterial species may be involved.

With minor differences in associated risk factors, we also sought to determine any differences in bacterial composition between cutaneous abscess and cellulitis. Recently,

we characterized 40 abscess microbiomes in military trainees at Fort Benning (144). We found that while most abscesses were dominated by *S. aureus*, polymicrobial infections were prevalent. Given this interesting phenomenon, we enrolled an additional 18 trainees suffering from cutaneous abscess and found remarkably similar microbial compositions; while *S. aureus* dominated most abscesses, there were 4 mixed infections. Given that polymicrobial SSTIs are typically associated with severe disease such as necrotizing fasciitis (76; 194), it would perhaps be interesting in future studies to document the progression of a polymicrobial abscess as opposed to a monomicrobial infection.

Unlike purulent abscesses, we observed cellulitis microbiomes rich in Proteobacteria, particularly from the *Rhodanobacter/Dyella* genera. Of note, there are no prior reports that suggest that *Rhodanobacter* or *Dyella* infect humans or carry virulence factors. Thus, given the study population of military trainees, perhaps *Rhodanobacter* gains entry into the skin via minor cuts and abrasions and subsequent contact with the soil. These findings further add to the ongoing debate regarding the bacterial etiology of cellulitis. In a pediatric study that utilized needle aspirates, 3 of the 20 patients cultured (15%) yielded a pathogen, all of which were MRSA (231). A separate study in adults found that only 5 of 50 (10%) cellulitis aspirates were culture positive (129). Given the limited success of aspirate culture, more sensitive detection methods such as PCR and DNA sequencing are being considered. Similar to our study, a recent investigation also subjected needle aspirates to high-throughput sequencing (63). Interestingly, they found a wide array of atypical bacteria that were not observed in our study such as *Acidovorax*, *Enterococcus*, and *Lactococcus*. There are numerous reasons that could explain this discrepancy; these include differences in study populations, site of needle aspiration

(center versus leading edge), or sequencing data analysis pipelines. Despite these differences, both studies suggest a potential role of atypical bacterial in cellulitis infections. We can hypothesize that cellulitis pathology may be an immune response to the presence of the atypical bacterium (*i.e. Rhodanobacter*). This idea is supported by previous reports that suggest an inflammatory component to cellulitis infections (65) and the observation that cellulitis is often resolved by the use of NSAIDS and steroids (23; 65). Thus, cellulitis may be a nonspecific immune response to atypical bacteria rather than an actual infection.

Despite conflicting results exploring cellulitis etiology, culture-based techniques favor *S. aureus* as the principal cause over beta-hemolytic streptococci (BHS) (49). While only one aspirate in our study yielded CNS, 8 of the cellulitis patients had elevated ASO titers at the acute and/or convalescent time points: a 36.4% seroconversion rate indicative of BHS exposure. In an investigation assessing serologic conversion, 73% of non-purulent cellulitis was suggested to be caused by BHS and 27% was not identifiable (142). Another study yielded a similar percent serconversion rate (74%) for cellulitis and erysipelas (171). Together these reports suggest the bulk of non-purulent cellulitis may be caused by BHS. While the ASO seroconversion rate in our study was lower than previous reports, it still represents a significant proportion of our total population. While our pyrosequencing data did detect *Streptococcus* within cellulitis infections (Figure 12D), it represented a minor fraction of the total number of reads (approximately 2-5% average abundance) and was greatly outnumbered by the atypical bacteria discussed above.

As with most clinical studies, there are limitations to our study. For our protocol, needle aspiration was performed on cellulitis patients and not healthy controls; a

limitation set forth by the Institutional Review Board. It should be noted that a few studies have begun to characterize the “healthy subcutaneous tissue microbiome,” and have detected bacteria such as *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Pseudomonas spp.* in both the reticular and dermal adipose layers (213).

Interestingly, all three of these organisms were also detected in our cellulitis aspirates (Table 10 (S2)). While it is premature to speculate on the role of these subcutaneous bacteria in cellulitis, our data will be of use in the future as researchers continue to investigate cellulitis etiology. Secondly, for the risk factor analysis, self-reporting can be error prone if the patient either cannot accurately recall specific events in their medical history or chose to withhold information. However, given our military study population, we expect minimal error due to self-reporting. We also note that while elevated ASO titers suggest BHS exposure, it is difficult to determine if they are associated with cellulitis or prior exposure to BHS (ex. strep throat). It is also possible given the low abundance of bacteria observed in cellulitis infections that ASO titers may not yet be detectable (false-negative). Additionally, our study population is composed entirely of young, healthy males stationed at Fort Benning and may not accurately represent SSTIs in the general public or in non-healthy/immunocompromised patients. Lastly, we acknowledge that participation was suboptimal for the body site colonization data, particularly for the inguinal and perianal locations. However, this data will be valuable in the future as we continue to sample military trainees at Fort Benning as part of additional ongoing research projects.

In conclusion, our results emphasize that cutaneous abscess and cellulitis represent two distinct diseases. While purulent abscesses are rich in *S. aureus* and

associated with MRSA nasal colonization, non-purulent cellulitis is composed of atypical bacteria such as *Rhodanobacter/Dyella* and linked to white ethnicity and prior blister formation. Thus, while both infections are considered SSTIs, they are dramatically different than one another and treatment should be uniquely tailored to either cutaneous abscess or cellulitis.

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CHAPTER FOUR: Recurrent Methicillin-Resistant *Staphylococcus aureus* Cutaneous Abscesses and the Selection of Reduced Chlorhexidine Susceptibility During Chlorhexidine Use

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The work presented in this chapter is the sole work of R.C. Johnson with the following exceptions: K. Crawford performed pulsed field gel electrophoresis and PCR for toxin/resistance genes; study oversight at Fort Benning provided by C.D. Schlett and M.W. Ellis; strain isolation performed by J.B. Lanier.

ABSTRACT

We describe the selection of reduced chlorhexidine susceptibility during chlorhexidine use in a patient with two episodes of cutaneous USA300 methicillin-resistant *Staphylococcus aureus* (MRSA) abscess. The second clinical isolate harbors a novel plasmid that encodes the QacA efflux pump. Greater use of chlorhexidine for disease prevention warrants surveillance for resistance.

CASE REPORT

An 18 year-old man undergoing Infantry basic training at Fort Benning, Georgia, presented to the outpatient clinic in July complaining of a painful skin lesion on his left knee. On physical examination, the patient was afebrile (37° C) and examination was remarkable only for a pre-patellar nodule that was erythematous, warm, indurated, tender, and fluctuant. The patient had no other skin lesions and no lymphadenopathy. The remainder of his exam was normal and the patient did not report a history of cutaneous abscesses. The patient was diagnosed with a cutaneous abscess without joint involvement and underwent incision and drainage. The purulent material underwent standard wound culture and yielded methicillin-resistant *Staphylococcus aureus* (MRSA) using the BD Phoenix automated microbiology system (Becton Dickinson, Sparks, MD). The isolate was resistant to oxacillin and erythromycin and had inducible clindamycin resistance as determined by double-disk diffusion (95), but was susceptible to trimethoprim-sulfamethoxazole (TMP-SMX), doxycycline, levofloxacin, linezolid, daptomycin, and vancomycin (Table 11). With no known medication allergies, the patient was treated with a ten day course of twice-daily Double Strength (DS) TMP-SMX and underwent serial follow-up examinations and wound care with complete resolution of his abscess within 14 days.

Nine weeks after his initial presentation, the patient returned to the clinic. This time he complained of a similar painful lesion on his left foot. On physical examination, the patient was again afebrile (37° C) and examination was remarkable only for an inflamed and fluctuant nodule on the dorsum of his left foot. The patient was again

Table 11. Molecular characteristics and antimicrobial susceptibilities of clinical MRSA isolates

| Characteristic | Clinical Isolate 1 (C01) | Clinical Isolate 2 (C02) |
|-----------------------|-------------------------------------|-------------------------------------|
| Sequence type | ST8 | ST8 |
| Pulsed-field type | USA300 | USA300 |
| Oxacillin | Resistant | Resistant |
| Erythromycin | Resistant | Resistant |
| Clindamycin | Inducible resistance | Inducible resistance |
| TMP-SMX | Susceptible | Susceptible |
| Doxycycline | Susceptible | Susceptible |
| Daptomycin | Susceptible | Susceptible |
| Vancomycin | Susceptible | Susceptible |
| Levofloxacin | Susceptible | Resistant |
| <i>qacA gene</i> | Negative | Positive |
| <i>mecA</i> | Positive | Positive |
| <i>SCCmec</i> Type | IV | IV |
| <i>PVL</i> | Positive | Positive |
| <i>mupA</i> | Negative | Negative |
| <i>norA</i> | Positive | Positive |
| Chlorhexidine MIC | 0.3 µg/mL | 0.8 µg/mL |

diagnosed with a cutaneous abscess and underwent incision and drainage with standard wound cultures yielding MRSA. This second clinical isolate shared the same antibiotic susceptibility pattern as the first MRSA isolate; however, it was resistant to levofloxacin (Table 11). The patient was treated in a similar fashion as the first episode and recovered without additional recurrences.

The patient was a soldier participating in a prospective cluster-randomized trial aimed at preventing skin and soft-tissue infections, which are common in this population (83). The patient was in a study group that received chlorhexidine for weekly showering (4% chlorhexidine gluconate, Hibiclens[®], Mölnlycke Heath Care, Norcross, Georgia). As part of the protocol, the patient completed a questionnaire at the time of his second episode that queried his chlorhexidine use; he reported using the agent every other week throughout his training. In terms of chlorhexidine use, the patient would have used the agent 1-2 times before his first episode, and 4-5 times prior to the second episode.

The two MRSA isolates underwent molecular analysis including typing with pulsed-field gel electrophoresis (PFGE) (193), multilocus sequence typing (MLST) (84), and polymerase chain reaction (PCR) to assess for toxin (98) and resistance genes (45; 189). PFGE findings were resolved and analyzed using BioNumerics (Applied Math, Austin, TX). Both MRSA isolates were sequence type 8 (ST8), USA300, staphylococcal cassette chromosome *mec* type IV (SCCIV), positive for Panton-Valentine leukocidin (*lukS-PV*) and negative for high level mupirocin resistance (*mupA*). The first clinical isolate (C01) was negative for the chlorhexidine resistance genes (*qacA/B*), but the second clinical isolate (C02) was positive for *qacA/B*. As part of the research protocol

(2), anterior nares sampling at the second episode revealed that the patient was colonized with a separate, unrelated methicillin-susceptible *S. aureus* strain (sequence type 30).

Both clinical isolates underwent chlorhexidine susceptibility testing. Briefly, about 4×10^4 colony forming units (CFUs) from an overnight culture were inoculated into 1 mL of Cation Adjusted Mueller Hinton II Broth (BD BBL) containing purified chlorhexidine (Sigma) that ranged in concentration from 0 to 1 $\mu\text{g/mL}$. The cultures were grown overnight with shaking (220 rpm). Upon visual analysis (Figure 13), the minimum inhibitory concentration (MIC) of chlorhexidine for the C01 isolate was 0.3 $\mu\text{g/mL}$ while the C02 isolate had a MIC of 0.8 $\mu\text{g/mL}$: approximate 2.7 fold increase over C01.

Previous reports have found that mutations in the promoter of the *norA* efflux pump can lead to increases in *norA* transcription, which result in increased resistance to antiseptic agents such as chlorhexidine (99; 220). The promoter region of *norA* was PCR amplified and sequenced using the 5'-GTCTTGGTCATCTGCAAAGGTTG-3' and 5'-GACTGGTATTACTAAACCGATACC-3' primers. Additionally, the 5'-GGTGGTATGAGTGCTGGTATGG-3' and 5'-GCATACGATGTGAACTTCTGCC-3' primers were used to assess *norA* transcription via RT-PCR. Total RNA was extracted from the C01 and C02 isolates using the Qiagen EasyRNA kit. Prior to RNA extraction, *S. aureus* was lysed by incubation in lysis buffer containing buffer TE, lysostaphin (20 $\mu\text{g/mL}$), and proteinase K (200 $\mu\text{g/mL}$) for 1 hour. cDNA was synthesized from total RNA using the QuantiTect Reverse Transcription kit (Qiagen). All PCR and sequencing reactions were performed as previously described (80). RT-PCR was performed in 10 μL reactions containing 1X SYBR Green (Qiagen) and 1.5 μM of each primer. Reaction mixtures were incubated for 5 minutes at 95°C followed by 35 cycles of 95°C for 10 s

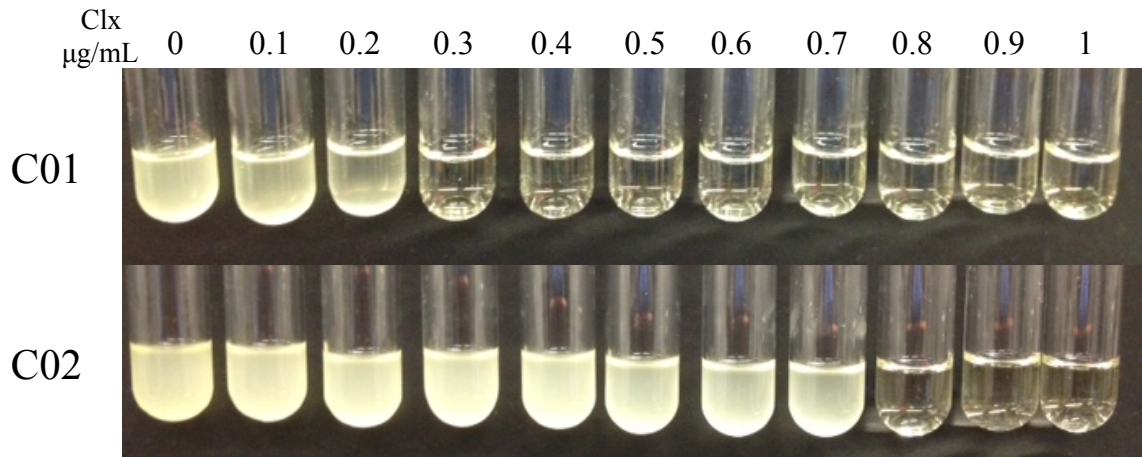


Figure 13. Minimum inhibitory concentration (MIC) of chlorhexidine (Clx) for the two *Staphylococcus aureus* abscess isolates (C01 and C02). The concentration of Clx tested ranged from 0 to 1 μg/mL. All cultures were inoculated with approximately 4×10^4 colony forming units (CFUs) and grown overnight at 37°C with shaking at 220 rpm. The MIC for the C01 strain was determined to be 0.3 μg/mL while the C02 isolate had a MIC of 0.8 μg/mL.

then 50°C for 10 s. Fluorescence readings were acquired at the end of each cycle using the Qiagen Rotor-Gene Q RT-PCR Machine. Sequencing data analysis revealed that the C01 and C02 *norA* promoters were identical in nucleotide composition. Not surprisingly, *norA* expression in the C02 isolate was indistinguishable from C01.

Plasmid extraction using the Qiagen Plasmid Purification kit and subsequent PCR amplification (5'-GCTGCATTTATGACAATGTTTG-3' and 5'-AATCCCACCTACTAAAGCAG-3') (304) and visualization on a 1% agarose gel revealed that the *qacA* gene was only detectable in the C02 isolate. Given the high level of nucleotide similarity between the *qacA* and *qacB* genes, we sequenced and then compared the DNA sequence of *qacA* from the C02 plasmid to the canonical *qacA* (accession number: GU565967.1) and *qacB* (accession number: AF053772.1) sequences. Of the seven amino acid differences described by Paulsen *et al.* that distinguish *qacA* from *qacB*, 6 of the *qacA* residues were observed in the *qacA* gene from C02; this includes a key aspartic acid residue carried at amino acid position 323 (232). The one amino acid residue that differed from the *qacA* consensus occurred at the first position, where an alternative lysine start codon was found. In total, the data suggest that C02 harbors the *qacA* gene. Finally, to ensure that the C02 isolate was the only strain that expressed the *qacA* efflux pump, we utilized cDNA as a template for *qacA* PCR amplification using the same *qacA* detection/sequencing primers as mentioned beforehand. Amplicon detection on a 1% agarose gel confirmed that the C02 isolate actively expressed the *qacA* gene while the C01 isolate did not.

Previous reports have found that *qacA* is typically encoded on the pSK1 family of plasmids (188; 218). We therefore sequenced approximately 550 base pairs upstream of

the *qacA* gene using the 5'-CTCCAATCCTTATAGACCGTGC-3' primer; we found a high level of nucleotide similarity to the pSK1 DNA sequence (Genbank accession number NC_014369) (>99% nucleotide similarity). To determine if the plasmid was indeed pSK1, we next used the pSK1 plasmid sequence to design a series of 10 PCR primer pairs that span the entire plasmid (Table 12). We found that only the primer pair that encompassed the *qacA* gene (primer pair 9) yielded a PCR amplicon of the correct size in the C02 isolate. This led us to conclude that the *qacA* gene found in the C02 isolate may be encoded on a pSK1-like plasmid, but not on pSK1 itself.

To assess clonality between the two isolates, total DNA was prepared from both isolates via phenol-chloroform extraction and was subjected to Pacific Biosciences RS II SMRT whole-genome sequencing (University of Maryland, School of Medicine, Institute for Genome Sciences). A single closed circular chromosome and plasmid were obtained for each isolate. A comprehensive list of chromosome and plasmid characteristics is included (Table 13). Genome analysis revealed that the C01 isolate contained 2,770 chromosomal open reading frames (ORFs) and was approximately 2.92 megabase pairs in length. Conversely, the C02 isolate had a reduced chromosome of 2.86 megabase pairs encoding 2,704 ORFs. In addition to whole gene changes, we observed over 140 single nucleotide polymorphisms (SNPs) between the two chromosomes, which suggests that the two isolates are genetically distinct. Of note, two nonsynonymous SNPs in the C02 *gyrA* and *griA* genes (C251T and T239A, respectively) were detected. These SNPs result in a S84L amino acid mutation in GyrA and a F80Y mutation in GriA that have previously been shown to contribute to quinolone resistance (271; 290; 317) and therefore likely explain the levofloxacin resistance of the C02 isolate.

Table 12. pSK1-like plasmid PCR primer panel

| Primer Pair | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|--------------------|-------------------------------|-------------------------------|
| 1 | ggagcactagtagcaacttcatc | ccagagccgatgctacgc |
| 2 | gccttaaaattccaggcgc | gctgaaagttatagagcggc |
| 3 | gaagcactgcatcacgatagtg | gctcacgctataccgacattc |
| 4 | ctaacgtgcgatcagatgcttg | gcaccctcagaagccattc |
| 5 | cgcagttggagcaagtgag | ctttatcttcgactctatcacgaac |
| 6 | catcatagcaccagtcacag | gtgtgcgatcatcgcgtctattc |
| 7 | caattaccttggcacttaccaaatg | ggttggaagaacgcacatatg |
| 8 | cttagatagtagccaacggctac | catcgtatc gatcttgtgtcc |
| 9 | cgatcgcacggtctataagg | gctttgaatctcttcgctttcag |
| 10 | cgaagacgccttcaatataccg | cctagagcttgccatgtatatg |

Table 13. Genome and plasmid statistics

| Chromosome | Plasmid | Size (bp) | % GC | # of ORFs | Average Gene Length (bp) | % Coding |
|-------------------|----------------|------------------|-------------|------------------|---------------------------------|-----------------|
| C01 | - | 2,918,599 | 32.8 | 2,770 | 879 | 84.5 |
| C02 | - | 2,864,998 | 32.8 | 2,704 | 882 | 84.4 |
| - | pC01 | 27,044 | 30.6 | 32 | 592 | 70.1 |
| - | pC02 | 61,537 | 29.5 | 71 | 677 | 78.2 |

The pC01 and pC02 plasmid sequences were analyzed using the Basic Local Alignment Software Tool (BLAST, NCBI). While the pC01 sequence shared near perfect identity to known *S. aureus* plasmids such as SAP046A (GenBank accession no. NC_013294.1), the pC02 plasmid was significantly larger and less than 40% of the plasmid contained regions of similarity to other sequences in the NCBI database. Annotation of the pC02 plasmid revealed the presence of numerous proteins that range in cellular processes including DNA replication (Ssb, TopB), transcriptional regulation (QacR) and substrate translocation (CadC). The fully annotated pC02 map is depicted in Figure 14 (59). The *qacA*-containing pC02 plasmid appears to be novel and may represent an additional class of antimicrobial resistance plasmids in *S. aureus*. The C01 and C02 genomes and plasmids were submitted to NCBI and given the following accession numbers: CP012118, CP012119, CP012120, and CP012121.

DISCUSSION

Chlorhexidine is a broad-spectrum topical biguanide cationic antiseptic agent with activity against *Staphylococcus aureus* (192; 200). Although it has been used for decades in various roles, ranging from hand washing to preoperative skin preparation, chlorhexidine has been increasingly employed for the prevention of both nosocomial (46; 52; 87; 133) and community-associated infections (83; 103; 198; 325). Evidence from large randomized-control trials points to the importance of chlorhexidine in prevention of the spread of drug-resistant organisms and hospital acquired infections (52; 133; 199).

Indeed, chlorhexidine has also been an integral component of strategies aimed at prevention of recurrent MRSA SSTI in individuals and households (104; 198).

Despite its widespread use, the prevalence of chlorhexidine resistance in the U.S. is low (approximately 1%) (103; 190; 268); this is in contrast to observations in other countries (304; 314). When used in large trials, both in community and hospital settings, chlorhexidine resistance has been only rarely reported (52; 133; 268; 325). Nevertheless, with the widespread and increasing use of this agent, experience has shown that there is appropriate concern for the potential emergence of chlorhexidine resistance (314). Additional studies that investigate the frequency of chlorhexidine use and selection of chlorhexidine resistant strains must be conducted to ensure proper chlorhexidine stewardship.

The plasmid-borne *qacA* gene in particular encodes an efflux pump that has been shown in numerous reports to confer resistance to numerous hydrophobic compounds, including cationic biocides, such as chlorhexidine (131; 169; 192). While there are no established breakpoints for chlorhexidine resistance, the presence of these genes has been associated both with increased MIC and with untoward clinical outcomes (20; 61; 168; 225). Interestingly, multiple reports have identified chlorhexidine resistant *S. aureus* isolates with MICs of ≥ 4 $\mu\text{g}/\text{mL}$ (61; 190; 268). The isolate described in this report, however, showed a reduced MIC (≤ 0.8 $\mu\text{g}/\text{mL}$). While the reason for the lower MIC is not clear, this may be due to reduced translation efficiency due to the alternate start codon found in the C02 *qacA* gene (221). The presence of *qacA/B* and increased MIC are sometimes poorly correlated (190); however, in our isolates, an increase in MIC was clearly observed for the *qacA*-positive C02 strain. We cannot determine the overall

clinical impact of this reduced chlorhexidine susceptibility in our patient other than to note that he developed a second USA300 MRSA abscess.

Chlorhexidine has residual antibacterial activity, which may be beneficial in reducing bacterial burden or preventing the spread of resistant organisms (238); however, this residual activity may also contribute to an environment that ultimately fosters resistance (304). Since our patient utilized chlorhexidine every other week, this may have played a part in the selection of reduced chlorhexidine susceptibility in the patient. Evidence suggests that the *qacA* gene may be able to be horizontally transferred across various staphylococcal species (131; 219). This typically is plasmid mediated since numerous reports have shown that the *qacA* gene is often encoded on a plasmid from the pSK1-family of vectors (159; 169). Although we do not know its original source, *qacA* in our identified clinical MRSA isolate was carried on a large, uncharacterized plasmid, which shows limited similarity to pSK1. This finding suggests that transmission of *qacA* is not limited to the pSK1-like vectors. Furthermore, the identification of this novel *qacA*-containing plasmid combined with the now ubiquitous use of chlorhexidine, highlights the need for increased surveillance programs that would seek to understand the evolution of *qacA* transmission across MRSA isolates and potentially across other staphylococcal species.

In summary, to our knowledge, this is the first report of the selection for increased chlorhexidine MICs while using chlorhexidine in a community-based patient with recurrent USA300 MRSA SSTI. In light of recent clinical trials that show the benefit of chlorhexidine in prevention of drug-resistant infections, the medical community should anticipate greater use of this agent, and consequently increased resistance. Further study

and surveillance for the emergence of chlorhexidine resistance should be considered in healthcare and community settings that use chlorhexidine for disease prevention.

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CHAPTER FIVE: Discussion

Despite the advances in medicine, the incidence rate of SSTIs among the human population continues to rise. Furthermore, SSTI prevention measures and treatments are repeatedly unsuccessful; a clear indication that our understanding of SSTIs is profoundly limited. While there are numerous factors that contribute to the complexity of SSTIs, two overarching unknowns include SSTI etiology and associated risk factors. As such, the primary goal of this dissertation was to investigate these two variables at the molecular and microbiome levels. Specifically, the studies described herein concentrate on the contribution of *Staphylococcus aureus* to SSTI formation and susceptibility.

In Chapter 2, we utilized high-throughput sequencing to characterize the nasal microbiomes of military trainees that either did or did not harbor a purulent abscess. We determined that bacteria from the Proteobacterial phylum were more abundant in the nares of individuals that did not have an SSTI. Additionally, we detected differences in microbial diversity between the SSTI and healthy nasal microbiomes that were independent of *S. aureus* colonization status. After looking at the nasal microbiome, we also determined the bacterial composition of purulent abscesses; while most purulent abscesses were dominated by *S. aureus*, polymicrobial infections were frequently observed. Finally, we demonstrated that the presence or absence of *S. aureus* in the nose and abscess had a profound impact on microbiome composition. Together, these data emphasize the complexity of the nasal and abscess microbiomes and provide a novel correlation between SSTI development and nasal microbiome composition.

The bacterial etiology of SSTIs is a debated topic in medicine. Specifically, the microbial species responsible for the often uncultivable cellulitis infections remains unclear. In Chapter 3, we sought to characterize the microbiome of both purulent and non-purulent (cellulitis) infections using a high-throughput sequencing strategy. We showed that the bacterial composition between the two SSTIs was remarkably different. We also employed an epidemiological analysis to assess risk factors associated with either purulent or non-purulent SSTIs. These results demonstrate the variability between SSTI disease types and emphasize the need for bacterial culture when treating SSTIs.

Finally, in Chapter 4, we employed molecular techniques in conjunction with whole-genome sequencing to characterize two strains of *S. aureus* that caused sequential SSTIs in a single military trainee. Interestingly, the patient was an active participant of a chlorhexidine soap hygiene trial. We found that the second isolate of *S. aureus* exhibited decreased susceptibility to chlorhexidine potentially due to the acquisition of the QacA efflux pump. Of epidemiological interest, the *qacA* gene was encoded on a large, previously uncharacterized plasmid. This clinical study demonstrates that in an era of increased chlorhexidine use, resistant strains are present in the population and are capable of causing SSTIs.

THE FUTURE OF HUMAN MICROBIOME RESEARCH

The study of the human microbiome has undoubtedly altered our perception of human health. Given the enormous numbers of colonizing bacteria, viruses, fungi, and protozoans, humans are now considered to be multi-organismal and should be treated as such. Research in the human microbiome field, while still in its infancy, is progressing at

a rapid rate, and there are elevated expectations of novel therapeutics and disease cures. Similar to the human microbiome project, the human genome project was initially created with high expectations; by merely analyzing an individual's DNA, scientists would be able to predict future disease susceptibilities and fine-tune personalized treatments. After more than 10 years since the first draft human genome was published, there have been numerous breakthroughs that have linked specific DNA signatures to human disease (114; 161). Interestingly, studies indicate that the variability between any two human genomes (~0.5%) is dwarfed by the variation between human microbiomes (173). Furthermore, this variability in microbiota can dramatically fluctuate over time (97), which sets the stage for hypothesis driven research projects that attempt to elucidate the mechanisms behind this microbial dysbiosis. Additionally, therapeutics that alter microbiome composition should prove easier, both technically and ethically, than genome manipulation. However, as with the human genome project, researchers and clinicians will likely need to be patient when it comes to microbiome therapies; while our current understanding of the human microbiome has exploded over the last decade, this growth in knowledge should not be misconstrued as a complete understanding of the interactions of humans with their bacterial inhabitants. The human microbiome is still an emerging science with major advances yet to come. As such, it is our hope that this thesis will serve as an important building block that will help researchers start to understand how the human microbiome influences our health. Furthermore, by studying the human microbiome and its association with SSTI formation, we provide the foundation required for future microbiome related therapies aimed at SSTI prevention.

CAN WE IDENTIFY A “MARKER” MICROBIOME?

One of the most exciting aspects of microbiome research is the potential for utilizing microbial composition as a predictor of disease susceptibilities. From a public health perspective, the ability to identify specific microbiome perturbations or signatures that can be exploited as a simple diagnostic would be ideal. While numerous studies have investigated how specific diseases alter microbial composition (50; 113; 204; 242), significantly fewer studies have performed longitudinal analyses that determine if a particular microbiome signature makes an individual more or less susceptible to future disease development. Indeed, the data from our microbiome related studies are limited to a single time point. Consequently, whether a reduction of Proteobacteria in the nares (Chapter 2) or presence of *Rhodanobacter* in the skin (Chapter 3) is a marker for future SSTI formation cannot be determined without assessing bacterial abundance levels prior to SSTI. Unfortunately, longitudinal analyses aimed at measuring changes in microbiome composition before and after disease formation are confounded by the uncertainty that a sufficient number of individuals will develop the disease being studied. Thus, the study of many diseases is hampered by the sample size required to obtain meaningful results. This can partially be overcome by looking within “at risk” populations. For example, given the high rates of SSTIs among military trainees at Fort Benning (3; 82), this population may be ideal for identification of a marker microbiome associated with future SSTI development. To this end, our laboratory is currently in the midst of a 14-week longitudinal analysis in which samples from trainees will be collected at 5 time points beginning upon initial admission to Fort Benning. In addition to the anterior nares, samples will be collected from other common *S. aureus*-associated body sites such as

oropharynx, inguinal, and perianal region. By enrolling a large number of trainees (~600), it is predicted that a substantial number of participants will develop a SSTI. Thus, it is our hope that we can build upon the data in this dissertation in order to identify a “marker” microbiome that may be used to identify those trainees that are at highest risk for SSTI development.

Given the difficulty associated with the identification of disease-predicting microbiomes, a growing body of research has instead concentrated on using the microbiome to diagnose diseases that, if not detected early, can have severe manifestations. For instance, it is estimated that nearly half of the individuals with Type 2 diabetes are undiagnosed, and may go undetected for up to a decade (123; 124). Recent evidence suggests that early detection of Type 2 diabetes can vastly improve disease outcome and quality of life (126). Indeed, microbiome analysis was successfully used to identify approximately 60,000 microbial markers for Type 2 diabetes (246). In particular, it was shown that key butyrate-producing bacteria were less abundant and opportunistic pathogens were enriched in the gut of diabetics compared to healthy controls (246). Similar approaches have been implemented to identify microbial biomarkers associated with the early diagnosis of colorectal, oral, and esophageal cancers (182; 184; 339). Furthermore, for patients currently suffering from diseases such as HIV and idiopathic pulmonary fibrosis, specific fluctuation in the microbiome may indicate future disease progression (121; 312). While SSTI formation has been the main clinical outcome assessed in this dissertation, it may be valuable to assess microbiome alterations for any individuals that progress to more severe disease such as necrotizing fasciitis or bacteremia. Furthermore, as shown in Chapter 2 and 3, many of the purulent abscesses

analyzed were comprised of mixed infections. While it remains unclear whether polymicrobial SSTIs impact disease prognosis, this is an obvious microbiome signature that warrants further investigation. Together, the identification of specific marker microbiomes is of great interest to the medical community and will likely influence how clinicians diagnosis and treat patients in the future.

**THE INTERACTIONS BETWEEN HUMANS AND AN EVOLVING PATHOGEN:
*STAPHYLOCOCCUS AUREUS***

In 1880, a Scottish surgeon by the name of Alexander Ogston became interested in a bacterium isolated from wound infections that appeared as “a bunch of grapes” under the microscope (217). 135 years later, *S. aureus* is still considered an important bacterial pathogen associated with human infections. Despite advances in hygiene practices and antibiotic therapy, *S. aureus* continues to evolve in order to efficiently colonize and infect humans. Of the most notable *S. aureus* evolutions, the acquisition of methicillin resistance has resulted in an ongoing epidemic of methicillin resistant *S. aureus* (MRSA)-associated infections in both the community and hospital settings. Interestingly, while antibiotic resistance acquisition often results in diminished bacterial fitness (157; 186; 197), MRSA, particularly community-associated MRSA, appears to efficiently compensate for any possible fitness defect (58; 135). In fact, numerous reports suggest that antibiotic resistant strains of *S. aureus* exhibit enhanced virulence and pathogenesis (66; 176; 316). This is particularly worrisome given the small window between antibiotic introduction and corresponding detection of resistant *S. aureus* strains: less than 4 years for penicillin and 2 years for methicillin (43; 229). In Chapter 2, all study participants were co-enrolled in a hygiene trial where they were instructed to use a chlorhexidine

body wash weekly (82). Interestingly, as described in Chapter 4, one of the study participants developed a SSTI that contained a strain of *S. aureus* that harbored a large (>60kb), novel plasmid that encoded the QacA efflux pump. This pump has been shown to confer resistance to quaternary ammonium compounds (QACs) and cationic agents such as chlorhexidine (131; 169; 192). Whether this plasmid-containing strain of *S. aureus* exhibits altered fitness or virulence has yet to be determined. Interestingly, in *Listeria monocytogenes*, exposure to sublethal levels of QACs increased expression of virulence genes (146). Conversely, growth in subinhibitory concentrations of chlorhexidine leads to decreased exotoxin synthesis in *Streptococcus agalactiae* (107). Although we do not currently know how widespread this novel *qacA*-containing plasmid is among *S. aureus* isolates, reports have shown that the prevalence of chlorhexidine resistant isolates is low (~1-2%) (268). Nevertheless, this vector may present a potential threat to human health and should be closely monitored.

Colonization with *S. aureus* is a complicated matter. From a clinical and epidemiological standpoint, individuals fall into one of three groups: persistent carriers, non-carriers, and those that are intermittently colonized (nonpersistent carriers) (306). Interestingly, these three groups appear to harbor unique microbial and immunological signatures. For example, in the anterior nares, a decrease in bacterial diversity was observed in nonpersistent carriers as compared to carriers and non-carriers (334). Furthermore, non-carriers appear to have the unique ability to resist experimental colonization by *S. aureus* (34). Although the mechanism behind this “*S. aureus*-colonization resistance” is still unknown, there is likely an immune system component. Surprisingly, some research also suggests that *S. aureus* carriers are not at a complete

health disadvantage. While persistent carriers are at increased risk for numerous *S. aureus* related infections, *S. aureus* bacteremia is remarkably more frequently fatal in non-carriers (323). Thus, colonization with *S. aureus* may actually “prime” the immune system so that it is better prepared to combat *S. aureus* bacteremia. Together, these data emphasize key health differences among the three *S. aureus* colonization groups. Unfortunately, as mentioned above, our studies are limited to a single time point which makes discerning between *S. aureus* carriers, non-carriers, and nonpersistent carriers impossible. Although we show that the nasal microbiota can differ dramatically depending on the presence or absence of *S. aureus* (Chapter 2), how the nasal microbiota varies in response to SSTIs among the various *S. aureus* colonization groups has yet to be determined. To address this question, culture data from the aforementioned longitudinal study will be used to assign military trainees into the various *S. aureus* colonization groups. By controlling for *S. aureus* colonization status, subsequent analysis of body site microbiomes should prove more accurate and may reveal key microbiome signatures that would otherwise be masked by the variability associated with the dynamic nature of *S. aureus* colonization.

While *S. aureus* preferentially colonizes the anterior nares, it is capable of colonizing many additional anatomical locations: inguinal, perianal, oropharynx, etc. (74; 336). The impact of *S. aureus* colonization on the microbiome at these various body sites, as well as alterations due to SSTI formation, are currently under investigation in our laboratory. Preliminary data suggest that if *S. aureus* is found at one anatomical location, it is typically found throughout the body (J. Singh, R.C. Johnson, C.D. Schlett, J.B. Lanier, E.R. Hall, N. Teneza-Mora M.W. Ellis, D.S. Merrell, unpublished data). These

data indicate that *S. aureus* is an incredibly robust organism that is ubiquitous not only at the interpersonal level, but the intrapersonal level as well.

SSTI INTERVENTIONS FOR THE MILITARY: FORT BENNING RESEARCH SITE

Poor hygiene, crowded living conditions, and frequent skin abrasions make up the perfect epidemiological recipe for future SSTI formation. Unfortunately, all of these conditions apply to members of the military, especially those undergoing basic training. As such, our study population concentrated exclusively on military trainees stationed at Fort Benning, one of the nations largest military training facilities. While the research described herein has direct implications for Fort Benning military trainees, how accurately these data represent SSTIs in the general population is unclear. With nationwide representation, it can be argued that Fort Benning is a geographically diverse training facility and thus may represent SSTI infections throughout the United States. However, with respect to patient demographics, our study population was composed predominately of young, white/Caucasian males in good physical condition. Studies have shown that SSTIs may present differently in other populations such as the elderly and immunocompromised (70). Therefore, it would be interesting to assess any differences in body site and SSTI microbiome composition among these alternative populations. Furthermore, while some studies have suggested a potential difference between various male and female microbiomes (115; 340), it is unknown if the results described in this thesis would also apply to women. Nonetheless, given the extremely high prevalence of SSTIs within the military, further characterization of trainee specific SSTIs should prove valuable.

SSTI INTERVENTIONS FOR THE MILITARY: TRAINEES AT HIGH RISK FOR SSTI

There have been numerous studies aimed at SSTI prevention in the military. One Fort Benning study investigated the effect of enhanced hygiene education and chlorhexidine body wash on combating SSTI development (82). Although SSTI rates remained the same among the various hygiene groups, a similar study in military trainees at a separate training facility observed a significant reduction in SSTI rates with the use of chlorhexidine body wash (205). Although the SSTI interventions described in these two studies were largely similar, there did exist minor procedural variations that may explain the differences in body wash efficacy such as frequency of use as well as when the body wash was administered (82; 205). Nevertheless, the discordance between these two studies emphasizes our limited understanding of SSTI development and may also suggest that novel prevention measures are needed.

In order to reduce SSTI rates in the military, it could be beneficial to group trainees into a “high risk” or “low risk” category. However, categorization would require initial screening upon basic training admission. Ideally, screening tools should be cheap, quick, and accurate. The detection of *S. aureus* in the nares via nasal swab and plating on selective media would be a beneficial first line screening tool to identify “high risk” individuals (79). However, given that *S. aureus* non-carriers also routinely develop SSTI, additional screening tools should be utilized in conjunction with *S. aureus* detection. For example, the nasal microbiota may provide additional microbial cues that may be indicators of future SSTI development. In Chapter 2, we show that Proteobacteria were significantly more abundant in the nares of trainees that did not harbor a SSTI as

compared to those that did have a SSTI (Chapter 2). This data may suggest that SSTI-predictive microbial signatures may reside within the Proteobacterial phylum. Thus, the rapid identification and quantification of non-*S. aureus* SSTI risk factors in the nares is of great interest to the military.

Previous research suggests that approximately 80% of the time, the infecting strain of *S. aureus* is identical to the colonizing strain in the nares (67; 153; 322). In theory, eradication of *S. aureus* from the nares could prevent endogenous infections and therefore reduce SSTI rates. To this end, numerous groups have explored methods to convert *S. aureus* carriers (high risk) to non-carriers (low risk) (53; 274). Indeed, it is recommended in the UK that individuals colonized with MRSA receive nasal mupirocin (56). Although mupirocin is a potent decolonizing agent, patients routinely become recolonized or harbor a mupirocin resistant strain of *S. aureus* (141; 236; 313). To overcome the transient effect of mupirocin, additional research has concentrated on using competing bacterial species that will not only reduce *S. aureus* levels, but will also confer long lasting protection from recolonization (179; 302; 334). Of particular interest, artificial inoculation of various *Corynebacterium* species has been shown to clear *S. aureus* from the nasal cavity (302). Nasal isolates of *Corynebacterium* have also been shown to inhibit the growth of *S. aureus in vitro* (334) (B. Hardy, D.S. Merrell, unpublished data). As shown in Chapter 2, this inverse correlation between *S. aureus* and *Corynebacterium* in the nares was also observed among trainees at Fort Benning. The effect of *Corynebacterium* implantation in the nares of trainees upon admission to basic training on subsequent SSTI rates merits further investigation. Furthermore, elucidation

of the mechanisms behind this bacterial antagonism could provide novel insight into *S. aureus* physiology and pathogenesis.

In addition to screening for *S. aureus* carriers, further characterization of the colonizing strain may prove useful. Strains of *S. aureus* are incredibly diverse and not all express the same virulence factors (208). For instance, a trainee colonized with PVL-positive CA-MRSA may be placed in the “high risk” group compared to a MSSA or coagulase-negative *S. aureus* carrier. Strain characterization can also be used to monitor the spread of specific strains within the military setting. This is especially relevant given the novel *S. aureus qacA*-containing plasmid described in Chapter 4 that confers decreased chlorhexidine susceptibility. In depth surveillance of resistance determinants will not only provide important information for disease prevention, but will also serve as the basis for hypothesis-driven research aimed at molecular interventions to combat spread of resistant pathogens.

While it is important to identify individuals at “high risk” and “low risk” for future SSTI development, we must acknowledge the fact that the term “SSTI” represents a wide array of skin infections, with some being significantly more common than others. For example, abscesses and cellulitis infections are by far the two most prevalent SSTIs in the military (3). Given the differences in bacterial etiology, physical presentation, and treatment options between abscesses and cellulitis, the identification of specific “SSTI type” risk factors would be very useful to the military. As shown in Chapter 3, nasal colonization with MRSA may be associated specifically with abscess formation while white ethnicity and antecedent blister formation were potential risk factors for cellulitis. However, our study was limited in that there were relatively few participants (200)

compared to other risk factor assessment studies (81). Additionally, as mentioned above, these risk factors may only apply to our male-dominated military population. Thus, a larger, more demographically diverse study is needed. Despite these limitations, these data can be used in conjunction with other established SSTI risk factors to identify and monitor those trainees that are highly susceptible to future abscess and/or cellulitis infections.

SSTI INTERVENTIONS FOR THE MILITARY: IMPORTANCE OF SSTI RESEARCH

Unfortunately, SSTIs represent just one disease that is rampant in members of the U.S. military. While SSTI remains the most common infectious cause of hospital admission within the first two year of military service (5), trainees are routinely admitted for other conditions such as mental disorders, traumatic brain injury, alcohol and drug abuse, and sexually transmitted diseases (5; 331; 332). Why then should the military continue to concentrate on SSTI prevention? From a financial standpoint, it is estimated that the average cost of hospitalization for *S. aureus*-associated SSTIs in the US in 2009 was approximately \$11,622 (284). By factoring in the number of hospitalization in 2008 due to bacterial skin infection in the U.S. Armed Forces (~1,500), SSTIs alone cost the military over \$17 million per year (4). This is likely a conservative approximation given that not all SSTIs are caused by *S. aureus*. Thus, SSTIs represent a significant financial burden to the military. While not all SSTIs require admission to the hospital, a much larger proportion of trainees with SSTI are placed on limited duty. In a recent SSTI-prevention clinical trial at Fort Benning, of the 1,377 trainees that developed SSTI, 67% of them were given work/duty limitations for an average length of 6.9 days (82).

Combined with the nearly 20,000 new trainees per year at Fort Benning and predicted SSTI incidence rate of 1 in 10, this translates to over 9,000 combined days of limited service per year (3). Therefore, further research aimed at SSTI prevention in the military is necessary to help alleviate the financial and medical hardships imposed by SSTIs.

LOOKING FORWARD: THE FUTURE OF SSTI RESEARCH AND TREATMENT

Despite the seemingly dire state of SSTIs in the military and throughout the world, our understanding of SSTI causes/pathogenesis/etiology is rapidly progressing. This is in part a reflection of new and improved research technologies such as high-throughput sequencing. The ability to characterize SSTI and body site microbiomes, as well as the ability to sequence the human genome, has nearly limitless applications when addressing important research questions. Consequently, the generation of enormous amounts of data requires development of analytical tools that can manipulate DNA sequences on a large scale while differentiating between quality DNA reads and potential contaminants. Although a few of these tools have been created (38; 270), they will require continual modification in order to keep up with the rapid advancement of new sequencing technologies. Thus, while it is important to study the microbiological and epidemiological aspects of SSTIs, it is equally important that new molecular and computational tools be developed in order to advance SSTI research.

Despite the findings described in this dissertation, there are still numerous unanswered questions. Not surprisingly, the vast majority of these questions are derived from the ever-increasing complexity associated with SSTI etiology. What exactly is the cause of cellulitis? Is cellulitis even caused by a bacterium or several bacteria? How do

polymicrobial infections differ from monomicrobial infections? With the aid of high-throughput sequencing and careful study design, the answers to these conundrums are within reach.

In addition to SSTI etiology, there are other microbiological, epidemiological, and clinical questions that continue to elude researchers and physicians. How does reduced susceptibility to antibiotics/antimicrobials affect SSTI pathogenesis? What is the prevalence of antibiotic resistance markers among SSTI *S. aureus* isolates? Can we potentially treat or prevent SSTIs with strains of bacteria that have anti-*S. aureus* properties? With such a large number of unresolved questions, this makes SSTI research a very important but equally exciting field to investigate in the future.

CONCLUSION

Skin and soft-tissue infections are inherently difficult to study given the wide range in clinical presentation and the heterogeneous nature of *S. aureus*. This is further complicated by the fact that not all SSTIs are caused by *S. aureus*. Despite our increased understanding of SSTI causes and pathogenesis, the incidence rates continue to rise, especially in the military (4). In order to combat the surging SSTI rates, numerous groups have attempted to develop vaccines that effectively prevent individuals from becoming carriers of *S. aureus*, particularly in the nares (62; 267). Unfortunately, the various tested vaccines have been largely unsuccessful. Thus, there is an increased pressure to develop other means to prevent future SSTI formation. As highlighted in this dissertation, significant consideration should be given to the human microbiome. In particular, how can the microbiota at various body sites, as well as at the site of infection, be exploited to

assess SSTI susceptibility and disease progression? The answer to these questions holds the potential to dramatically influence how we treat and prevent SSTIs in the future.

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APPENDIX A: Dynamic HypA Zinc site is Essential for Acid Viability and Proper Urease Maturation in *Helicobacter pylori*

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The work presented in this chapter is the sole work of R.C. Johnson with the following exceptions: H.Q. Hu created the constructs for the site-directed mutagenesis of *hypA* Zn-binding site and performed the urease activity assays.

ABSTRACT

Helicobacter pylori requires urease activity in order to survive in the acid environment of the human stomach. Urease is regulated in part by nickelation, a process that requires the HypA protein, which is a putative nickel metallochaperone that is generally associated with hydrogenase maturation. However, in *H. pylori*, HypA plays a dual role. In addition to an N-terminal nickel binding site, HypA proteins also contain a structural zinc site that is coordinated by two rigorously conserved CXXC sequences, which in *H. pylori* are flanked by His residues. These structural Zn sites are known to be dynamic, converting from Zn(Cys)₄ centers at pH = 7.2 to Zn(Cys)₂(His)₂ centers at pH = 6.3 in the presence of Ni(II) ions. In this study, mutant strains of *H. pylori* that express zinc site variants of the HypA protein are used to show that the structural changes in the zinc site are important for the acid viability of the bacterium, and that a reduction in acid

viability in these variants can be traced in large measure to deficient urease activity. This in turn leads to a model that connects the $\text{Zn}(\text{Cys})_4$ coordination to urease maturation.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram negative bacterium that colonizes the human gastric mucosa (15). Infection causes chronic inflammation and is a major risk factor for development of peptic ulcers and cancer (19; 20; 23). *H. pylori* is a prevalent pathogen that colonizes approximately one-third to one-half of the worldwide adult population (10). Successful colonization of the acidic environment of the stomach requires the activity of urease (7-9), a nickel-dependent enzyme that catalyzes the hydrolysis of urea to ammonia and carbon dioxide (5; 27). Urease is highly expressed in *H. pylori*, representing up to 10% of the total protein (2). However, the enzyme remains inactive without maturation through nickel insertion into the active site (5; 27).

H. pylori urease maturation is dependent on accessory proteins UreIEFGH (5; 27). Additionally, HypA and HypB, accessory proteins normally involved in the maturation of NiFe-hydrogenases, also aid in urease maturation; deletion of *hypA* or *hypB* results in urease deficiency in *H. pylori* despite the presence of the full *ureIEFGH* cascade (4; 17; 18). Urease deficient phenotypes in *hypAB* deletion mutants can be compensated for by addition of Ni to the media, implicating the role of HypA and HypB in nickel delivery to the urease maturation pathway (4; 18). The traditionally hydrogenase-specific nickel metallochaperone, HypA, has been shown to interact directly with a urease-specific nickel metallochaperone, UreE, *in vitro*, providing a possible link

for the role of HypA in urease maturation (4). Several studies have interrogated the interaction between HypA and UreE. Biophysical characterizations show that HypA can outcompete UreG for interaction with UreE (3). Additionally, nickel transfer from HypA to UreE has been shown to involve the interaction of the HypA nickel-binding domain and the UreE nickel-binding C-terminus (26).

As shown in Figure 15, the overall protein structure of HypA has been shown by nuclear magnetic resonance (NMR) to have a distinct nickel-binding domain and structural zinc-binding domain [PDB: 2KDX] (24). In addition to the two conserved CXXC motifs in the HypA structural zinc site, the *H. pylori* zinc binding motifs also have His residues closely flanking the conserved motifs. The structural zinc site has also been extensively characterized by X-ray absorption spectroscopy (XAS), demonstrating that the WT HypA protein structural zinc site is dynamic (11; 14). The average Zn coordination at pH 7.2 is $\text{Zn}(\text{Cys})_4$, which changes to $\text{Zn}(\text{Cys})_2(\text{His})_2$ at pH 6.3 (the estimated internal pH of *H. pylori* under acid shock conditions) with nickel bound in the nickel site (11; 14). The dynamic nature of the HypA zinc site is lost when any one of the four cysteine residues in the CXXC motif is mutated to alanine or aspartate, and results in locking of the zinc coordination in the $\text{Zn-Cys}_2\text{His}_2$ structure (acidic conformation) (11). The dynamic nature of the HypA zinc site is also lost when either of the histidine residues flanking the CXXC motifs are mutated to alanine; this change results in locking of the zinc coordination in the Zn-Cys_4 structure (neutral conformation) (11). These structure-locking mutants of HypA are ideal for interrogation of the role of the HypA zinc site and corresponding protein conformations in urease maturation and acid resistance in *H. pylori*, and are the focus of this study. Herein we create a series of isogenic *H. pylori*

strains that each express zinc site mutant *hypA* variants under the control of the endogenous promoter, and then utilize these mutants to define the contribution of the conserved HypA motifs to *H. pylori* acid viability and urease activity. These studies connect the point mutations in the structural zinc site of a nickel metallochaperone, HypA, to changes in acid viability and urease maturation *in vivo*.

MATERIALS AND METHODS

Bacterial growth conditions

All *H. pylori* strains were maintained at -80°C in brain heart infusion broth (Becton Dickinson) supplemented with 10% fetal bovine serum (FBS) and 20% glycerol and were cultivated on horse blood agar (HBA) medium containing 4% columbia agar base (Neogen Corp), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β -cyclodextrin (Sigma), 10 $\mu\text{g}/\text{mL}$ of vancomycin (Amresco), 2.5 U/mL of polymyxin B (Sigma), 5 $\mu\text{g}/\text{mL}$ of trimethoprim (Sigma), and 5 $\mu\text{g}/\text{mL}$ of amphotericin B (Amresco). Where required, 5% sucrose was added to HBA for selection of sucrose sensitive strains. Liquid growth of *H. pylori* was performed in brucella broth (Neogen Corp) with 10% FBS and 10 $\mu\text{g}/\text{mL}$ of vancomycin. All *H. pylori* cultures were grown under microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂) at 37°C with 100rpm shaking for liquid cultures. *H. pylori* strain G27 was used for all experiments (1).

Escherichia coli Top10 cells were either grown on LB agar or in LB liquid medium with shaking at 225rpm. Kanamycin (25 $\mu\text{g}/\text{mL}$) and ampicillin (100 $\mu\text{g}/\text{mL}$) were used for bacterial selection.

***hypA* mutant construction**

H. pylori G27 genomic DNA was used as the template for mutant construct development. A *hypA* mutant strain containing a *kan-sacB* cassette insertion was constructed as follows. The HypA_Up_F and HypA_Up_R primers (Table 14) were used to amplify a 556 base pair segment of DNA that spanned 398 base pairs upstream of *hypA* (HPG27_832) and 158 base pairs into the *hypA* coding region. Additionally, the HypA_Dn_F and HypA_Dn_R primers (Table 14) were used to amplify a 532 base pair region that began at nucleotide position 160 of *hypA* and spanned 337 base pairs downstream of the *hypA* stop codon. The HypA_Up_R and HypA_Dn_F primers were designed to contain identical flanking sequences, which harbored XhoI and XbaI restriction enzyme sites. Thus, the upstream and downstream amplicons were next fused together utilizing splicing by overlap extension (SOE) PCR (12). The resulting spliced product was cloned into the pGEM-T easy vector (Promega) and transformed into *E. coli* Top10 cells. The *kan-sacB* cassette (6), which confers resistance to kanamycin and sensitivity to sucrose, was amplified from pKSF-II (6) using primers Kan_SacB_F and Kan_SacB_R, which were designed to incorporate a XhoI and XbaI restriction site, respectively. The resulting fragment was digested with XhoI and XbaI and was then inserted between the spliced *hypA* fragments that had been similarly digested. The resulting construct was then transformed into *H. pylori* G27 and transformants were selected on HBA plates containing kanamycin. Successful insertion of the *kan-sacB* cassette into the *hypA* gene was confirmed by PCR and sequencing using the

Table 14. Strain, plasmids, and primers used in this study

| Strains | Description | Reference |
|---------|---|------------------|
| DSM1 | G27 WT | (1) |
| DSM43 | G27 Δ <i>ureB</i> Kan ^R | (13), This study |
| DSM1283 | G27 <i>hypA::kan-sacB</i> Kan ^R Suc ^S | This study |
| DSM1295 | G27 <i>hypA</i> restorant | This study |
| DSM1296 | G27 <i>hypA</i> C74A | This study |
| DSM1297 | G27 <i>hypA</i> C94D | This study |
| DSM1298 | G27 <i>hypA</i> C91A | This study |
| DSM1299 | G27 <i>hypA</i> C91D | This study |
| DSM1300 | G27 <i>hypA</i> H95A | This study |
| DSM1301 | G27 <i>hypA</i> C74D | This study |
| DSM1363 | G27 <i>hypA</i> C77A | This study |
| DSM1364 | G27 <i>hypA</i> C77D | This study |
| DSM1365 | G27 <i>hypA</i> H79A | This study |
| DSM1366 | G27 <i>hypA</i> C94A | This study |

| Plasmids | Description | Reference |
|-------------------|--|----------------------|
| pDSM3 | pKSF-II | (6) |
| pDSM32 | pEJ22 | (13) |
| pJ110 | pET22b(+) vector with <i>hypA</i> WT coding sequence | (11; 14), This study |
| pET22b-HypA(C74A) | pET22b(+) vector with <i>hypA</i> C74A coding sequence | This study |
| pET22b-HypA(C74D) | pET22b(+) vector with <i>hypA</i> C74D coding sequence | This study |
| pET22b-HypA(C77A) | pET22b(+) vector with <i>hypA</i> C77A coding sequence | This study |
| pET22b-HypA(C77D) | pET22b(+) vector with <i>hypA</i> C77D coding sequence | This study |
| pET22b-HypA(H79A) | pET22b(+) vector with <i>hypA</i> H95A coding sequence | This study |
| pET22b-HypA(C91A) | pET22b(+) vector with <i>hypA</i> C91A coding sequence | This study |
| pET22b-HypA(C91D) | pET22b(+) vector with <i>hypA</i> C91D coding sequence | This study |
| pET22b-HypA(C94A) | pET22b(+) vector with <i>hypA</i> C94A coding sequence | This study |
| pET22b-HypA(C94D) | pET22b(+) vector with <i>hypA</i> C94D coding sequence | This study |
| pET22b-HypA(H95A) | pET22b(+) vector with <i>hypA</i> H95A coding sequence | This study |

| Primers | Sequence (5'-3') | Reference |
|----------------|--|------------------|
| HypA_Up_F | CCGCTTGGATTGAGATGGGGTG | This study |
| HypA_Up_R* | <i>T</i> TAGAAAGCTTGGATCGCTCGAGACTCTAAAAGTCTCAAACGCGCTC | This study |
| HypA_Dn_F* | <i>C</i> TGAGCGATCGCAAGCTTCTAGAGAATCTTGGTGTGTAAAGACGC | This study |
| HypA_Dn_R | GCAAAACGCTGCGGTATTGC | This study |
| Kan_SacB_F* | GTGGGCTCGAGCCCGGGCGAACCATTTGAGGTGA | This study |
| Kan_SacB_R* | GCGCGTCTAGATATAAGCCCATTTTCATGC | This study |
| HypA_Confirm_F | GGCTAACGAGCGTGGATAAG | This study |
| HypA_Confirm_R | GCACTCACTAAAATCGTGGGC | This study |
| HypA_seq_F | CTAAAGCGGTAACCACATCCG | This study |
| HypA_seq_R | GACTTGCTTCAATTCAAAACGG | This study |
| HypA_C74A_F | GGTTGAATTAGAAGCCAAGGATTGTTTCGCATGTTTTTAAGCCTAACGCG(11) | (11) |
| HypA_C74A_R | CGCGTTAGGCTTAAAAACATGCGAACAATCCTTG <u>G</u> CTTCTAATTCAACC(11) | (11) |
| HypA_C74D_F | GGTTGAATTAGAAGCAAGGATTGTTTCGCATGTTTTTAAGCCTAACGCG(11) | (11) |
| HypA_C74D_R | CGCGTTAGGCTTAAAAACATGCGAACAATCCTTG <u>I</u> CTTCTAATTCAACC(11) | (11) |
| HypA_C77A_F | GAATTAGAATGCAAGGATGCTTCGCATGTTTTTAAGCCTAACGCGC(11) | (11) |
| HypA_C77A_R | GCGCGTTAGGCTTAAAAACATGCGAAGCATCCTTGCAATTCAATT(11) | (11) |
| HypA_C77D_F | GAATTAGAATGCAAGGATGATTCGCATGTTTTTAAGCCTAACGCGC(11) | (11) |
| HypA_C77D_R | GCGCGTAGGCTTAAAAACATGCGAATCATCCTTGCAATTCAATT(11) | (11) |
| HypA_H79A_F | GCAAGGATTGTTCCGCTGTTTTTAAGCCTAACGCGCTAG(11) | (11) |
| HypA_H79A_R | GTTAGGCTTAAAAACAGCCGAACAC(11), This study | (11), This study |
| HypA_C91A_F | GCGCTAGATTATGGGGTGGCTGAGAAATGCCACAGC(11) | (11) |
| HypA_C91A_R | GCTGTGGCATTCTCAGCCACCCATAATCTAGCGC(11) | (11) |
| HypA_C91D_F | CGCCGTAGATTATGGGGTGGATGAGAAATGCCACAGC(11) | (11) |
| HypA_C91D_R | GCTGTGGCACTTTCTCA <u>T</u> CACCCATAATCTAGCGCG(11) | (11) |
| HypA_C94A_F | GGTGTGTGAGAAAGCCACAGCAAG(11) | (11) |
| HypA_C94A_R | AACATTCTGCTGTGGGCTTTCTCAC(11) | (11) |
| HypA_C94D_F | GGGGTGTGTGAGAAAGACCACAGCAAGAATGTTATTATCAC(11), This study | (11), This study |
| HypA_C94D_R | GTGATAATAACATTCTTGTGTGGICTTTCTCACACACCC(11), This study | (11), This study |
| HypA_H95A_F | GTGTGTGAGAAATGCGCCAGCAAGAATGTTATTATC(11), This study | (11), This study |
| HypA_H95A_R | GATAATAACATTCTTGTGGCGCATTCTCACACAC(11), This study | (11), This study |

*Restriction enzyme sites are italicized (XhoI or XbaI); Underline denotes nucleotide changes made to the pJ110 (wild type *hypA*) plasmid

HypA_Confirm_F/R and HypA_seq_F/R primers, respectively. The resulting mutant strain was named DSM1283.

Site-directed mutagenesis of *hypA* Zn-binding site

Mutations of the cysteines in the HypA CXXC motifs (Cys71, Cys74, Cys91 and Cys94) to alanine or aspartic acid and the flanking histidines (His79 and His95) to alanine were constructed by polymerase chain reaction (PCR) using the wild type *hypA* sequence carried on a plasmid (pET-22b(+)) as a template (11). The plasmid carrying the wild type *hypA* was transformed into NovaBlue (Novagen) competent cells and then reisolated using the Axyprep Plasmid MiniPrep Kit (Axygen) and used as the DNA template in all subsequent PCR reactions. PCR primers were designed to incorporate the desired mutations and are listed in Table 14. Reactions were carried out in 50 μ L volumes using 1ng of template DNA and 2.5ng or 2 μ M of each primer per reaction. Successful PCR amplifications were confirmed by 0.8% agarose gel electrophoresis. Products were then cloned into the pET-22b(+) vector, and transformed into NovaBlue competent cells. Single colonies were selected and grown to saturation in 5mL liquid cultures of LB-Miller (Fisher Scientific) media supplemented with ampicillin at 37°C. Cells were pelleted by centrifugation at 6,000 g for 5 minutes and plasmids were isolated using the Axyprep Plasmid MiniPrep Kit. Successful mutations were confirmed by plasmid sequencing (GENEWIZ, Inc.).

We utilized the wild-type or mutant HypA pET-22b(+) plasmids, which each contained the entire HypA coding region, to move the mutant constructs of interest into the *H. pylori* chromosome. Each of the constructs were individually transformed into

DSM1283, and double crossover events in which the *kan-sacB* cassette was replaced by the mutagenized *hypA* gene carried on the pET-22b(+) vector were selected for based on sucrose resistance. Sucrose resistant transformants were screened for kanamycin sensitivity and proper integration of the *hypA* construct was confirmed by PCR and sequencing using the HypA_Confirm_F/R and HypA_seq_F/R primers, respectively. In total, *H. pylori* mutant strains were made that contained the following *hypA* mutations: C74A (DSM1296), C74D (DSM1301), C77A (DSM1363), C77D (DSM1364), H79A (DSM1365), C91A (DSM1298), C91D (DSM1299), C94A (DSM1366), C94D (DSM1297), and H95A (DSM1300). In addition, a *hypA*-restorant (*hypA*-R; DSM1295) in which the *kan-sacB* cassette was replaced by the wild type *hypA* gene was also created in order to control for any defects that may have resulted due to genetic manipulation.

Acid resistance testing

Each of the ten *hypA* Zn-site *H. pylori* mutants were tested for acid resistance. Additionally, the wild type *H. pylori* strain, the *hypA::kan-sacB* mutant, and the *hypA* restorant were included as controls. Furthermore, a urease-deficient mutant of *H. pylori* (DSM43) in which the kanamycin resistance cassette replaced the *ureB* subunit was used as a positive control for acid sensitivity; DSM43 was created by transforming wild type *H. pylori* with vector pEJ22 as previously described (13). Assays were conducted as follows: 20mL liquid cultures of *H. pylori* were inoculated to an optical density (600nm) of 0.05 from overnight liquid grown bacterial cells and then allowed to grow for approximately 19 hours. 1mL aliquots were removed from the culture and pelleted by centrifugation. The supernatants were removed and the bacterial pellet was resuspended

in 1mL of phosphate buffered saline (PBS) at pH 6 or 2.3, with or without supplementation with 5mM urea. Immediately after the bacterial pellets were resuspended, an aliquot was removed, serially diluted in brucella broth and plated on HBA plates to determine colony forming units (CFU) per milliliter. The cultures were then incubated in 1.5mL capped tubes for 1 hour at 37°C. At this point, a second aliquot was removed from the cultures, immediately serially diluted, and plated to determine CFU/mL as described above. Percent survival after 1 hour incubation in the various PBS solutions was determined for each bacterial strain. At least three biological replicates were performed for each strain.

Urease activity assay

Urease activities were determined for each of the ten *hypA* Zn-site variants, the wild type *H. pylori* strain, the *hypA::kan-sacB* mutant, and the *hypA* restorant strain. For each strain, 8mL liquid cultures of *H. pylori* were inoculated to an optical density (600nm) of 0.05 from overnight liquid grown bacterial cells and then allowed to grow for approximately 22 hours. At that point, 1mL aliquots were removed from the culture and pelleted by centrifugation ($\sim 10^8$ cells). The supernatants were removed and the bacterial pellets were stored at -20°C until ready for urease assays. The frozen cells were thawed and then resuspended in 750 μ L of ice cold HEPES buffer (pH 7.0), 1mM phenylmethanesulfonyl fluoride (PMSF) (MP Biomedicals, LLC), and 1X protease inhibitor cocktail (Sigma-Aldrich) and then lysed by sonication at 70% power for 6 pulses (2-second each) on ice. Lysate was centrifuged at 15,000-g for 10 minutes to remove insoluble fractions from soluble whole cell extracts. Soluble whole cell extracts

were kept at 4°C for up to one month and insoluble fractions were stored at -20°C. Total protein concentration in soluble whole cell extract was assessed by Bradford Assay using the Coomassie Protein Assay Kit (Thermo Scientific).

Urease activities for each strain were determined using a modified phenol-hypochlorite method to assay the amount of ammonia released in the soluble whole cell extract of *H. pylori* lysate in the presence of urea (16; 22). For each strain, 5µL of whole cell extract was added to 245µL of urease reaction buffer (50mM HEPES, 25mM Urea, pH 7.0), and incubated at 37°C for 20 minutes to allow for ammonia production. The reaction was quenched with the sequential addition of 375µL of phenol-hypochlorite buffer A (100mM phenol, 167.8µM sodium nitroprusside) and then the addition of 375µL of phenol-hypochlorite buffer B (125mM NaOH, 0.044% NaClO); samples were mixed with quick vortexing after the addition of each buffer. The assay mixture was incubated at 37°C for 30 minutes to allow for color development (the conversion of ammonia to indophenol) and the absorbance was evaluated at 625nm. Assays were performed alongside a standard curve created using known amounts of ammonium chloride (0.24 – 500nmol) in place of whole cell extract. The urease activity of $\Delta ureB$ strain was set as background and subtracted from the activity of all other strains. Urease activity for the various mutants was normalized to the *hypA*-restorant (*hypA*-R; DSM1295) *H. pylori* as 100%. All experiments were performed in triplicate with two independently grown cultures.

RESULTS

HypA Zn-binding sites are important for acid survival

To determine whether changes in the zinc binding sites of HypA affected acid resistance of *H. pylori*, we created strains of *H. pylori* in which the Cys residues found in the two CXXC motifs associated with zinc binding (Cys74, Cys77, Cys91 and Cys94) and the two His residues (His79 and His95) that flank these two CXXC motifs were mutated. Cys residues were changed to Asp and Ala, while His residues were changed to Ala. We then utilized these 10 HypA mutant strains in combination with the wild type strain, a *hypA* mutant (*hypA::kan-sacB*), a *hypA*-restorant (*hypA-R*) and the *ureB* mutant ($\Delta ureB$) control strains to assess acid resistance. All 14 of these *H. pylori* strains showed similar robust survival profiles when exposed to pH 6 in the presence or absence of 5mM urea (Figure 16A, B). Conversely, when exposed to pH 2.3, all of the *H. pylori* strains, including the wild type, showed a dramatic decrease in viability; less than 0.01% of the inoculum survived at pH 2.3 in the absence of urea (Figure 16C). However, when urea was supplemented to the pH 2.3 buffer, we began to detect differences in acid resistance across the various strains (Figure 16D). As expected, the wild type *H. pylori* strain and the *hypA*-restorant were able to efficiently utilize the supplemented urea as a substrate for the urease system and survive the acidic challenge. Also as expected, the urease deficient $\Delta ureB$ strain was incredibly acid sensitive; no surviving bacteria were detected (limit of detection 500 CFU/mL equating to 0.0001% survival). The $\Delta hypA$ strain that carried an insertion in the *hypA* coding sequence was also deficient in its ability to resist acidic stress (< 3% survival). This result confirmed previous studies that indicated that HypA is necessary for efficient urease activity (4; 17; 18). For the various Zn-binding site mutants, mutation of Cys77, His79, and His95 of HypA resulted in no changes in acid

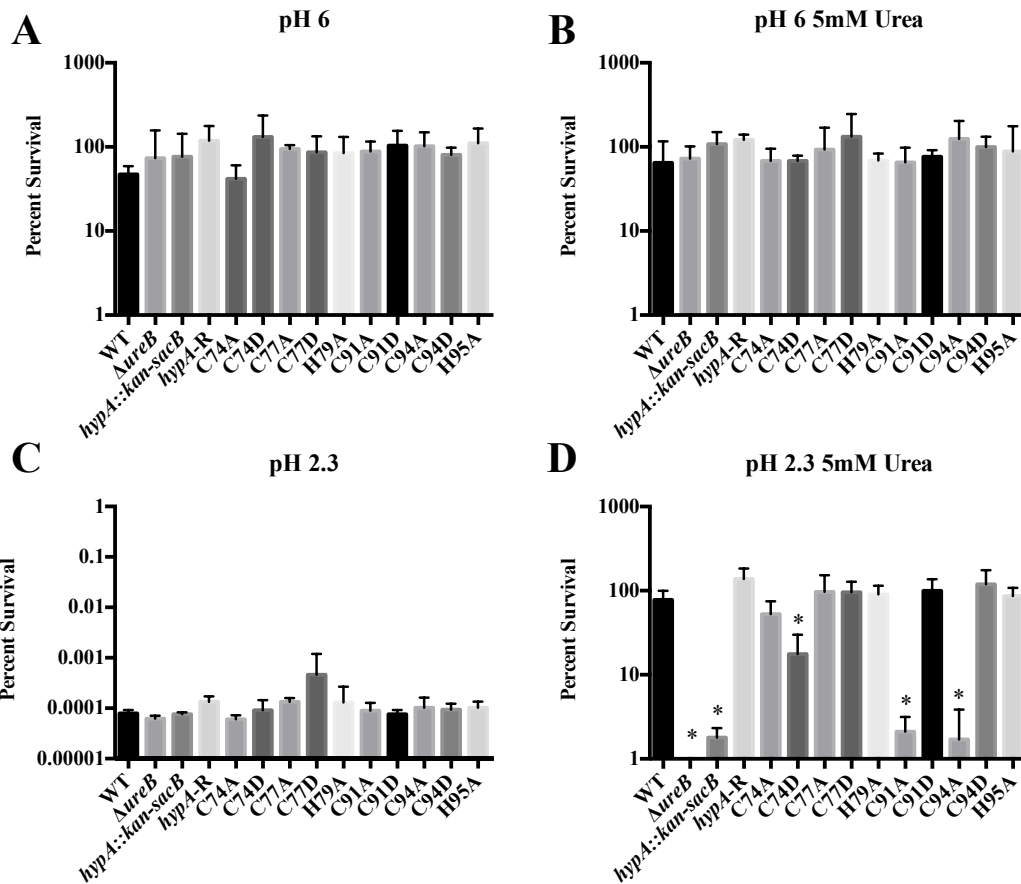


Figure 16. Specific amino acid mutations within the zinc-binding site of the HypA protein in *Helicobacter pylori* result in decreased acid resistance. The ten *hypA* mutants, as well as the wild type (WT), *ureB* knockout ($\Delta ureB$), *hypA* interrupted mutant (*hypA::kan-sacB*), and the *hypA*-restorant (*hypA-R*) were exposed to various environments for 1 hour: pH 6 (A), pH 6 containing 5mM urea (B), pH 2.3 (C), and pH 2.3 containing 5mM urea (D). Percent survival was calculated for each strain. Data represent mean \pm standard deviation. * = Acid resistance was significantly reduced when compared to wild type ($p < 0.01$, one-way ANOVA followed by Dunnett's test for multiple comparisons).

resistance. Conversely, significant decreases in acid resistance, as compared to the wild type strain, were observed for the C74D, C91A, and C94A mutant strains: 17.6%, 2.1% and 1.7% average survival, respectively. This finding suggests that these zinc-binding site residues play a critical role in acid viability, presumably by affecting the ability of HypA to provide nickel to the urease maturation pathway.

Mutation to HypA Zn-binding sites impacts urease activity

The acid survival data suggested that the C74, C91 and C94 residues play a critical role in the ability of HypA to facilitate urease maturation (4; 17; 18). We next directly investigated urease activity in each of the ten Zn-binding site mutants of *hypA*, as well as in the $\Delta ureB$, the *hypA::kan-sacB*, the *hypA*-restorant and the wild type strains. The *hypA*-restorant showed three-fold less urease activity than wild type *H. pylori*, which may be a consequence of genetic manipulation. Consequently, urease activities were normalized to the *hypA*-restorant strain. Since the $\Delta ureB$ strain of *H. pylori* is missing the nickel-containing β subunit of the urease enzyme and should have no urease activity, the average urease activity of the $\Delta ureB$ strain was subtracted from measurements of all strains to correct for any background levels of ammonia present in whole cell extract.

As shown in Figure 17, we identified two classes of mutations of the HypA structural zinc site, those that had no effect on urease activity (WT-like), and those that decreased urease activity. This later class can be subdivided into moderately deficient and severely deficient ($\Delta hypA$ -like) subclasses. Mutations of His residues (H79A and H95A) flanking the HypA CXXC motifs, resulted in strains that retained WT-like activity, suggesting that these His residues are not important for urease maturation. In

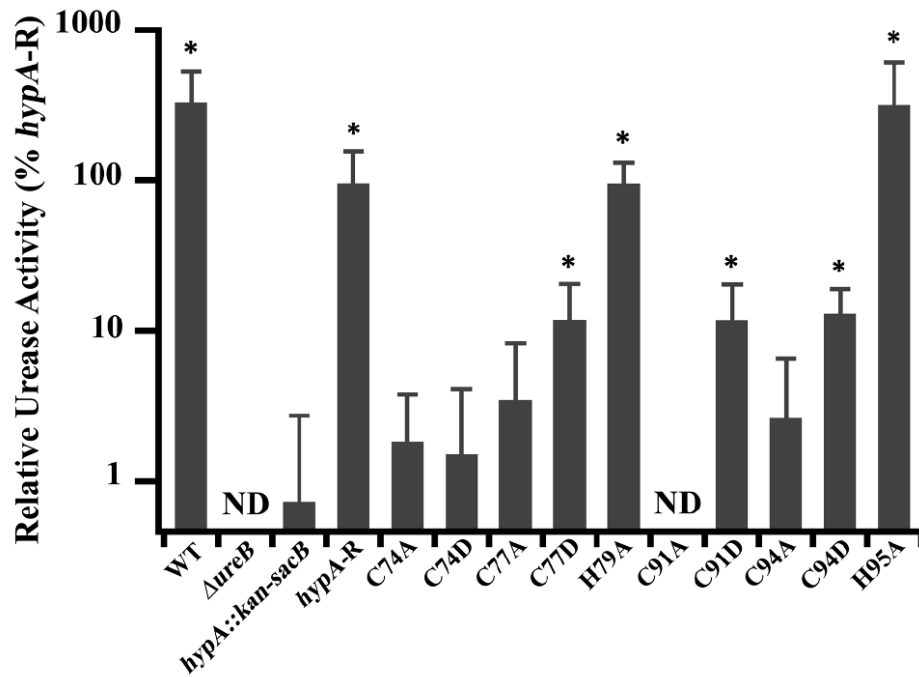


Figure 17. Zn-site mutations of *hypA* in *H. pylori* lead to deficiencies in urease activity. Soluble whole cell extracts were obtained from lysis of ten *hypA* Zn-site mutants strains, as well as the wild type (WT), *ureB* knockout ($\Delta ureB$), *hypA* interrupted mutant (*hypA::kan-sacB*), and the *hypA*-restorant (*hypA-R*) strains. The phenol-hypochlorite method was used to assay the ammonia production (urease activity) of the strains. Amount of ammonia produced was quantified by comparison with a standard curve constructed with known amount of ammonium chloride. The $\Delta ureB$ strain activity was subtracted from each strain as background ammonia in cell extracts and all activity was normalized to *hypA-R* strain. * = Urease activity was significantly different from the *hypA::kan-sacB* strain ($p < 0.05$). ND = none detected.

contrast, all of the mutations involving Cys residues in the HypA CXXC motifs were deficient in urease activity to some extent (less than 15% compared with the *hypA*-restorant strain), indicating the importance of the Cys residues in the proper function of HypA in the urease maturation pathway. Comparisons of the urease activities in the Cys mutant strains with the $\Delta hypA$ strain revealed more subtle phenotypes. The activity of C77D, C91D, and C94D were statistically different ($p < 0.05$) from the $\Delta hypA$ strain (12 – 13% compared with the *hypA*-restorant strain) constituting a moderately deficient phenotype (Figure 17). The remaining Cys mutants, C74A, C74D, C77A, C91A, and C94A, showed activity levels similar to the $\Delta hypA$ strain ($> 4\%$ compared with *hypA*-restorant strain). These urease activity results corroborate the acid survival phenotypes, where C74D, C91A, and C94A mutants were found to be both acid-sensitive and severely deficient in urease activity. Contrary to the acid survival results, C74A and C77A mutants were not acid sensitive under our test conditions, but were found to be severely deficient in urease activity. These findings suggest that, although all of the Cys residues in the CXXC motifs are important for proper HypA function in the urease maturation pathway, there is a position-dependent gradient of importance based on the residue position as well as the type of mutation.

DISCUSSION

The *H. pylori* HypA protein is a putative nickel metallochaperone that is involved in both hydrogenase maturation (as are all HypA proteins) and urease maturation, a function that is unique to *H. pylori*. NMR structural studies of a monomeric construct of *H. pylori* HypA revealed that it contains a structural zinc site associated with two

rigorously conserved CXXC motifs in addition to a nickel-binding site associated with the N-terminus (24). The amino acid sequence of *H. pylori* reveals that the zinc-binding CXXC motifs are unique among other HypA proteins in that each is flanked by a His residue (11). The structural Zn-sites of HypA were previously shown to change coordination in a pH and nickel-dependent manner, adopting a $\text{Zn}(\text{Cys})_4$ conformation at neutral pH (7.2), and a $\text{Zn}(\text{Cys})_2(\text{His})_2$ conformation at more acidic pH (6.3) (Figure 15) in the presence of Ni(II) (11; 14). These two structures were correlated with other physical properties of dimeric HypA, including nickel binding stoichiometry and thermal stability, with the lower pH form associated with greater thermal stability (11). Mutagenesis studies revealed that altering any Cys residue in the two CXXC motifs (to Ala or Asp) resulted in adoption of the acidic conformation ($\text{Zn}(\text{Cys})_2(\text{His})_2$), and any mutation of the flanking His residues resulted in the neutral conformation ($\text{Zn}(\text{Cys})_4$), in the presence of Ni(II) at both pH values, as determined by EXAFS analysis of data collected at $\sim 50\text{K}$ (11). These pH-insensitive and structure-locking mutants of HypA were important tools employed in the present study to probe the role of HypA in urease maturation and acid viability *in vivo*.

In vivo urease activities of *H. pylori hypA* Zn site variant strains correlate well with previous *in vitro* studies employing addition of purified HypA proteins to whole cell extract from a ΔhypA mutant of *H. pylori* (11). Results of the *in vitro* studies show a general decrease in urease activity upon addition of the zinc site variants relative to addition of wild type HypA protein, and a generally greater effect associated with Cys mutants as compared to His mutants (11). The *in vivo* studies presented here reveal two distinct phenotypes: WT-like urease activity, represented by His mutants, and urease-

deficient strains associated with Cys-mutants. These two classes of mutants correspond to the $\text{Zn}(\text{Cys})_4$ or $\text{Zn}(\text{Cys})_2(\text{His})_2$ conformations, respectively. Our data suggest that all the Cys residues in the HypA Zn site are important in the maturation of urease in *H. pylori*, a result that is consistent with a model in which the conformation associated with $\text{Zn}(\text{Cys})_2(\text{His})_2$ sites, or in which access to the $\text{Zn}(\text{Cys})_4$ structure is inhibited in a dynamic sense, leads to lower levels of urease activation. Among the Cys mutations, there were two levels of urease activity observed, a moderate decrease associated with C77D, C91D, and C94D, all Cys \rightarrow Asp mutations, and a severely deficient level associated primarily with Cys \rightarrow Ala mutations, which have activity levels similar to the ΔhypA strain (C74A, C74D, C77A, C91A, and C94A). With the exception of C74D, Cys \rightarrow Asp mutations to give rise to more active variants than Cys \rightarrow Ala mutations, suggests that at least part of the difference lies in coordination of the CXXC motifs. A Cys \rightarrow Ala mutation results in a loss of a ligand in the CXXC motif, while a Cys \rightarrow Asp mutation results in the potential substitution of a Cys thiolate ligand by a carboxylate ligand. The latter, although uniformly deleterious, appears to preserve greater HypA activity, presumably by providing a mechanism to access a $\text{Zn}(\text{Cys})_3\text{Asp}$ site that resembles the wild type-like $\text{Zn}(\text{Cys})_4$ conformation. A similar trend can be discerned in the study of acid viability: His mutations have no effect, whereas C91A and C94A mutations are associated with low acid survival (comparable to the ΔhypA strain), and C91D and C94D have wild type survival under the assay conditions employed. Interestingly, mutations in the more N-terminal CXXC motif lead to strains that are significantly less acid sensitive. Taken together with the urease activity assays, this suggests that the ability of HypA to deliver Ni to the urease maturation pathway may lie

with the dynamic nature of the structural zinc sites. While no clear reason is apparent for the functional differences observed for the two CXXC motifs, it is noteworthy that two Cys residues are substituted by His at low pH, whereas two are retained and anchor the Zn site. This pair-wise difference in function is one possible explanation for the differences between the more N- and C- terminal CXXC sequences. Further elucidation of this difference awaits structural studies of the low pH form of HypA.

It is clear from both the urease assay data and acid viability data that Cys74 is a special position among the Cys variants. It is the only position where the Cys → Asp mutation results in a less acid viable strain than the Cys → Ala mutation, and the only position where urease activity is not higher for the Asp variant than for the Ala variant. Examination of the NMR structure (Figure 15) shows that the Zn site is formed by a loop that begins with Cys74 and ends with Cys94. However, the terminal Cys residues differ in that Cys94 is flanked by a His residue and Cys74 is not. In the HypA dimer crystal structure from *Thermococcus kodakorensis* KOD1 (PDB ID: 3A44) (21), Cys74 and 94 have a similar relationship, though these residues are now derived from different monomers in the strand-swap dimer (21). The lack of an alternative ligand could make Cys74 a more vulnerable position, particularly if it plays an anchoring role, the loss of which may result in a more severe phenotype due to destabilization of the entire structural zinc site.

HypA and HypB are accessory proteins that are generally associated with hydrogenase maturation, but in *H. pylori* have been shown to be important for the maturation of both NiFe-hydrogenase (17; 18) and urease (4; 17; 18). In addition to interacting with HypB (17; 25), HypA has also been shown to interact directly with the

urease-specific Ni metallochaperone, UreE (3; 4; 26). Interaction of HypA with both HypB (25) and UreE (26) has been demonstrated to involve the N-terminal Ni-binding domain of HypA and Ni-transfer between proteins. In this study we demonstrated that altering the HypA structural zinc site can also severely affect acid viability and urease maturation *in vivo*. These findings suggest that protein structural dynamics, reflected in the HypA zinc site structure, may result in subtle alterations of the overall HypA fold and differential interactions between HypA and partnering proteins, ultimately leading to a gradient in urease and hydrogenase enzymatic activities that is a function of cellular pH and nickel availability.

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APPENDIX B: Fur-Dependent Activation of Two Divergently Transcribed Genes (*HpG27_51* and *HpG27_52*) in *Helicobacter pylori*

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The work presented in this chapter is the sole work of R.C. Johnson with the following exceptions: O.Q. Pich created strains DSM1180 and DSM1182.

ABSTRACT

The human bacterial pathogen, *Helicobacter pylori*, colonizes the gastric mucosa of approximately one-half of the world's population. In order to survive such a harsh environment, *H. pylori* is equipped with numerous regulatory pathways that allow the microbe to adapt to external stimuli such as iron deprivation and low pH. One *H. pylori* master regulatory protein that is required for *in vivo* adaptation is the ferric uptake regulator (Fur). In *H. pylori*, Fur has been shown to both activate and repress gene transcription in both its iron-bound and iron-free (*apo*) form. This occurs via DNA binding at specific nucleotide sequences known as Fur boxes. Conserved and semi-conserved Fur boxes are found throughout the *H. pylori* genome; those located in the promoter region may suggest direct Fur regulation of the corresponding gene. In this study, we characterize two divergently transcribed genes (*HpG27_51* and *HpG27_52*) that contain numerous intergenic Fur boxes. While gene *HpG27_51* encodes the *putA* homologue of *H. pylori*, which is important for proline utilization, gene *HpG27_52* encodes a hypothetical protein that we show harbors urease enhancing properties. At the

transcriptional level, both genes were up regulated in response to iron chelation in a manner that suggests *apo-Fur* activation. Preliminary animal studies suggest that gene *HpG27_52* is also important for proper colonization of the gastric mucosa. These findings further expand our ever-growing knowledge of the *H. pylori* Fur regulon and suggest additional ways in which *H. pylori* senses and responds to its fluctuating environment.

INTRODUCTION

The Gram-negative bacterium *Helicobacter pylori* is considered by some to be the most successful pathogen in human history; nearly 50% of the world's population is currently colonized (15; 26). This translates to nearly 3.5 billion individuals that are at increased risk for the development of severe gastric maladies such as gastric cancer (15). Indeed, over 60% of new gastric cancer cases can be directly attributed to *H. pylori* colonization (45). Despite the enormous impact on human health, it wasn't until 1982 that "S-shaped bacilli" were initially observed in gastric biopsy specimens (38). At the time, the noxious environment of the human stomach was considered a sterile body site. However, it is now clear that despite the low pH and limited nutrients available in this niche, *H. pylori* not only survives, but thrives within the human stomach.

To combat the harsh conditions of the human stomach, *H. pylori* is equipped with a vast array of regulatory networks that are called upon in response to environmental cues (18). One cue, of particular interest, is iron availability. Iron is essential to *H. pylori* physiology as it is a common cofactor required by numerous proteins (25; 48; 56).

Conversely, too much iron results in free radical formation through the Fenton and Haber-Weiss reactions (43; 60). To ensure proper uptake and utilization of iron, *H. pylori* has evolved a complex iron-responsive regulon that is centralized around the master regulatory protein known as the ferric uptake regulator (Fur) (59). Classically, in response to high levels of iron, iron-cofactored Fur will bind the promoter region of a gene and repress transcription via RNA polymerase (RNAP) interference (20). In *H. pylori*, Fur has also been shown to bind up stream of the promoter and to activate expression of some genes (20; 29). Additionally, upon iron limitation, iron-free (*apo*) Fur has been shown to both activate and repress expression of different sets of genes (3; 21; 25). Interestingly, while some bacteria possess a Fur homologue that performs multiple regulatory functions, *H. pylori* harbors the only documented Fur protein that can both activate and repress gene expression in both its iron-bound and *apo* forms (10). Additionally, not only is *H. pylori* Fur required for iron uptake, but it also responds to a wide array of environmental stresses including acid, osmotic, oxidative, and nitrosative stresses (4; 12; 25; 28; 49).

In order to define Fur regulated genes in *H. pylori*, we previously characterized the DNA sequences that are crucial for Fur-DNA binding (Fur boxes) (8; 47). A whole genome search revealed the presence of conserved and semi-conserved variants of the iron-bound Fur box (5'-TAATAATnATTATTA-3') that were interspersed throughout the *H. pylori* genome (47). Of particular interest was a semi-conserved Fur box (5'-TAATAATGCTTTTTTA-3') located in the intergenic region between the two divergently transcribed genes *HpG27_51* and *HpG27_52*. While gene *HpG27_51* is believed to be involved in proline utilization (*putA*) (2), gene *HpG27_52* encodes a small hypothetical

protein of unknown function. The region containing the Fur box was confirmed to be bound by iron-bound Fur by electrophoretic mobility shift assays (EMSA) (47). In addition to the iron-bound Fur box, DNA sequences that are involved in *apo*-Fur binding (5'-AAATGA-3') were also identified within the *HpG27_51*-*HpG27_52* intergenic region (8). We therefore hypothesized that Fur binds to the Fur box(es) located in the promoter regions of genes *HpG27_51* and *HpG27_52* and alters gene expression in response to iron availability.

In this study, we show that expression of both *HpG27_51* and *HpG27_52* is influenced by iron availability in a Fur-dependent manner. However, initial analysis suggests that this occurs independent of the identified predicted Fur-binding site; Fur-dependent regulation still occurs when the Fur box is mutated. We also provide preliminary biological characterization of gene *HpG27_52* both *in vitro* and *in vivo*. The data provided herein will help investigators better understand the ever-growing complexity of the Fur regulon in *H. pylori*. Given Fur's importance to *H. pylori* adaptation *in vivo* (42) and the fact that it is a bacterial specific protein, Fur may be an attractive target for future drug development (11).

MATERIALS AND METHODS

Bacterial strains and growth conditions

All strains, plasmids, and primers utilized in this study are listed in Table 15. Wild-type *H. pylori* strain G27 (DSM1), a common laboratory strain originally isolated

Table 15. Strains, plasmids, and primers used in this study

| Strains | Description | Reference |
|---------|---|------------|
| DSM1 | G27 WT | (2; 14) |
| DSM43 | G27 $\Delta ureB::kan$ Kan ^R | (32) |
| DSM46 | J166 WT | (53) |
| DSM48 | 7.13 WT | (27) |
| DSM300 | G27 $\Delta fur::cat$ Cm ^R | (9) |
| DSM343 | G27 $\Delta fur::cat$ (pDSM340) Cm ^R | (9) |
| DSM1139 | DH5 α (pHP8080) Cm ^R | (39) |
| DSM1180 | G27 $\Delta HpG27_52::kan$ Kan ^R | This study |
| DSM1182 | DH5 α (pHP8080 + pBS:: <i>HpG27_52</i>) Cm ^R Amp ^R | This study |
| DSM1228 | 7.13 $\Delta VDI6_RS08305::kan$ Kan ^R | This study |
| DSM1271 | G27 $\Delta HpG27_51::kan$ Kan ^R | This study |
| DSM1302 | G27 $\Delta HpG27_52::kan$ complement Kan ^R Cm ^R | This study |
| DSM1369 | 7.13 $\Delta VDI6_RS08305::kan$ complement Kan ^R Cm ^R | This study |
| DSM1396 | DH5 α (pHP8080 + pBS) Cm ^R Amp ^R | This study |
| DSM1412 | DH5 α (pHP8080 + pUEF1004) Cm ^R Amp ^R | (39) |
| DSM1413 | DH5 α (pHP8080 + pUEF1004-511) Cm ^R Amp ^R | (39) |
| DSM1435 | J166 $\Delta EGG65_00320::kan$ Kan ^R | This study |
| DSM1436 | J166 $\Delta EGG65_00320::kan$ complement Kan ^R Cm ^R | This study |
| DSM1437 | G27 $\Delta P_{52}::kan-sacB$ Kan ^R Suc ^S | This study |
| DSM1438 | G27 $\Delta fur \Delta P_{HpG27_52}::kan-sacB$ Cm ^R Kan ^R Suc ^S | This study |
| DSM1439 | G27 $\Delta P_{HpG27_52}::scrambled$ iron-bound Fur Box | This study |
| DSM1440 | G27 $\Delta fur P_{HpG27_52}::scrambled$ iron-bound Fur Box Cm ^R | This study |
| DSM1444 | G27 $\Delta P_{HpG27_52}::scrambled apo$ Fur Box | This study |
| DSM1445 | G27 $\Delta fur P_{HpG27_52}::scrambled apo$ Fur Box Cm ^R | This study |

| Plasmids | Description | Reference |
|----------|---|------------|
| pDSM3 | pKSF-II | (13) |
| pDSM101 | pUC18k-2 | (40) |
| pDSM324 | pRDX-C | (16) |
| pDSM340 | p199:: <i>fur</i> | (9) |
| pDSM745 | pKJMSH | (33) |
| pDSM775 | pBluescript (pBS) modified MCS (EcoRV only) | This study |
| pDSM1139 | pHP8080 | (39) |

| Primers | Sequence (5'-3')* | Reference |
|-----------------|--|------------|
| 51_upF | CAATATGAGTGCCACTCATAAG | This study |
| 51_upR | GTTAGTCACCCGGGTACCGAGCTCGGCTTGATTGTAGCATGT TTAAG | This study |
| 51_dnF | CCTCTAGAGTCGACCTGCAGGCACACAAATCATGCTGTGCTG | This study |
| 51_dnR | GCGCTCATCATCAAAGCC | This study |
| 52_upF | GGTTACTGATGCTATCTTGC | This study |
| 52_upR | GTTAGTCACCCGGGTACCGAGCTCGAGTGTGCCAATCATAAT ACC | This study |
| 52_dnF | CCTCTAGAGTCGACCTGCAGGCCAAATTAGAAGACCTTGAAC CC | This study |
| 52_dnR | GTTTTTCTGTGGTTAGCGC | This study |
| Kan_F | GAGCTCGGTACCCGGGTGACTAAC | (40) |
| Kan_R | TGCCTGCAGGTCGACTCTAGAGG | (40) |
| 51confirmF | GGGTAGAAGCAGTCGTCTTGTG | This study |
| 51confirmR | CGACTACGCTAGAGCCTTGC | This study |
| 52confirmF | CTCAATCATTTCAAAGCGGGC | This study |
| 52confirmR | GTTGATAGCGCTGTGTTAGG | This study |
| 52_upF_J166 | CCGATCCATAAGCTCTATGAGC | This study |
| 52_upR_J166 | GTTAGTCACCCGGGTACCGAGCTCCCTTTGTTTTGTAAAATTT GTTATAAATCC | This study |
| 52_dnF_J166 | CCTCTAGAGTCGACCTGCAGGCACGGAGATATTCTTATGAGTG GCACTC | This study |
| 52_dnR_J166 | CTTGCTGAGATTGTTCTAACTC | This study |
| 52confirmF_J166 | GCGGGCTTTATTGTCTAGGC | This study |

| | | |
|-----------------|---|------------|
| 52confirmR_J166 | CGGCTAGCTTTTGGATTTTCTCC | This study |
| 52compF | CGCGCGGATCCGTAGCATGTTTTAAGCGGG | This study |
| 52compR | CGCGCCTGCAGGCCCTCTTTATAAACCCC | This study |
| HP0203-F | GATTGACTTGGGGTTCAGCGTTGGTG | (33) |
| HP0204-R | GTTTGAGCTTGCTAATGATAAGCGG | (33) |
| P52_Up_F | GACCATAGCTTTCGTGTCG | This study |
| P52_Up_R | <u>TCTAGAAGCTTGC</u> GATCGCTCGAGGACCGCTTAAAAACCCGC | This study |
| P52_Dn_F | <u>CTCGAGCGATCGCAAGCTTCTAGAGTGGTAAGTATTTACCGCT</u> TAAAAAC | This study |
| P52_Dn_R | CCCTCTTATAAACCCCTAGAATG | This study |
| Kan_SacB_F | GTGCTCGAGCCCGGGCGAACCATTGAGGTGA | This study |
| Kan_SacB_R | GCTCTAGACCCGGGTATAAGCCCATTTTCATGC | This study |
| P52_Confirm_F | CCGCTCTCTTAATTGGCTC | This study |
| P52_Confirm_R | GAGTGCCACTCATAAGAATATCTCC | This study |
| P52_Up_R1 | CAATTTTAATAATTAATCGCGCGAGTATGAAAACAACCAC | This study |
| P52_Dn_F1 | GTGGTTGTTTTCATACTCCGCCGATTTAATTATTTAAATTG | This study |
| 51_RT_PCR_F | CGCCGGTATTGTGTTGCAAGC | This study |
| 51_RT_PCR_R | CTTCGCTCTCCATGTTTCGCTCC | This study |
| 52_RT_PCR_F | ATTATGATTGGCACACTCGG | This study |
| 52_RT_PCR_R | AGAATGTAGAATCCCAGTGG | This study |
| 52_seq_F | GTGGTAAGTATTTACCGCTTAAAAAC | This study |
| 52_seq_R | GCCCTTAGGCATGTATTGCG | This study |
| 52_P.ext | CTGAGTATCAAAGCTAGAACAACC | This study |
| 52_RBS_F | CAAACAAAGGATATAAAATG | This study |
| 52_RBS_R | ATAAACCCCTAGAATGTAGA | This study |

*XhoI and XbaI restriction sites are underlined

by gastric biopsy from Grosseto Hospital (Tuscany, Italy) (2; 14), was used for all molecular experiments in this study. Additionally, the animal adapted *H. pylori* strains 7.13 (DSM48) and J166 (DSM46) were used for *in vivo* colonization studies (27; 53). All *H. pylori* strains were maintained at -80°C in brain heart infusion broth (Becton Dickinson) supplemented with 10% fetal bovine serum (FBS, Gibco) and 20% glycerol and were routinely cultivated on horse blood agar (HBA) containing 4% Columbia agar base (Neogen Corp), 5% defibrinated horse blood (HemoStat Laboratories, Dixon, CA), 0.2% β -cyclodextrin (Sigma), 10 $\mu\text{g}/\text{mL}$ of vancomycin (Amresco), 2.5 U/mL of polymyxin B (Sigma), 5 $\mu\text{g}/\text{mL}$ of trimethoprim (Sigma), and 5 $\mu\text{g}/\text{mL}$ of amphotericin B (Amresco). When required, 5% sucrose was added to the HBA medium. Liquid *H. pylori* cultures were grown in brucella broth (Neogen Corp) containing 10% FBS and 10 $\mu\text{g}/\text{mL}$ of vancomycin with shaking at 115 rpm. All *H. pylori* cultures were grown under microaerobic conditions (5% O₂, 10% CO₂, 85% N₂) at 37°C. *Escherichia coli* strains were maintained at -80°C in LB broth (MO BIO laboratories Inc.) containing 40% glycerol and were grown at 37°C on 1.5% LB agar or in LB broth with shaking at 225 rpm. When necessary, bacterial selection was performed using the following antibiotics: kanamycin (Kan) (25 $\mu\text{g}/\text{mL}$), ampicillin (Amp) (100 $\mu\text{g}/\text{mL}$), and chloramphenicol (Cm) (25 $\mu\text{g}/\text{mL}$).

Mutant construction

Deletion of the *fur* gene in *H. pylori* G27 (DSM300) and plasmid complementation of the Δfur mutant (DSM343) were previously performed in our laboratory (9). Deletion of gene *HpG27_51* in *H. pylori* was accomplished through

splicing by overlap extension (SOE) mutagenesis (31). Briefly, the 51_upF/51_upR and 51_dnF/51_dnR primer pairs were used to PCR amplify the up stream and down stream regions of the *HpG27_51* coding region, respectively; note the 51_upR and 51_dnF primers were designed with regions of homology to the non-polar kanamycin resistance cassette. The up stream and down stream regions were then sequentially fused to the kanamycin resistance cassette originally PCR amplified from pDSM101 using the Kan_F and Kan_R primers. The “up stream-kanamycin-down stream” SOE construct was then integrated into the chromosome of wild-type *H. pylori* by natural transformation. Mutant selection was performed on HBA Kan. Successful replacement of the *HpG27_51* gene was confirmed by PCR and sequencing using the 51confirmF and 51confirmR primers. Replacement of *HpG27_52* in strain G27 and the *HpG27_52* homologue *VD16_RS08305* in strain 7.13 with the kanamycin cassette was performed in a similar fashion using the 52_upF and 52_upR and 52_dnF and 52_dnR SOE primers followed by confirmation using the 52confirmF and 52confirmR primers. The G27 Δ *HpG27_51* and Δ *HpG27_52* mutant strains were named DSM1271 and DSM1180, respectively. The 7.13 Δ *VD16_RS08305* mutant was named DSM1228.

Since it was determined by sequencing that the *HpG27_52* up stream region was not conserved between the G27 and J166 strains, new SOE primers were designed for gene replacement of the *HpG27_52* homologue *EG65_00320* in J166: 52_upF_J166 and 52_upR_J166 and 52_dnF_J166 and 52_dnR_J166. SOE was conducted as described above. The resulting J166 Δ *EG65_00320* mutant strain was confirmed by PCR and sequencing (primers: 52confirmF_J166 and 52confirmR_J166) and was named DSM1435.

Complementation of the $\Delta HpG27_{52}$ mutation in G27 and the $\Delta VDI6_{RS08305}$ mutation in 7.13 was accomplished via chromosomal insertion of the *HpG27_52* or *VDI6_RS08305* gene into an intergenic region of their respective mutant genomes as previously described (33). The gene coding region and endogenous promoter were PCR amplified (primers: 52compF and 52compR) and were individually cloned into the pDSM745 vector after digestion of both the vector and insert with BamHI and PstI. The resulting vectors were transformed into Top10 *E. coli* and transformants were selected on LB Cm. Successful insertion of the genes into pDSM745 was confirmed by PCR (primers: 52compF and 52compR). The confirmed vectors were then transformed into the G27 $\Delta HpG27_{52}$ and 7.13 $\Delta VDI6_{RS08305}$ mutants (DSM1180 and DSM1228, respectively) and transformants were selected for on Cm. Complemented mutant strains were confirmed by PCR and sequencing using the HP0203-F and HP0204-R primers. The G27 $\Delta HpG27_{52}$ and 7.13 $\Delta VDI6_{RS08305}$ complementation strains were named DSM1302 and DSM1369, respectively.

The J166 $\Delta EG65_{00320}$ mutant was complemented via chromosomal insertion of the *EG65_00320* gene into the *rdxA* locus (52). The J166 *EG65_00320* coding region and endogenous promoter were PCR amplified (primers: 52compF and 52compR), digested with BamHI and PstI, and inserted into the similarly digested pDSM324 plasmid. Vector confirmation and transformation into strain DSM1435 were performed as described above. Successful insertion of the *EG65_00320* gene into the *rdxA* locus was confirmed by PCR and sequencing.

DNA mutagenesis of Fur-binding sites

Markerless mutations of either the iron-bound or *apo*-Fur putative DNA-binding regions (Fur boxes) were created by sequential insertion (selection) and replacement (counter selection) of the *kan-sacB* cassette by DNA fragments containing the desired mutated Fur boxes. Briefly, DNA regions flanking the iron-bound and *apo*-Fur box #2 (Figure 18) were PCR amplified and spliced together using SOE (primers: P52_UpF and P52_UpR and P52_DnF and P52_DnR). The resulting construct was then cloned into the pGEM-T easy vector (Promega) and transformed into *E. coli* Top10 cells. Importantly, the spliced construct was designed to contain centralized XhoI and XbaI restriction enzyme sites. The *kan-sacB* cassette, which confers resistance to kanamycin and sensitivity to sucrose, was next amplified from pDSM3 using the Kan_SacB_F and Kan_SacB_R primers. The *kan-sacB* cassette was then inserted between the two spliced DNA fragments following digestion of both insert and vector by XhoI and XbaI. The resulting construct was transformed into wild-type G27 (DSM1) and G27 Δfur (DSM300) strains and transformants were selected on HBA Kan. Chromosomal insertion of the *kan-sacB* cassette was confirmed by PCR and sequencing (primers: P52_Confirm_F and P52_Confirm_R). The wild-type and Δfur P₅₂::*kan-sacB* mutant strains were named DSM1437 and DSM1438, respectively.

Next, the *kan-sacB* cassette was replaced with DNA fragments that harbored scrambled Fur DNA-binding sequences. For both the iron-bound and *apo*-Fur boxes, the sequences were mutated to a run of alternating guanines and cytosines. We utilized G-Block technology (Integrated DNA Technologies) to synthesize a 909 base pair DNA fragment that contained the mutated iron-bound Fur box in the center (Figure 18). The G-Block was transformed into DSM1437 and DSM1438 and homologous recombination

5'...GACCATAGCTTTCGTGTCGCTTCTGATTTTATTGACAAAGAAAGGCACGCTCATATCAGGGAGCATTTTCCCAAAGCTTAAAAACCCCATTA
 GCCATTTTCAAACGGAGAAAAAATCTCACGGCTTTTGTATTTGTCTAAAAACATGCTCAATCATTCAAAGCGGGCTTTATTGTCCAAGCATCTAAACT
 CCGATCCATAAGCTCTATGAGCATGACTTTGTTTTTCAGGGTTGTTTAAAAGCTTTTGCATTTTAGAGTGGAACGCTTTTCTTGCTCGCTCAAATGGTTA
 CTGATGCTATCTTGCAGTTTTTTAGCTAATCTAATGAATCCTCAATGATTTTTTGCATGAGCTTACCTTTTATTTAAGAATTTGGCTTGATGTAGCAT
 GTTTTAAGCGGGTTTTAAGCGGTCATTTTAATAATGCTTTTTAATTGTTGGTTGTTTTCATACTAAATGAATTAATTAATAAATGATTTTTTAA
 AAGTGGTAAGTATTTACCGCTTAAACATAAAATTAGAGGAATTTTTATCGTTTGAAAAGTCATTTACTTTTCGTTTAAATGAATTAATAATTCG
 CATCGTGTAAATTTAAAGTTAGCTTATTCAATTAACGATTTTAAATTAAACGGATTATAACAAATTTTACAAACAAAGGATATAAAATGAAACAATT
 AAAAAAGTATTATGATTGGCACACTCGGTGCGTTGTTATTGAGCGGTTGTTCTAGCTTTGATACTCAGCGTTTCGCTTGTCTTCCATAAGACCATTCCT
 CAAAAGACGCTTCTACAAAAAAGAAGCGCAATACATGCCTAAGGCTTTTTTGACCCTTATTCCTTCTAACTTAAACCACGGGATTCTACATTCTAGGG
 GTTTATAAAGAGGG...3'

Figure 18. Key regulatory elements found within the intergenic region of genes *HpG27_51* and *HpG27_52*.

The sequence shown was extracted from the *Helicobacter pylori* G27 genome (NC_011333.1) beginning at nucleotide position 62,140 and ending at position 63,048. The green and blue DNA sequences correspond to the coding region of genes *HpG27_51* and *HpG27_52*, respectively. The arrows indicate the transcriptional start sites (TSS) and the -10 sequences are shown in red. The various putative *apo*-Fur boxes are underlined and numbered (1-4). The putative iron-bound Fur box is underlined with the dashed line. The sequence shown is identical to the G-block used to mutate the iron-bound Fur box except with TAATAATGCTTTTTA changed to GCGCGCGCGCGCGG.

events were selected for on HBA sucrose plates. Sucrose resistant colonies were subsequently screened for Kan sensitivity on HBA Kan. Colonies that were sucrose resistant as well as kanamycin sensitive were confirmed by PCR and sequencing (primers: P52_Confirm_F and P52_Confirm_R) to contain the mutated version of the iron-bound Fur box. The scrambled iron-bound Fur box strains were named DSM1439 (wild-type) and DSM1440 (Δfur). For the *apo*-Fur box, the mutated DNA fragment was created by SOE. Briefly, DNA regions up stream and down stream of *apo*-Fur box #2 (Figure 18) were amplified with the following primer pairs: P52_Up_F and P52_Up_R1 and P52_Dn_F1 and P52_Dn_R. Of note, the P52_Up_R1 and P52_Dn_F1 primers contain overlapping DNA sequences that encompass the mutated *apo*-Fur box. The two DNA fragments were spliced together (SOE) and subsequently transformed into DSM1437 and DSM1438 strains. Selection and confirmation of the mutant strains were performed in a similar fashion as described above. The wild-type and Δfur scrambled *apo*-Fur box strains were named DSM1444 and DSM1445, respectively.

Cell harvesting, RNA extraction, and RT-PCR

Overnight liquid starter cultures of *H. pylori* were used to inoculate a 25 mL culture to an OD₆₀₀ of 0.05 in 125 mL flasks. Cultures were grown for various times ranging from 6 hours (early exponential growth phase) to 31 hours (stationary phase). 10 mLs was removed from the culture and harvested by vacuum filtration onto a 0.45 μ m nitrocellulose filter and immediately snap-frozen in liquid nitrogen. Another 10 mLs was simultaneously removed from the 25 mL culture and added to a fresh 125 mL flask. Iron chelation of the medium was then performed by the addition of 200 μ M 2, 2'-Bipyridyl

(DPP, Sigma). The chelated culture was incubated for 1 hour at 37°C under microaerobic conditions with shaking (115 rpm). The chelated cultures were then harvested onto nitrocellulose filters and snap-frozen as described above. TRIzol (Invitrogen) mediated extraction of RNA was performed as previously described (7). Next, approximately 500 ng of purified RNA per sample was converted into cDNA using the Quantitect reverse transcription kit (Qiagen). For each reverse transcription (RT) reaction, a corresponding tube lacking the reverse transcriptase enzyme was included (NoRT) to assess genomic DNA contamination. A 1 µl aliquot of the RT or NoRT reaction was added to a 20 µL real-time PCR (RT-PCR) reaction consisting of 1X SYBR Green RT-PCR master mix (Qiagen) and 3 pmol of each primer. All RT-PCR primers are listed in Table 15. The RT-PCR reactions underwent an initial activation step for 5 minutes at 95°C and were then subjected to 35 cycles of 95°C for 5 seconds followed by 50°C for 10 seconds using a Roto-gene Q thermocycler (Qiagen). RT-PCR primers were tested for specificity by single DNA band detection using DNA gel electrophoresis (2% agarose gel) as well as post RT-PCR melt curve analysis. Fold change in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method with the 16S rRNA gene as the internal control. All RT-PCR reactions were performed minimally in triplicate.

Primer extension and 5' transcriptional start site (TSS) mapping

A 375 base pair region encompassing the promoter region of gene *HpG27_52* in *H. pylori* G27 was PCR amplified using the 52_seq_F and 52_seq_R primers. This PCR fragment served as template for dideoxy sequencing reactions using the Sequenase PCR product sequencing kit v2.0 (USB) according to the manufacturer's recommendation.

Additionally, approximately 2.5 µg of total RNA served as template for primer extension analysis using the AMV reverse transcription kit (Promega). An additional NoRT control was performed in a similar manner as mentioned above. Sequencing and primer extension reactions were initiated from the same genomic location using the 52_P.ext γ-³²P-labeled DNA primer and resolved by urea polyacrylamide gel electrophoresis. The gels were subsequently exposed to phosphor screens overnight and scanned using a Storm 860 scanner (Molecular Dynamics). Gel images were analyzed using ImageQuant TL Software (GE). 5' TSS mapping was performed in triplicate with comparable results.

Urease activity analysis

To test the ability of gene *HpG27_52* to enhance urease activity in *H. pylori*, we utilized a colorimetric assay to measure ammonia production in both *H. pylori* as well as in a previously documented surrogate *E. coli* model (39). A fragment of DNA containing the *HpG27_52* ribosomal binding site (RBS) and coding region from *H. pylori* G27 were PCR amplified (primers: 52_RBS_F and 52_RBS_R) and cloned into the EcoRV digested pDSM775 vector. The pBS::*HpG27_52* construct was then transformed into strain DSM1139, which carries the pHP8080 vector that expresses the entire urease gene cluster (*ureABIEFGH*). An empty pBS vector (DSM1396) as well as two pBS vectors that each contained known urease enhancing factors (pUEF1004 (DSM1412) and pUEF1004-511 (DSM1413)) were included as negative and positive controls, respectively. All *E. coli* strains were grown overnight from a single colony in 1.5 mLs of M9 minimal medium-1X M9 salts, 0.4% glucose, 1 mM MgSO₄, 0.1 mM CaCl₂, 1.68 µM thiamine-HCl, 0.5% Casamino Acids, 1% L broth, and 1 µM NiCl₂ (39) containing either

Cm or Cm plus Amp to ensure plasmid retention. The following day, the bacteria were pelleted via centrifugation at 12,000 x g for 2 minutes at 4°C. The cells were washed twice with 50 mM HEPES buffer pH 7.5 and subsequently resuspended in 1 mL of the HEPES buffer in 1.5 mL eppendorf tubes. The resuspended bacteria were lysed on ice by sonication (3 x 20 second pulses at 40% intensity). Cytosolic proteins (supernatant) were separated from the cell debris (pellet) by centrifugation at 12,000 x g for 10 minutes at 4°C. The protein concentration in the supernatant was measured using the Pierce BCA protein assay (Life Technologies). 50 µgs of protein was then added to urease buffer (50mM HEPES, 25mM urea, pH 7.5) to a final volume of 1.5 mLs and incubated at 37°C for 20 minutes. 100 µLs of the reaction was then stopped by the addition of 1.5 mLs of solution A (10 g/liter phenol, 50 mg/liter sodium nitroprusside). An equal volume of solution B (5 g/liter NaOH, 0.044% NaClO) was added, vortexed, and incubated for 30 minutes at 37°C. Color development was measured by absorbance at 625 nm using a Genesys 20 spectrophotometer (Thermo Scientific). A standard curve of ammonium chloride ranging from 0 to 2.5 mM was implemented to quantify ammonia production. All data was normalized to DSM1139 (pHP8080).

A modified protocol was used to directly assess urease activity in *H. pylori*. For all strains of *H. pylori* used, 25 mL cultures were initially inoculated to an OD₆₀₀ of 0.05 and grown for 6 hrs. 1 mL was removed from the culture, pelleted, and washed 2X in HEPES buffer as described above. The *H. pylori* cells were then resuspended in 750 µLs of buffer containing HEPES (50 mM), pH 7, 1 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma) and protease inhibitor cocktail tablets (Roche) and lysed on ice by sonication (6 x 2 second pulses at 70% intensity). Cell debris was removed by

centrifugation (15,000 x g for 10 minutes at 4°C) and the supernatant was collected. 10 µg of total protein from the supernatant was added to the urease buffer (50 mM HEPES, 25 mM urea, pH 7) to a final volume of 1.5 mLs and incubated at 37°C for 20 minutes. 50 µLs of the reaction was removed and added to solution A (same recipe as above) to a final volume of 1.5 mLs. Addition of solution B, incubation, and quantification of ammonia was performed as described above. A previously created G27 $\Delta ureB$ mutant (DSM43) served as a negative control to compensate for any background ammonia production. Ammonia production for the various *H. pylori* mutant strains was normalized to wild-type G27.

Competitive animal infections

H. pylori infection of Mongolian gerbils (6-12 week-old male) was performed using the 7.13 and J166 *H. pylori* strain backgrounds. Wild-type *H. pylori* was mixed with mutant bacteria or the complementation strain in a 1:1 ratio. Gerbils were fasted for 12 hours and then infected with approximately 10^9 total colony forming units (CFUs) of the bacterial mixture via oral gavage. Animals were sacrificed at 1, 2, and 3 weeks post infection and the glandular portion of the stomachs were excised, cleared of food bits, and weighed. After mechanical homogenization of the stomach (Bullet Blender 5, Next Advance), a portion was plated onto HBA containing nalidixic acid (10 µg/mL) (Sigma), bacitracin (100 µg/mL) (USB), and vancomycin (5 µg/mL) (Amresco) and an equal portion was plated on HBA containing Kan and/or Cm to select for mutant strain growth. Wild-type colonization levels were determined by subtracting the number of antibiotic resistant colonies from the total number of colonies that grew on plain HBA medium.

Competitive index (CI) values were calculated by dividing the ratio of mutant to wild-type bacteria recovered from the gerbil's stomach by the inoculum mutant to wild type ratio. A total of 5 animals were included in each coinfection group.

Statistical analysis

Differences in gene expression between the various strains for the RT-PCR experiments were calculated using the Student's *t* test on log₁₀-transformed fold change ratios. Additionally, the Student's *t* test was used to compute differences in ammonia production between the various strains tested for the urease activity assays.

RESULTS

***HpG27_51-HpG27_52* intergenic region contains Fur-binding sequences**

Previously, we identified a putative semi-conserved iron-bound Fur box located in the intergenic region between the divergently transcribed genes *HpG27_51* and *HpG27_52* in *H. pylori* G27 (47). A detailed diagram of this intergenic region is depicted in Figure 18. The Fur box contains two mismatches as compared to the canonical *H. pylori* iron-bound Fur box (5'-TAATAATGCTTTTTA-3'). Through the use of electrophoretic mobility shift assays (EMSA), it was previously determined that Fur does bind the fragment of DNA containing the predicted iron-bound Fur box (47). Additional analysis revealed four potential *apo*-Fur boxes (5'-AATGA-3') dispersed throughout the *HpG27_51-HpG27_52* intergenic region. We utilized *H. pylori* transcriptome data to identify the transcriptional start sites (TSS) for genes *HpG27_51* and *HpG27_52* (50).

Because the original transcriptome analysis was performed in the 26695 strain background, we experimentally verified the TSS in G27 for gene *HpG27_52* by 5'TSS mapping (Figure 19). As shown in Figure 18, the iron-bound Fur box lies 47 base pairs up stream of gene *HpG27_51*'s TSS and 161 base pairs up stream of gene *HpG27_52*'s TSS. Additionally, the first two identified *apo*-Fur boxes appear to flank the iron-bound Fur box and are in close proximity to the *HpG27_51* promoter region (40 and 83 base pairs up stream of the *HpG27_51* TSS, respectively). The other two predicted *apo*-Fur boxes are farther up stream of the *HpG27_51* promoter but are in the direct vicinity of the *HpG27_52* promoter (19 and 38 base pairs up stream of the *HpG27_52* TSS, respectively).

Fur regulation of genes *HpG27_51* and *HpG27_52*

Given the large number of potential Fur-binding sites, it stands to reason that these two genes may be directly regulated by Fur. To assess this, we utilized RT-PCR to measure transcript levels in response to iron chelation in the wild-type and Δfur backgrounds. We initially performed a time course analysis to determine at which growth phase the *HpG27_51* and *HpG27_52* genes were most responsive to iron chelation. As shown in Figure 20, at 6 hours of growth, we saw a significant increase in both *HpG27_51* and *HpG27_52* expression in wild-type G27 after iron chelation; a similar increase was not observed in the Δfur mutant ($p < 0.004$). Of note, the increase in expression was more pronounced for the *HpG27_52* gene (16.76 versus 3.75 average fold increase for *HpG27_51*, $p = 0.0011$). We also detected an increase in expression in the Δfur complemented strain compared to the Δfur mutant (Figure 21), thus

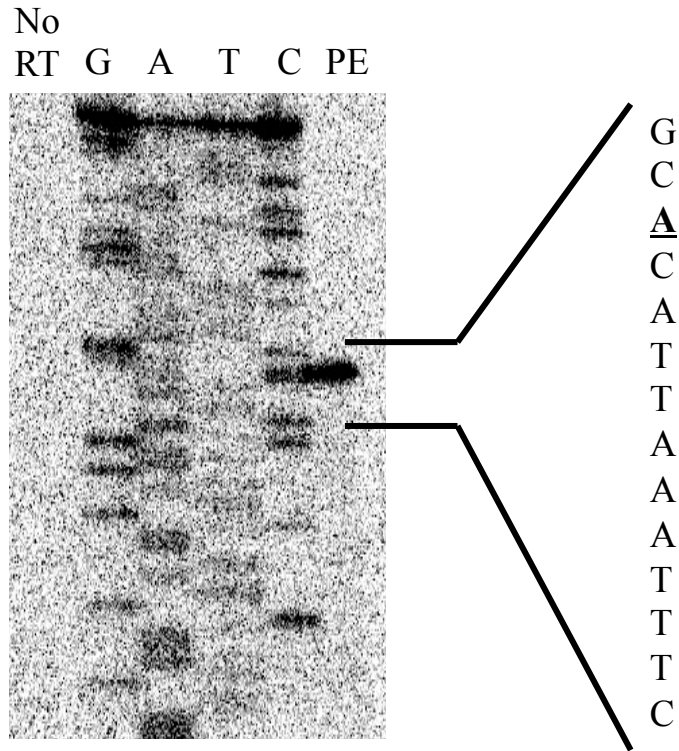


Figure 19. 5' Transcriptional start site (TSS) mapping of gene *HpG27_52*. Primer extension (PE) was performed on wild-type G27 RNA and the single DNA band is shown on the right hand side of the gel. A similar reaction in which no reverse transcriptase was added to the primer extension reaction (NoRT) was simultaneously performed and ran on the left hand side of the gel to control for any genomic DNA contamination. The DNA sequence surrounding the PE product is shown to the right of the gel. The nucleotide that aligns to the PE product (TSS) is underlined and in bold.

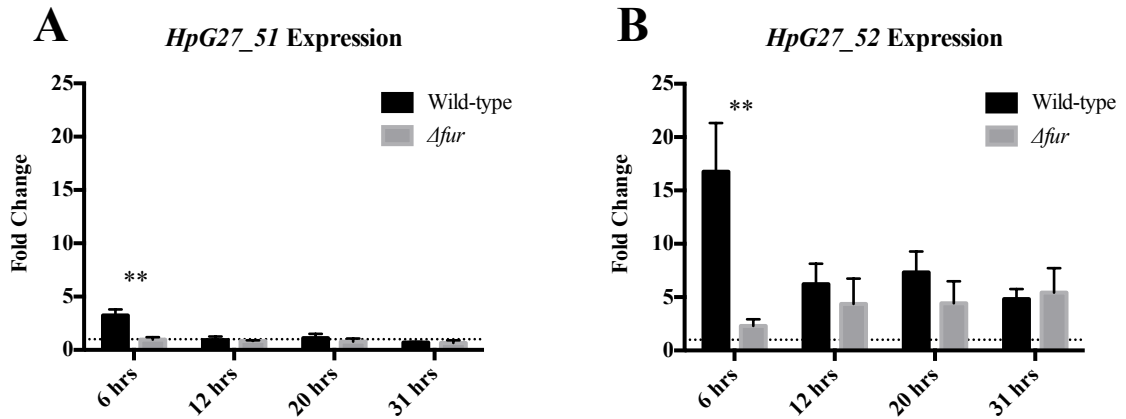


Figure 20. Fold change in gene expression after 1 hour iron chelation as assessed via RT-PCR.

Iron chelation was performed on wild-type (black) and Δfur (gray) cultures that were grown for 6, 12, 20 and 31 hours. Changes in expression were determined for genes *HpG27_51* (A) and *HpG27_52* (B). Double asterisk denotes significant differences in fold change between wild-type and Δfur ($p < 0.005$) as determined by Student's *t* test. Each bar corresponds to the mean fold change with the error bars representing the standard deviation. The dotted line at $y=1$ indicates no change in gene expression. 3 biological replicates were performed for each sample.

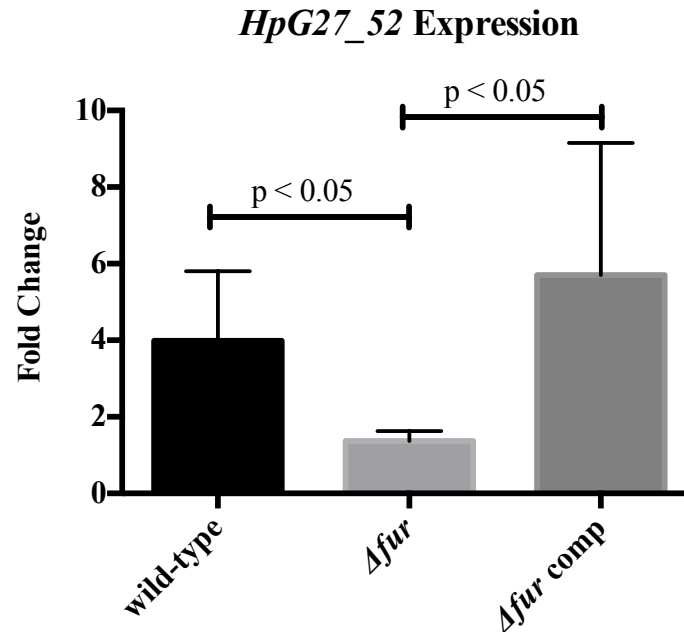


Figure 21. Fold change in *HpG27_52* expression after 1 hour iron chelation for wild-type, Δfur , and the complemented Δfur *H. pylori* G27 strains. RNA was extracted from cultures grown to early stationary phase (18-22 hours). Each bar represents the mean of 3 biologically independent experiments. Error bars represent the standard deviation. Statistical comparisons were conducted using the Student's *t* test.

confirming that the increase in *HpG27_52* expression was a Fur-dependent phenomenon.

To determine if the increase in *HpG27_51* and *HpG27_52* transcripts was a product of Fur activation or de-repression, we compared the ratio of transcripts in the Δfur strain to the wild-type before and after iron chelation (Figure 22). For both *HpG27_51* and *HpG27_52*, gene expression in wild-type and Δfur was similar prior to iron chelation at 6 hours. However, post iron chelation, transcripts levels were more abundant in the wild-type as compared to the Δfur mutant at 6 hours. Together, these data suggest that in early exponential growth phase (6 hours), iron chelation results in *apo*-Fur activation of expression of *HpG27_51* and *HpG27_52*. Of note, this phenomenon was exquisitely growth phase dependent since similar results were not seen at any other timepoints.

Fur box contribution to *HpG27_51* and *HpG27_52* expression

As mentioned previously, Fur typically influences gene expression via direct DNA binding at specific locations known as Fur boxes. To assess the contribution of the various putative Fur boxes identified in the promoter regions of *HpG27_51* and *HpG27_52*, we mutated the 15 nucleotide iron-bound Fur box as well as the 6 nucleotide *apo*-Fur box #2 (Figure 18) to a series of repeating guanines and cytosines. Fur box mutations were created in both wild-type G27 as well as the Δfur mutant. Despite evidence that Fur directly binds the iron-bound Fur box (47), we did not detect any significant differences in *HpG27_51* or *HpG27_52* expression between wild-type and the scrambled iron-bound Fur box counterpart (Figure 23). Additionally, disruption of *apo*-

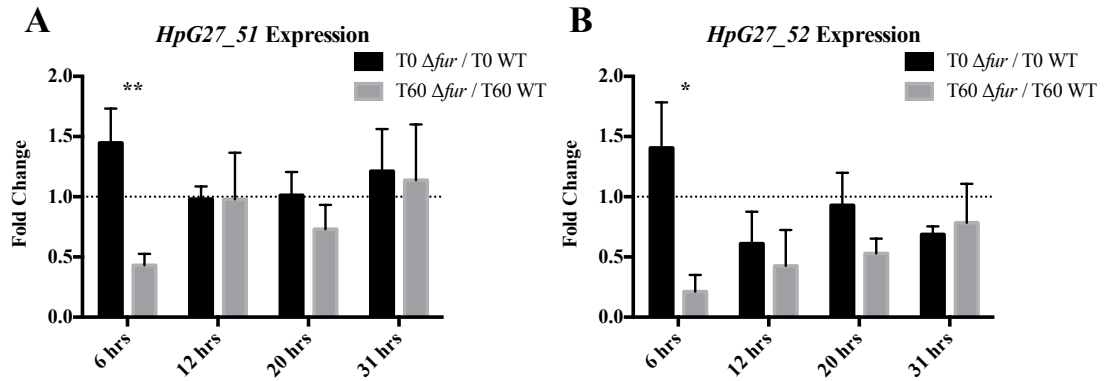


Figure 22. Expression of genes *HpG27_51* (A) and *HpG27_52* (B) in the Δfur mutant compared to wild-type either before (T0, black) or after (T60, gray) 1 hour iron chelation.

Ratios were computed for cultures grown for 6, 12, 20, and 31 hours. The dotted line at $y=1$ represents equal transcript levels in the Δfur and wild-type strains. Bars represent the mean of 3 biologically independent experiments. Error bars correspond to standard deviation. * = $p < 0.05$, ** = $p < 0.01$.

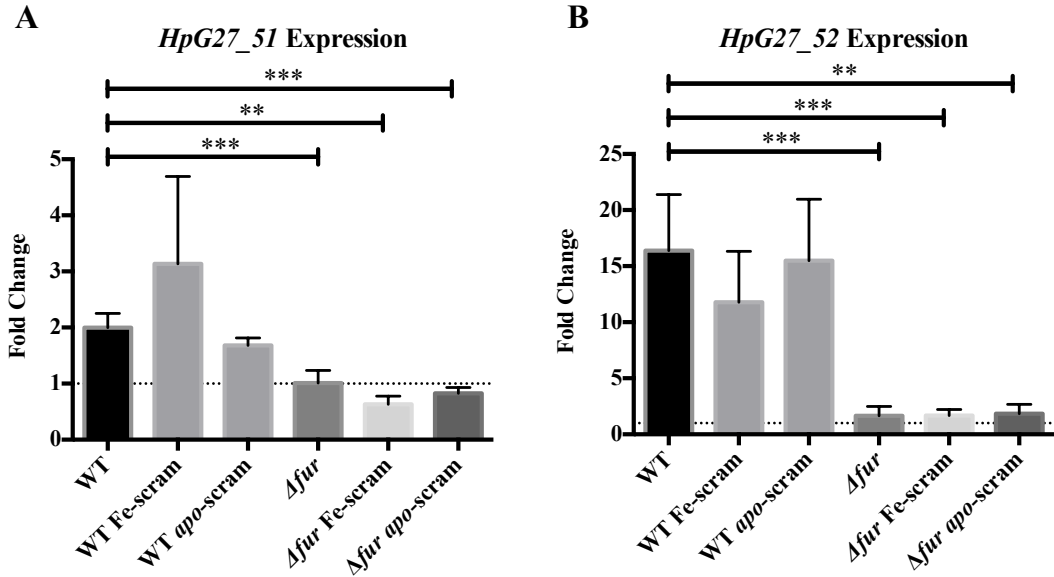


Figure 23. Fold change in gene *HpG27_51* (A) and *HpG27_52* expression after 1 hour iron chelation for the various Fur box mutants. Both the iron-bound (Fe-scram) and *apo* Fur box (*apo-scram*) mutants were generated in the wild-type (WT) and Δfur strain backgrounds. RNA used for cDNA synthesis was extracted from cultures grown for 6 hours. The dotted line at $y=1$ indicates no change in gene expression. Bars represent the mean of 3 biologically independent experiments. Error bars correspond to standard deviation. No significant differences among the various wild-type or Δfur strains were detected for either gene *HpG27_51* or *HpG27_52* as determined by the Student's *t* test. ** = $p < 0.01$, *** = $p < 0.001$.

Fur box #2 did not alter either gene's expression pattern after iron chelation. This was true in both wild-type and Δfur strains.

When transcript levels were compared between the various Fur box mutant strains prior to iron chelation, we found that mutation of the iron-bound Fur box resulted in a significant reduction in *HpG27_51* transcript levels as compared to the non-mutated Fur box strains (Figure 24). Because this effect was observed both in the wild-type and Δfur mutant strains, it is likely a product of promoter-structure disruption rather than actual Fur regulation.

***HpG27_52* encodes a potential urease enhancing factor**

According to the annotated *H. pylori* G27 genome (NC_011333.1), *HpG27_51* encodes an enzyme involved in proline utilization (proline/delta1-pyrroline-5-carboxylate dehydrogenase). On the other hand, *HpG27_52* encodes a hypothetical protein of unknown function. To associate a function to the protein encoded by *HpG27_52*, we searched the *H. pylori* G27 genome for potential gene paralogs using the Basic Local Alignment Search Tool (BLAST). We found that the amino acid sequence of *HpG27_52* showed high similarity to a known urease enhancing factor (UEF) (*HpG27_469*) (43% amino acid identity, 58% similarity). We therefore utilized a previously described *E. coli* surrogate colorimetric assay to test the ability of *HpG27_52* to enhance urease activity. As shown in Figure 25A, when the *HpG27_52* gene was introduced into an *E. coli* strain expressing *H. pylori* urease, a significant increase in ammonia production was seen as compared to the same strain containing an empty pBS vector. While the increase in

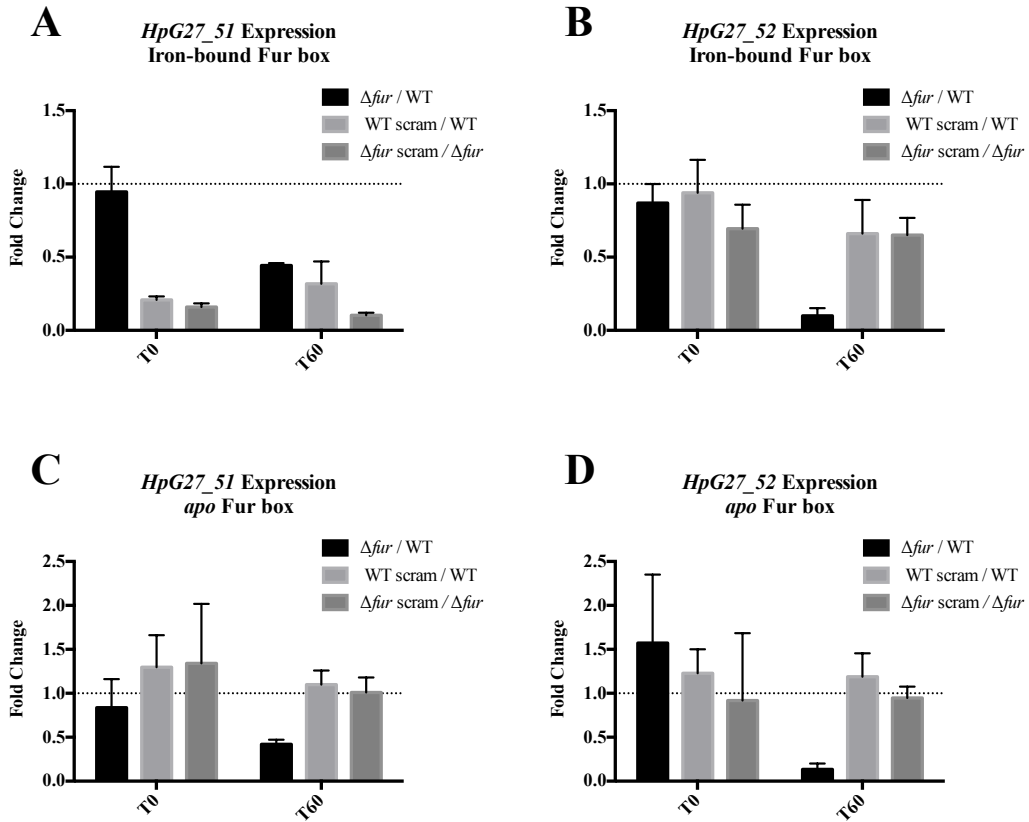


Figure 24. Expression of genes *HpG27_51* (panels A and C) and *HpG27_52* (panels B and D) in the scrambled Fur box mutant strains compared to wild-type or Δfur either before (T0) or after (T60) 1 hour iron chelation.

Fold change data was calculated for the iron-bound Fur box mutants (top panels, A and B) as well as the *apo* Fur box mutants (bottom panels, C and D) at 6 hours. Fur box mutations were created in both wild-type (WT scram) as well as Δfur (Δfur scram). The dotted line at $y=1$ represents equal transcript levels between the two strains. Bars represent the mean of 3 biologically independent experiments. Error bars correspond to standard deviation.

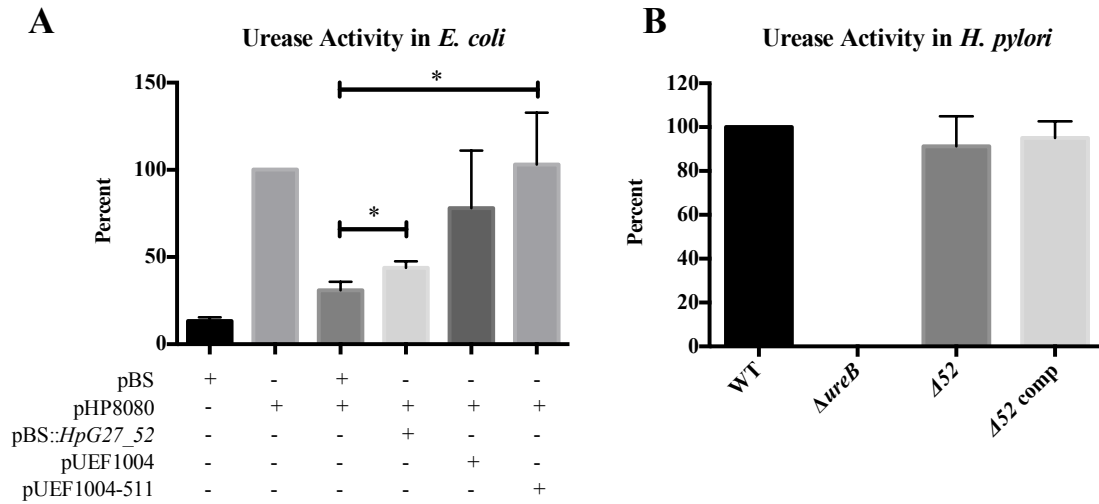


Figure 25. Urease enhancing activity of gene *HpG27_52*.

The ability of gene *HpG27_52* to enhance urease activity was measured in a surrogate *E. coli* model (A) as well as in *H. pylori* G27 (B). For the various *E. coli* strains tested in panel A, the plus symbol (+) indicates the presence of the listed plasmid. Data is normalized to pHP8080 in panel A and wild-type (WT) in panel B. As a negative control, a $\Delta ureB$ strain was included to detect background ammonia levels. Bars represent the mean of 3 biologically independent experiments. Error bars correspond to standard deviation. Statistical differences were determined using the Student's *t* test (* = $p < 0.05$).

ammonia production was smaller than with other known UEFs (pUEF1004-511), these data support the notion that *HpG27_52* has a role in urease function.

Although the *E. coli* system is an accepted model for measuring urease activity (39), we felt it necessary to directly assess urease activity in *H. pylori*. To this end, we used a similar protocol to measure ammonia production in wild-type *H. pylori* as well the Δ *HpG27_52* mutant and its respective complemented strain. Additionally, we included a urease-deficient strain of *H. pylori* (Δ *ureB*) as a means to assess background ammonia levels produced by other systems in the cell. With the exception of Δ *ureB*, We did not detect any differences in ammonia production among the various strains tested (Figure 25B), which suggests that within the context of *H. pylori*, *HpG27_52* has little to no detectable urease enhancing activity in the conditions tested.

***HpG27_52* homologues in 7.13 and J166 may contribute to *in vivo* colonization**

While elucidation of the molecular function of *HpG27_52* is important, we also wished to ascertain any role gene *HpG27_52* may play in *in vivo* colonization. This was accomplished through competitive animal infections using the 7.13 and J166 gerbil adapted *H. pylori* strains. For our model, we infected male Mongolian gerbils with a 1:1 mixture of wild-type and mutant bacteria via oral gavage. The bacteria were allowed to colonize the stomach for up to three weeks before euthanasia and subsequent recovery of *H. pylori*. As shown in Figure 26, for both 7.13 and J166, the mutant strain was dramatically outcompeted by wild-type for all time points tested. In fact, the mutant strain was cleared from the stomach in the vast majority of infected animals.

Unfortunately, the complemented strains were also outcompeted by wild-type at two

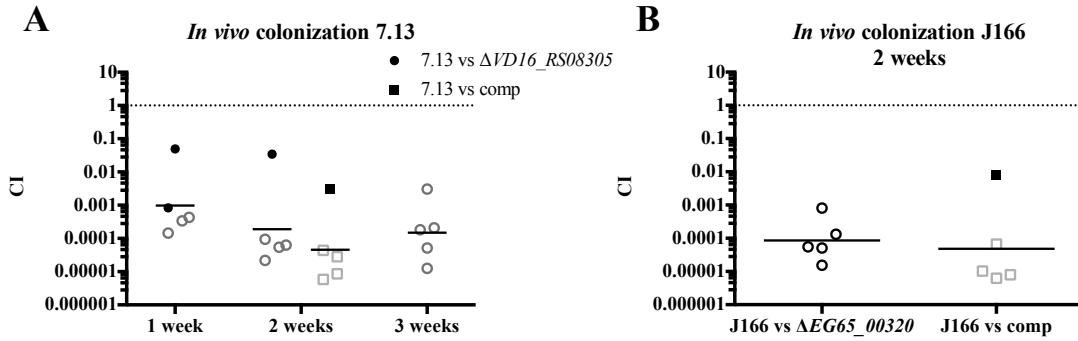


Figure 26. Competitive index (CI) of the various *HpG27_52* homologue mutant strains ($\Delta VD16_RS08305$ and $\Delta EG65_00320$) and complemented strains (comp) versus wild-type *H. pylori*.

(A) CI of wild-type 7.13 versus $\Delta VD16_RS08305$ (circles) and wild-type versus the complemented $\Delta VD16_RS08305$ mutant (squares) after 1, 2, and 3 weeks post infection. (B) CI of wild-type J166 versus $\Delta EG65_00320$ (circles) and wild-type versus the complemented $\Delta EG65_00320$ mutant (squares) after 2 weeks post infection. Mongolian gerbils in which no mutant bacteria were recovered are depicted as open symbols. Lines represent geometric mean. Dotted line indicates no colonization advantage for either strain (CI = 1).

weeks post infection. Thus, there appears to be a secondary mutation affecting these results. *In vivo* competition analyses between the mutant and its complement are currently planned in order to elucidate any role of the *HpG27_52* gene homologues in colonization.

DISCUSSION

Helicobacter pylori is a unique bacterial pathogen for numerous reasons. First, *H. pylori* is capable of surviving within the harsh environment of the human stomach and, if left untreated, can persist in the stomach for decades (36). Secondly, the genome of *H. pylori* is relatively small (~1.7 Mb) and encodes surprisingly few regulatory proteins (55). In fact, of the 1,590 open reading frames identified in the *H. pylori* 26695 genome, only four encode proteins with a perfect helix-turn-helix DNA-binding motif (55). Lastly, of the few regulatory proteins encoded by *H. pylori*, many have evolved multiple regulatory functions that influence expression of numerous genes throughout the genome (24; 37; 44). In support of these three unique bacterial facets, the *H. pylori* Fur protein is a master regulatory protein that is not only important for *in vivo* survival (42), but also influences an impressively large number of genes that span an array of physiological functions that range from iron acquisition and storage to oxidative stress response (4; 12; 24; 28; 49). Further elucidation of the Fur regulon is imperative as it may reveal susceptibilities in the microbe that can be exploited for development of antimicrobial compounds. Despite numerous reports that have characterized portions of the Fur regulon (4; 24; 28; 48; 59), significant work still remains. To this end, we characterize a set of

two divergently transcribed genes, *HpG27_51* and *HpG27_52*, that are regulated by Fur in response to iron availability.

According to the annotated G27 genome, *HpG27_51* encodes an enzyme involved in proline utilization (*putA*) (proline/delta1-pyrroline-5-carboxylate dehydrogenase). For most organisms, proline is converted to glutamate via a two-step oxidation process that requires two distinct enzymes: initial oxidation of proline to delta1-pyrroline-5-carboxylate (P5C) via proline dehydrogenase followed by NAD⁺-dependent oxidation of P5C to glutamate through P5C dehydrogenase activity (41). In certain Gram-negative bacteria, including *H. pylori*, this dual enzymatic activity is coupled into a single protein known as PutA (1; 34; 41; 54). In regards to *Helicobacter* physiology, PutA oxidation of proline generates reactive oxygen species as a bi-product that can be harmful to the cell (35). In fact, high levels of proline were shown to be toxic to wild-type *H. hepaticus* (35). Interestingly, Fur has been shown to directly regulate the expression of antioxidant genes that are responsible for combating oxidative stress (12). In our study, we show that expression of the *HpG27_51* gene is influenced by Fur (Figure 20). Thus, the balance between proline utilization and antioxidant production is likely controlled by Fur. Alternatively, as pointed out by Pich and Merrell (48), the reduced flavins generated by PutA may also be used to convert extracellular ferrous iron to ferric iron via iron reductases, a process also influenced by Fur (61). Thus, by placing *putA* expression under the control of Fur, *H. pylori* can efficiently modulate both proline and iron metabolism within the cell.

HpG27_52 is predicted to encode a protein of unknown function. Initial characterization of the gene revealed a high similarity to a known urease enhancing factor

(UEF) in *H. pylori* (*HpG27_469*). While the ability of *HpG27_52* to enhance urease activity in *H. pylori* was undetectable, the gene did possess UEF qualities when tested in *E. coli* (Figure 25). Urease is essential for *H. pylori* survival within the human stomach as it hydrolyzes urea to ammonia, thus neutralizing the surrounding gastric acid (22; 23). Besides urease, Fur is also involved in acid tolerance in *H. pylori* (4) as well as in other bacterial pathogens (30). In fact, mutation of the *fur* gene in *H. pylori* results in diminished acid-responsive induction of the two major subunits of the urease enzyme, UreA and UreB (58). Given that *HpG27_52* was also shown to be Fur-regulated (Figure 20), it is possible that *H. pylori* can further fine tune urease activity via levels of UEFs in the cell. Preliminary data in our lab suggests no survival defect at low pH for the *HpG27_52* mutant, however, additional experiments need to be conducted. We also acknowledge that multiple UEF's may exist within the *H. pylori* genome (39) and that deletion of one UEF may be compensated for by redundancy or by overexpression of additional UEFs. This may explain why we did not detect differences in urease activity between the wild-type and Δ *HpG27_52* mutant strain in *H. pylori*. Regardless, these data reveal additional facets of the Fur regulon that warrant further investigation.

While both *HpG27_51* and *HpG27_52* are up regulated in response to iron chelation in a Fur dependent manner, the nature of the interaction of Fur with the promoter regions remains unclear. It is not entirely surprising that mutations in the iron-bound Fur box did not alter either *HpG27_51* or *HpG27_52*'s iron-responsive Fur regulation (Figure 23) as both genes have regulatory profiles indicative of *apo*-Fur activation. Subsequent mutation of an *apo*-Fur box in the promoter regions also did not elicit changes in iron responsiveness. We do note, however, that three additional *apo*-Fur

boxes were identified in the intergenic region and may contribute to *apo*-Fur activation of *HpG27_51* and *HpG27_52*. Mutational studies to address this possibility are currently underway. We are also in the process of conducting DNA footprinting assays under iron-deplete and replete conditions to ascertain where Fur binds.

In addition, we also must consider the possibility that Fur may not be the only regulatory protein at play, especially in regards to *HpG27_52* gene regulation. *HpG27_52* was demonstrated to enhance urease activity and thus, may have a direct role in *H. pylori*'s response to acid stress. In addition to Fur, the metal-dependent regulatory protein, NikR, and the two-component signal transduction system, ArsRS, are both involved in regulation of genes important for acid tolerance, including urease (6; 46; 58). Previous reports have shown that some Fur regulated genes in the *H. pylori* genome are co-regulated by NikR and/or ArsR (17; 19; 46). We hypothesize that *HpG27_52* transcription is likely up regulated in response to low pH, however, this possibility and the role of the three acid-responsive regulators (Fur, ArsRS, and NikR) would need to be investigated to determine if this is in fact the case.

If *HpG27_52* is pH responsive, this could be important for *in vivo* colonization of the gastric mucosa (Figure 26). Logically, we can hypothesize that in the acidic environment of the stomach, *H. pylori* up regulates *HpG27_52*, which in turn increases urease activity. Proper urease activity has long been known to be required for gastric colonization (23; 51; 57). Unfortunately, we were unable to restore the colonization defect in the 7.13 and J166 mutant strains upon chromosomal reintroduction of the *HpG27_52* gene homologue (Figure 26). While we expect that mutant complementation with the gene coding region and its endogenous promoter would yield wild-type

transcript levels, it is possible that genomic location may also affect gene transcription. A recent study found that in *E. coli*, gene location on the bacterial chromosome can alter gene expression as much as 300 fold (5). While we can test *HpG27_52* expression *in vitro*, we currently have no way to assess transcript levels while the bacterium actively colonizes the gastric mucosa.

In summary, we demonstrate that two genes, one involved in proline utilization (gene *HpG27_51*), and another involved in urease activity (gene *HpG27_52*), are activated in response to iron limited conditions in a Fur-dependent fashion. This activation did not appear to be the result of Fur binding to the iron-bound or one of the tested *apo*-Fur boxes identified in the intergenic/promoter regions of *HpG27_51* and *HpG27_52*. Lastly, through *in vivo* competitive index studies, we show that *HpG27_52* may be necessary for proper colonization of the gastric mucosa. Significant work yet remains in order to tease out the details regarding the exact role of Fur in this system and how it may influence *H. pylori* physiology.

ACKNOWLEDGMENTS

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APPENDIX C: Identification of additional conserved Fur binding sites on the *Helicobacter pylori* chromosome

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The work presented in this chapter is the sole work of R.C. Johnson.

The ferric uptake regulator, Fur, is a bacterial specific regulatory protein that controls expression of key iron-homeostasis genes in response to fluctuating iron levels (4; 12; 16). When iron levels are elevated, iron-cofactored Fur will bind to the promoter region of iron acquisition genes and repress their expression (4; 12; 16). When iron levels decrease, this repression is alleviated and transcription is restored. The region of DNA that is bound by Fur (Fur box) appears to be sequence specific and has been defined for numerous organisms (1; 7; 9; 11). While some Fur boxes are structured as hexameric repeats (9; 11), others consist of two AT-rich inverted repeats separated by a single nucleotide in a 7-1-7 or 9-1-9 pattern (1; 7; 13). Post elucidation of the Fur box sequence, subsequent bacterial genome searches for this consensus sequence have led to the identification of additional genes that may be directly regulated by Fur (1; 13; 15). For example, in the Gram-negative gastric pathogen *Helicobacter pylori*, a total of 33 semi-conserved Fur boxes were previously identified. These Fur boxes were interspersed throughout the genome, and some were located upstream of transcriptional start sites, which is indicative of direct Fur regulation (13). Of the various potential Fur regulated genes identified in *H. pylori*, some are predicted to be involved in iron metabolism: *fecA1* and *frpB1*. Interestingly, Fur boxes were also found upstream of genes involved in non-

iron related processes including virulence (*cagA*), proline utilization (*putA*), and pyridoxine synthesis (*pdxJ*). The Fur regulon in *H. pylori* is exceptionally complex (4; 6; 8; 14), and despite years of research and numerous published studies (2; 3; 5; 10), our current understanding of Fur regulation in *H. pylori* likely encompasses only the “tip” of the regulatory iceberg.

The iron-bound Fur box in *H. pylori*, as characterized by Pich *et al.*, is a 7-1-7 motif with dyad symmetry (5'-TAATAATnATTATTA-3') (13). Characterization of this Fur box, as mentioned previously, has been instrumental in the identification of additional Fur regulated genes on the *H. pylori* chromosome. However, given the palindromic nature of this Fur box, it is probable that Fur also binds the reverse complement sequence (5'-ATTATTAnTAATAAT-3'). Because this sequence was not originally considered in the search, the number of Fur boxes in the *H. pylori* genome may be vastly underestimated. Therefore, we set out to develop a computer program (Fur Box Finder) that could identify the 5'-TAATAATnATTATTA-3' sequence and its reverse complement in the *H. pylori* genome. Additional Fur boxes identified by this program may reveal additional Fur regulated gene that would warrant further investigation.

The Fur Box Finder program was written in the Python programming language and executed using Python version 2.7.8 (www.python.org). The entire script is shown in Figure 27. The program can essentially be broken down into 3 segments: fasta sequence preparation, Fur box identification, and Fur box location reporting. To demonstrate the functionality of the Fur Box Finder program, we initially downloaded the *H. pylori* G27 genome fasta file (*H. pylori_G27.fasta*, GenBank: NC_011333.1) from the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>). As shown

```

import re
genome_seq = file("Hp_G27.fasta").readlines()
genome_seq = "".join(genome_seq[1:]).replace("\n", "")
file = open("Furboxes_in_Hp.txt", "w")
def compare(furbox):
    for N in range(0, (len(genome_seq)+1)):
        start = N
        stop = N + 15
        DNA_seq = genome_seq[start:stop]
        check_len = len(DNA_seq) == 15
        if check_len == True:
            score = 0
            for num in range(0, 7):
                if DNA_seq[num] == furbox[num]:
                    score = score + 1
                else:
                    score = score
            for num1 in range(8, 15):
                if DNA_seq[num1] == furbox[num1]:
                    score = score + 1
                else:
                    score = score
            if round(((score/14) * 100), 2) == 100:
                match = re.search(DNA_seq, genome_seq)
                print DNA_seq + " 0 mismatches at position " + str(match.start()+1)
                file.write(DNA_seq + " 0 mismatches at position " + str(match.start()+1) + "\n")
            elif round(((score/14) * 100), 2) == 92.86:
                match = re.search(DNA_seq, genome_seq)
                print DNA_seq + " 1 mismatch at position " + str(match.start()+1)
                file.write(DNA_seq + " 1 mismatch at position " + str(match.start()+1) + "\n")
            elif round(((score/14) * 100), 2) == 85.71:
                match = re.search(DNA_seq, genome_seq)
                print DNA_seq + " 2 mismatches at position " + str(match.start()+1)
                file.write(DNA_seq + " 2 mismatches at position " + str(match.start()+1) + "\n")
            else:
                score = score
compare("TAATAATNATTATTA")
compare("ATTATTANTAATAAT")
file.close()

```

Figure 27. Fur Box Finder program written in Python.

The program is designed to search a single fasta file (“Hp_G27.fasta”) for the presence of the iron-bound Fur box sequence (5’-TAATAATnATTATTA-3’) and its reverse complement. Output from the program include: identified Fur box sequence, number of mismatches from canonical Fur binding sequence, and location of Fur box within the fasta file.

in Figure 27, the initial step of the program converts the fasta sequence into a single line of nucleotide characters. This obviates any issues with “tab” or “return” characters located within the fasta sequence. The next step opens a blank text file (“Furboxes_in_Hp.txt”) that will be used later to document the various Fur boxes in the genome. In order to search the genome for potential Fur boxes (5'-TAATAATnATTATTA-3' or 5'-ATTATTAnTAATAAT-3'), we utilized a 15 base pair “sliding-window” that begins at nucleotide position 1 and slides one base pair at a time until it reaches the end of the genome. For each 15 base pair segment located within the “sliding window”, the sequence was compared to the canonical *H. pylori* Fur box. Because the middle nucleotide is not specified (“n”), the program was designed to sequentially analyze the first and last 7 nucleotides within the window. For each comparison, a scoring variable was created that was initially set to 0. For each nucleotide that matches the known Fur box sequence, the score was increased by 1. For example, if a perfectly conserved Fur box sequence is located within the genome, it will be given a score of 14. A Fur box with one mismatch will have a score of 13. The score is then converted to a percentage that represents the percent of nucleotides that match the canonical Fur box sequence. Fur boxes with 0, 1, or 2 mismatches will have a percent match of 100%, 92.86% and 85.71%, respectively. The program was designed to extract Fur boxes that contain 2 or fewer nucleotide mismatches. As output, Fur Box Finder will print the exact sequence of the identified conserved/semi-conserved Fur box, the number of mismatches to the established Fur box sequence, along with the location of the Fur box on the *H. pylori* genome. The genomic location data can then be used to determine if the Fur box is located in close proximity to regulatory elements of genes. The results of the

Fur Box Finder program for *H. pylori* G27 using the 5'-ATTATTAnTAATAAT-3' sequence are shown in Table 16.

Fur Box Finder successfully identified 46 additional Fur boxes on the *H. pylori* chromosome. As shown in Table 16, 14 of the identified Fur boxes lie in the promoter region of genes. The vast majority of potentially Fur regulated genes identified encode proteins of unknown function. Additionally, for the genes with described functions, these genes span a wide array of molecular tasks including protein folding (*groEL/groES*), oxidative stress response (catalase), and DNA recombination. Validation of the Fur Box Finder program via transcriptional and Fur binding studies are currently underway and will hopefully further expand our current knowledge regarding Fur regulation in *H. pylori*.

Table 16. Additional Fur boxes identified in *Helicobacter pylori* G27

| # of Mismatches ^a | Genomic Location ^b | Intergenic or Coding ^c | Coding Gene ^c | Flanking Gene 1 ^c | Flanking Gene 2 ^c |
|------------------------------|-------------------------------|-----------------------------------|--|--|-------------------------------|
| 2 | 9020* | intergenic | | <i>groEL</i> * | <i>groES</i> |
| 2 | 9023* | intergenic | | <i>groEL</i> * | <i>groES</i> |
| 2 | 16927* | coding | hypothetical protein | | |
| 2 | 62565 | intergenic | | <i>putA</i> * | hypothetical protein* |
| 2 | 67486* | coding | hypothetical protein | | |
| 2 | 68318* | coding | hypothetical protein | | |
| 2 | 79017* | intergenic | | <i>ureA</i> * | <i>tRNA-Val</i> |
| 2 | 135602* | intergenic | | outer membrane protein | hypothetical protein* |
| 2 | 218087* | coding | tRNA uridine | | |
| 2 | 222796 | intergenic | | hypothetical protein* | phospholipid-binding protein* |
| 2 | 258822* | coding | outer membrane protein (<i>hopG-1</i>) | | |
| 1 | 264689 | coding | type III adenine methyltransferase | | |
| 2 | 264940* | coding | type III adenine methyltransferase | | |
| 2 | 285743 | coding | hypothetical protein | | |
| 2 | 290509* | coding | hypothetical protein | | |
| 2 | 306594 | intergenic | | para-aminobenzoate synthase | <i>amiE</i> * |
| 2 | 323622 | coding | type II restriction enzyme | | |
| 2 | 389923* | coding | phosphatidyl serine synthase | | |
| 2 | 389926* | coding | phosphatidyl serine synthase | | |
| 2 | 389931 | coding | phosphatidyl serine synthase | | |
| 2 | 430480 | intergenic | | <i>fur</i> * | <i>rarA</i> |
| 2 | 449319* | coding | type I restriction enzyme S protein | | |
| 2 | 477843 | intergenic | | catalase* | <i>hofC</i> |
| 2 | 537753 | coding | <i>cagM</i> | | |
| 2 | 545139 | coding | <i>cagE</i> | | |
| 2 | 546269* | coding | <i>cagE</i> | | |
| 2 | 548097 | intergenic | | <i>cagB</i> * | <i>cagA</i> * |
| 2 | 548095* | intergenic | | <i>cagB</i> * | <i>cagA</i> * |
| 2 | 548100 | intergenic | | <i>cagB</i> * | <i>cagA</i> * |
| 2 | 579993* | coding | hypothetical protein | | |
| 1 | 588073 | coding | amino deoxy chorismate lyase | | |
| 2 | 603954* | coding | multidrug resistance protein | | |
| 2 | 616318* | coding | <i>vacA</i> like protein | | |
| 2 | 636769* | intergenic | | recombinant and DNA strand exchange inhibitor protein* | hypothetical protein |

| | | | | | |
|---|----------|------------|--|--|--|
| 2 | 636770 | intergenic | | recombinant and DNA strand exchange inhibitor protein* | hypothetical protein |
| 2 | 636773 | intergenic | | recombinant and DNA strand exchange inhibitor protein* | hypothetical protein |
| 2 | 636776 | intergenic | | recombinant and DNA strand exchange inhibitor protein* | hypothetical protein |
| 2 | 636781* | intergenic | | recombinant and DNA strand exchange inhibitor protein* | hypothetical protein |
| 2 | 669932* | intergenic | | hypothetical protein | protective surface antigen D15* |
| 1 | 703673 | intergenic | | <i>fecA1</i> | ligand gated channel* |
| 2 | 728269 | coding | <i>hopE</i> | | |
| 2 | 732049* | coding | <i>homB</i> | | |
| 2 | 732052* | coding | <i>homB</i> | | |
| 1 | 828366 | intergenic | | <i>ribBA*</i> | <i>lex2B</i> |
| 1 | 832471 | intergenic | | <i>facA2*</i> | holo-ACP synthase |
| 2 | 834782 | intergenic | | dihydroneopterin aldolase* | SAM-dependent methyltransferase |
| 2 | 834777* | intergenic | | hypothetical protein | hypothetical protein* |
| 1 | 834780* | intergenic | | hypothetical protein | hypothetical protein* |
| 2 | 834782 | intergenic | | hypothetical protein | hypothetical protein* |
| 2 | 834783* | intergenic | | hypothetical protein | hypothetical protein* |
| 1 | 834785 | intergenic | | hypothetical protein | hypothetical protein* |
| 2 | 892111 | intergenic | | CDP-diacylglycerol pyrophosphatase* | hypothetical protein* |
| 2 | 895819* | intergenic | | catalase* | Iron-regulated outer membrane protein* |
| 1 | 895824 | intergenic | | catalase* | Iron-regulated outer membrane protein* |
| 2 | 897622 | coding | <i>frpB1</i> | | |
| 2 | 930656* | intergenic | | DNA-dependent ATPase | outer membrane protein* |
| 2 | 930659* | intergenic | | DNA-dependent ATPase | outer membrane protein* |
| 2 | 950237* | coding | <i>vacA</i> like protein | | |
| 2 | 950240* | coding | <i>vacA</i> like protein | | |
| 2 | 980666 | coding | hypothetical protein | | |
| 2 | 997324* | coding | cobalt-zinc-cadmium resistance protein | | |
| 2 | 1001033 | coding | hypothetical protein | | |
| 2 | 1054951 | coding | comB9-like protein | | |
| 2 | 1057322* | coding | competence protein | | |
| 0 | 1057325* | intergenic | | competence protein | hypothetical protein |
| 2 | 1058383 | coding | virB11-like ATPase | | |
| 2 | 1067777 | coding | adenine-specific DNA methyltransferase | | |
| 2 | 1074922* | coding | adenine specific methyltransferase | | |

| | | | | | |
|---|----------|------------|-----------------------------------|--|--|
| 2 | 1078502* | coding | hypothetical protein | | |
| 2 | 1079627* | coding | hypothetical protein | | |
| 2 | 1139462* | coding | UDP-glucose4-epimerase | | |
| 2 | 1169453 | intergenic | | <i>ggt*</i> | <i>flgK</i> |
| 2 | 1181050* | coding | FOF1 ATP synthase subunit gamma | | |
| 2 | 1209065* | coding | <i>hopL</i> | | |
| 2 | 1374701* | intergenic | | ATP-binding protein | hypothetical protein |
| 2 | 1447119* | coding | hypothetical protein | | |
| 2 | 1465943 | intergenic | | <i>rrnA</i> methyltransferase A* | <i>hpn2*</i> |
| 2 | 1535497* | coding | type II methylase | | |
| 2 | 1536879* | coding | type II adenine methyltransferase | | |
| 2 | 1536887 | coding | type II adenine methyltransferase | | |
| 2 | 1571795* | intergenic | | periplasmic competence protein | chromosomal replication initiation protein |
| 2 | 1571800 | intergenic | | periplasmic competence protein | chromosomal replication initiation protein |
| 2 | 1645025 | intergenic | | methicillin resistance protein* | <i>pdxJ*</i> |
| 2 | 1648219 | intergenic | | threonylcarbomyl-transferase | <i>flgG*</i> |

^a Number of nucleotide mismatches from the canonical 5'-ATTATTAnTAATAAT-3' or 5'-TAATAATNATTATTA-3' Fur box sequence

^b Newly identified Fur boxes are denoted with an asterisk (*). Previously identified Fur boxes were originally described in Pich *et al.* 2012 (13). The number corresponds to the genomic location of the first nucleotide of the Fur box.

^c Location of Fur box with respect to gene coding regions. An asterisk (*) indicates that the Fur box is located within or upstream of the gene's promoter.

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