Maintaining the Genomic Integrity of *Staphylococcus aureus* in the Presence of Exogenously Induced DNA Damage

by

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Staphylococcus aureus is a major human pathogen that causes a variety of illnesses ranging from minor skin and soft tissue infections (SSTIs) to more severe systemic infections. Although the primary host immune response can typically clear pathogenic bacterial infections, *S. aureus* is uniquely resistant to this environment. Our lab has determined that nitric oxide (NO·), an important component of the innate immune response that plays a role in both immunomodulatory and antibacterial processes, is the effector to which *S. aureus* is specifically resistant. Additionally, NO· and its derivatives can lead to damage of *S. aureus* DNA, more specifically, deamination and/or oxidation of DNA bases; however, regulation and repair mechanisms of DNA in *S. aureus* is understudied. Thus, we hypothesize several DNA repair mechanisms may account for the replication fidelity of *S. aureus* and may contribute to fitness in the presence of NO·. Here we show the role of several DNA repair mechanisms in *S. aureus.* More specifically, we found recombinational repair gene, *recG*, may play a role in the repair of NO·-induced replication fork collapses. We also show a role of base excision repair pathway protein, MutY, in reducing NO·-mediated mutagenesis. Lastly, we show the role of the mismatch repair pathway in preventing illegitimate recombination. It is known that MMR proteins prevent RecA-mediated recombination between divergent sequences. *S. aureus* has three mismatch repair MutS homologues that work alongside an endonuclease, MutL. Only one has been studied and was shown to limit spontaneous mutagenesis but did not appear to have a role in preventing illegitimate homologous recombination. Here we confirm only one MutS homologue, MutS1, contributes to mutagenesis

in *S. aureus*. We also show a role of the MutS1 homologue in preventing illegitimate recombination between divergent sequences. Overall, our results suggest NO· leads to DNA damage, which subsequently induces activity of several DNA repair pathways, contributing to the replication fidelity and fitness of *S. aureus*. Although one mismatch repair homologue contributes to mutagenesis, no other combinatorial homologues play a role.

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Preface

I would like to thank the many people who've supported me throughout my time as a student in the IDM program. Firstly, I would like to thank the mentors I met during the program, specifically the program director, Dr. Robbie Mailliard, for his guidance as an advisor. I'd also like to thank Dr. Joshua Mattila who has not only guided my education but has also contributed to my critical thinking skills and overall growth as a researcher.

I'd like to sincerely thank my advisor, Dr. Tony Richardson, for his support since I joined his lab as a laboratory technician and throughout my time as a part-time student in the IDM program. Without his continued support and guidance (…and patience) throughout my time in his lab, I wouldn't be where I am now. I'd also like to thank the members of his lab, most importantly, I want to give a shout out to Amie Stephens and Srijon Banerjee. Srijon recently joined our lab as a post-doc but in the short period of time I've known him, he has been a huge asset to me. Our PhD student, Amie has been with me in the lab since day 1 and without her or her friendship I wouldn't have gotten this far.

I'd like to give a shout out to my friends and family for their support, especially my brother and my parents who still have no idea what I do but listen to me ramble on about my love-hate relationship with bacteria anyway. Lastly, to my #1 cheerleader and fiancée, Megan, who challenges me to be the best version of myself. She has quite literally seen me at my worst and continues to love and support me whole-heartedly.

1.0 Introduction

1.1 *Staphylococcus aureus* Background

Staphylococcus aureus is a gram-positive bacterium that is a highly invasive human pathogen. It is known to cause a variety of illnesses ranging from superficial skin and soft tissue infections to more severe systemic infections such as endocarditis, osteomyelitis, and sepsis (1). *S. aureus* typically colonizes the anterior nares and skin asymptomatically and is estimated ~30% of the human population are natural carriers (2). The treatment of infections has been increasingly difficult due to the ability of *S. aureus* to evolve antibiotic resistance. As such, Methicillin resistant *Staphylococcus aureus* (MRSA) has been the most common cause of infection since the 1960s (3). Historically, it is known that MRSA-related hospitalizations lead to severe morbidity and mortality globally. However, in recent decades, community-acquired MRSA (CA-MRSA) infections have been increasingly found in otherwise healthy populations (4). Additionally, CA-MRSA clones have been found to be phylogenetically distinct from hospital-associated MRSA (HA-MRSA) and have exhibited both hypervirulence and improved transmission within the host. Factors contributing to the evolution of *S. aureus* regarding healthcare cost, prevalence, virulence, and overall pathogenesis make it a major public health concern. One major factor contributing to *S. aureus* pathogenesis is resistance to the broad-spectrum antimicrobial immune radical, NO·. Resistance to NO· distinguishes *S. aureus* from other closely related Staphylococcal species and thus, understanding this unique trait is an important contribution to limiting the spread of *S. aureus* throughout both hospital and community settings as well as potentially designing novel antimicrobial therapeutics (5).

1.2 *S. aureus* is uniquely resistant to nitric oxide (NO·)

Nitric oxide is an important component of the host innate immune response and plays a role in antibacterial and immunomodulatory processes. During infection, NO· is produced by activated phagocytes through the inducible nitric oxide synthase (iNOS) and can react directly with invading organisms in surrounding inflamed host tissues (6,7). NO· and its derivatives are known to target heme, iron-sulfur clusters, thiols, lipids, and DNA (8, 9). Additionally, under high concentrations of NO·, the reversible binding of NO· to cytochrome heme centers results in aerobic respiration inhibition, which is restored once NO· is detoxified (10). The reactions of NO· and its targets subsequently interferes with many pathways, inducing metabolic and replicative stress. *S. aureus* therefore must evolve mechanisms to aid in survival under these conditions. Although inflammatory NO· is typically required for clearance of pathogenic bacterial infections, *S. aureus* is uniquely resistant to this immune radical, which distinguishes *S. aureus* from closely related coagulase negative Staphylococcal species (CoNS) that are unable to grow in the presence of NO· (5). The mechanism underlying *S. aureus* resistance to NO· is complex and consists of several metabolically regulated gene products. Thus far, it has been determined that NO· induces the activation of the SrrAB two-component system (TCS), which regulates several downstream metabolic genes and allows *S. aureus* to replicate in a high NO· concentrated environment (11). SrrAB also induces a flavohemoprotein, Hmp, which detoxifies NO·, as well as two terminal oxidases, Qox and Cyd, that play a role in overcoming the inhibitory effects of NO· on respiration (12). Additionally, a lactate dehydrogenase enzyme, Ldh1, unique to *S. aureus* was previously characterized to balance redox when respiration is inhibited under NO·stress (11). With that said, we are still lacking in understanding of what makes *S. aureus* effective resistance to NO· so unique.

As previously stated, it is known NO· targets *S. aureus* DNA, leading to DNA damage. More specifically, NO· leads to deamination of DNA bases; however, the regulation and repair mechanisms of DNA in *S. aureus* is understudied.

1.3 NO· leads to the induction of DNA damage in *S. aureus*

In bacteria, DNA is damaged due to several endogenous and exogenous factors such as radiation, chemical compounds, and environmental stress (13). This damage can in turn inhibit replication and downstream gene transcription, ultimately affecting cell survival and can lead to the accumulation of mutations (14). DNA damage can result from replication fork collapses, single-strand breaks, or exposure to metabolic byproducts such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) (15). Since *S. aureus* has uniquely evolved to survive in the presence of RNS, more specifically, NO·, we wanted to determine if there were any DNA repair pathways that enhanced the overall fitness of this pathogen in the host environment as well as contribute to replication integrity under NO· stress. We know NO· exposure results in deamination and/or oxidation of DNA bases in *S. aureus* but the regulation and repair mechanisms of this damage are unknown. Thus, we employed mutants from the nucleotide excision repair (NER), base excision repair (BER), recombination repair, and replication fork restart pathways to determine a role under NO· stress. Overall, DNA repair mechanisms may pose as a target for novel therapeutics that sensitize pathogen to effectors of the host defense and/or first line antibiotics.

1.4 Defects in Mismatch Repair System (MMR) play a role in *S. aureus* evolution

The DNA mismatch repair (MMR) system is responsible for correcting errors that occur during replication due to faulty DNA polymerase proofreading activity (16). These errors may result in base mismatches and/or extra nucleotides such as insertion or deletion loops. In gram-negative bacteria such as *E. coli*, the mismatch repair mechanism and its components are fully established (17). For instance, mismatched bases generated during DNA synthesis are recognized by the mismatch sensing protein, MutS, which elicits a conformational change, converting this protein into a sliding clamp. MutS then recruits MutL to activate a MutH endonuclease. Upon activation, MutH nicks the unmethylated strand with the mismatch at the hemi-methylated DNA site. The UvrD helicase unwinds the DNA toward the detected mismatch, allowing degradation by several exonucleases. Finally, DNA polymerase III generates nascent DNA to fill the ssDNA gap and a DNA ligase is able to seal the nick. This process is likely conserved in *S. aureus* and other gram-positive bacteria such as *B. subtilis*, although in a MutH- and methyl-independent pathway. In most bacteria lacking MutH, MutL homologues have instead been shown to exemplify endonuclease activity. Regardless, the MMR system has been shown to play an important role in reducing overall mutation rate (18). Both in *B. subtilis* and *S. aureus*, the inactivation of *mutS* and *mutL* has been shown to increase the overall mutation rate as well as create hypermutable phenotypes that play a role in the adaptation of bacterial populations in stressful environments (15, 19). Interestingly, in *E. coli* and *Salmonella sp.*, it has been shown that MMR prevents recombination between divergent sequences that occur during genetic exchange events such as conjugation, transduction, and transformation (20). However, when MMR is defective in these species, the recombination rate increases significantly between partially divergent sequences (21). Little is known about this phenomenon in *S. aureus.* A previous group not only confirmed the

inactivation of both *mutS* and *mutL* in *S. aureus* results in hypermutation but also tried to elucidate a link between this hypermutable phenotype and hyperrecombination (19). Although their results suggest a very limited effect of MMR in preventing homologous recombination between divergent sequences, this group has only employed 1 MutS homologue alongside the MutL endonuclease. Since we now know *S. aureus* has acquired 3 MutS homologues, we wanted to determine if this enhances the ability of this pathogen to control mutagenesis as well as if any single or combinatorial homologues play a role in preventing illegitimate recombination between divergent species. Altogether, occurrences of illegitimate recombination due to defects in the mismatch repair system could enhance the ability of *S. aureus* to incorporate divergent, exogenous DNA into its genome. This may in part explain the evolved antibiotic resistance and increased virulence characteristics exhibited by this pathogen, in turn, contributing to overall genomic evolution.

1.5 Public Health Statement of Relevance

Methicillin-resistant *Staphylococcus aureus* (MRSA) poses a serious threat to population health worldwide. As I previously mentioned, MRSA was typically isolated from hospital associated infections but recently, we have seen MRSA spread to the community, causing infections in otherwise healthy people. Since CA-MRSA exhibits increased virulence and host-to-host transmission, this necessitates the understanding of *S. aureus* evolution to determine the source of this pathogen's success. Additionally, MRSA has evolved as a multi-drug resistant pathogen. *S. aureus* has acquired resistance to most antibiotics designed to eliminate this pathogen, which highlights the need for alternative therapies. Since *S. aureus* has also uniquely evolved resistance to the primary host immune defense, inflammatory NO·, our lab sought to

determine the mechanisms *S. aureus* employs in order to thwart the activity of NO·. We found several DNA repair mechanisms that enhance *S. aureus* replicative fidelity and overall fitness in this NO·-concentrated environment. Thus, these DNA repair mechanisms could potentially act as a target for novel therapeutics that sensitize this pathogen to the primary host immune response. Finding alternative therapies to target this multi-drug resistant pathogen is essential to circumvent the global burden of antibiotic resistance.

2.0 Research Methods and Design

2.1 Bacterial Strains and Growth Conditions

S. aureus strains were grown in Brain Heart Infusion medium (BHI) and *E. coli* DH10B strains were grown in Luria-Bertani (LB) broth. *S. aureus* USA300 and *E. coli* DH10B were used in this study as background strains for mutant construction and plasmid construction, respectively (**Appendix Table 1**). Antibiotic selection in *E. coli* was carried out using ampicillin (100μg/mL). Antibiotic selection in *S. aureus* was carried out using the following concentrations: chloramphenicol (20μg/mL), kanamycin (50μg/mL), erythromycin (5μg/mL), tetracycline (6μg/mL), and spectinomycin (100μg/mL).

2.2 Mutant Construction-Cloning Technique

The inactivation of *mutS1* and *mutS2* was accomplished using an allelic exchange method by cloning flanking DNA sequence on either side of a selectable tetracycline or spectinomycin marker in the *E. coli* shuttle vector pBT2ts, pBTT or pBTS respectively, using primers listed in Table 1. These constructs were electroporated into *S. aureus* RN4220 with chloramphenicol (20μg/mL) selection at 30°C. The plasmid was then transduced using Φ-11 phage into a *S. aureus* USA300 background with chloramphenicol (20μg/mL) selection at 30°C. Cointegration was accomplished by growth overnight at 43°C, followed by plating on media with chloramphenicol (20μg/mL). Single colonies were picked and grown at 30°C for three consecutive days without the

presence of antibiotic. Cultures were then diluted 1:100 and grown at 37°C to mid-exponential phase followed by addition of chloramphenicol to inhibit growth of resolved cointegrates. Cycloserine (100μg/mL) was then added to kill chloramphenicol resistant cointegrates. Cultures were then incubated at 37°C for 5 hours and the surviving cells were plated on selective media and screened for successful allelic replacement by PCR.

Table 1. List of Primers/Sequences used for Cloning

Primer	Sequence	Use
uni_lqo.1A	GGGGGATCCAATGCAGGTACTGGACATGC	construction of KH01, KH02, KH03, and KH04
uni_lqo.1B	GGGGGATCCTCTAAAGCAACTGACACTGA	construction of KH01, KH02, KH03, and KH04
mutS_5'1.A	GGGGGATCCCAGTCGGAACATACCATTC	construction of Δ*mutS*::tet^R
mutS_5'1.B	GGGGGATCCGATGCCTCCTTGGCATCTTC	construction of Δ*mutS*::tet^R
mutS_3'1.A	GGGGAATTCCAGAGCTTTGCAGAAATTGC	construction of Δ*mutS*::tet^R
mutS_3'1.B	GGGGGATTCCTTAACTTCACCAATGCCTC	construction of Δ*mutS*::tet^R
mutS2_5'1.A	GGGGGATCCGATGAAACGATTGGTGTCG	construction of Δ*mutS2*::spc^R
mutS2_5'1.B	GGGGGATCCGTCTCATAAAATCCCTC	construction of Δ*mutS2*::spc^R
mutS2_3'1.A	GGGGAATTCGGATTTGGCGTTACCGTTGC	construction of Δ*mutS2*::spc^R
mutS2_3'1.B	GGGGAATTCTTAGCATCATGAGCACCC	construction of Δ*mutS2*::spc^R
mutS.1A	ATGTTACACCAATGATGCAGC	confirmation of transposon insertion
mutS.1B	TTTTCCCCATTTTGCAACACC	confirmation of transposon insertion
mutS2.1A	TCGCGTGAAGCTTTTAAAGAC	confirmation of transposon insertion
mutS2.1B	TCTGTTACTTTTACGATTGCC	confirmation of transposon insertion
mutL.1A	TGGGGAAAATTAAAGAACTCC	confirmation of transposon insertion
mutL.1B	CATCCTCTCTACATCACACGC	confirmation of transposon insertion
mutX.1A	AAGCAAACGAATTAGCTATCC	confirmation of transposon insertion
mutX.1B	GATTTAGCATTAAATGGGCAC	confirmation of transposon insertion

2.3 Plasmid Construction

A 1 kb fragment of the *mqo2* gene was amplified from *Staphylococcus aureus*, *Staphylococcus simiae*, *Staphylococcus epidermidis*, and *Staphylococcus saprophyticus* backgrounds using primers listed in Table 1. These fragments were subsequently cloned into pBTK, a thermosensitive shuttle *E. coli* plasmid containing a kanamycin cassette, with selection on ampicillin (100μg/mL). These plasmids were further phage transduced into various Staphylococcal strains to perform integration rate assays.

2.4 Mutation Rate Assay

S. aureus cultures were shaken at 250 rpm at 37°C overnight. Overnight cultures were serial diluted and plated on BHI agar either with or without exposure to NO·. The NO· donor we used in this experiment was diethylenetriamine NONOate (DETA/NO), which was resuspended in 0.01 N NaOH and has a half-life [$t_{1/2}$] of 20 hours. In cultures exposed to NO·, a disc was placed in the center of the plate and 20μL of 500mM DETA/NO was added to the disc. Plates were incubated at 37°C. The following day, 20 single colonies were picked from both NO· exposed and NO· unexposed plates and resuspended in 200μL of phosphate-buffered saline (PBS) within a 96-well plate. Each resuspension was serial diluted and subsequently plated on BHI agar plates containing rifampicin (100μg/mL) and BHI agar plates lacking antibiotic. Following incubation overnight at 37°C, we were able to calculate mutation rate by dividing the number of colonies found on the BHI + rifampicin plate by the CFU/mL on BHI agar plates lacking antibiotic.

2.5 Growth Rate Analysis

S. aureus cultures were grown overnight in BHI at 37°C shaking at 250 rpm. Overnight cultures were washed three times with PBS and inoculated at a 1:200 ratio in a 96-well plate containing Tryptic soy broth (TSB) (200μL/well). Cells were grown at 37°C and shaken in a BioTek microplate reader. For NO· growth curves, a mixture of 10mM NOC-12 [$t_{1/2}$=100 min]/1mM DEANO [$t_{1/2}$=2 min] was added at an OD_{660} of 0.20. Growth was monitored every 15 minutes for 24 hours.

2.6 Integration Rate Assay

S. aureus cultures containing thermosensitive *mqo2* plasmids were plated overnight at 30°C. A single colony of each plasmid-containing strain was picked and grown up at 43°C overnight, shaking at 250 rpm. The following day, each culture was serial diluted and plated on BHI agar without antibiotics at 30°C as well as BHI agar containing both kanamycin (50μg/mL) and chloramphenicol (20μg/mL) and incubated at 43°C overnight to observe the rate of integration. We calculated integration rate by dividing the number of colonies found on the BHI plate + antibiotics at 43°C by the CFU/mL on BHI plates lacking antibiotic at 30°C.

2.7 Mutation Accumulation Assay

S. aureus WT USA300 culture was struck out on 80 Tryptic Soy Agar (TSA) plates. Forty plates were exposed to NO· (500mM DETA/NO) and forty plates were unexposed. For forty consecutive days, a single colony was picked and struck out onto a fresh TSA plate either with or without exposure to NO·. For the NO· exposed plates, a dot was randomly drawn near the NO· disc prior to incubation at 37°C to ensure elimination of selection bias. Finally on day 40, a single colony was picked and struck out to create a lawn on a fresh TSA plate. The following day, the lawn was resuspended in 50% BHI + glycerol and stored at -80°C. Genomic DNA was extracted from the eighty isolates and subjected to sequencing analysis.

2.8 Whole Genome Sequencing Analysis

At the end of the mutation accumulation experiment, we extracted genomic DNA from the eighty isolates using the Epicentre MasterPure Gram Positive DNA Purification Kit (Qiagen). DNA was sequenced using Illumina NextSeq 500 at the Microbial Genomics Sequencing center (migscenter.com). We sequenced one clone from the final timepoint of every evolved lineage. Sequencing reads were trimmed and quality filtered using trimmomatic version 0.36 with the following criteria: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36 (41). Reads were aligned to a reference genome closely related to the ancestral strain (*Staphylococcus aureus* subsp. *aureus* USA300_FPR3757) and variants were called using breseq version 0.35.0 in consensus mode (42). Breseq was run with default parameters except that a minimum of 5 reads from each strand were required to support variant calls. Sample read depth ranged from 91-355X.

3.0 Results

3.1 Investigating the Role of DNA Repair Mechanisms in *S. aureus* NO· Stress

To determine if there were any DNA repair mechanisms that contribute to replicative fitness under NO· stress, we selected 15 DNA repair mutant strains that had previously been identified in *S. aureus* due to known homology in *B. subtilis.* We then performed growth curves with these 15 repair mutant strains alongside a WT control. Without the addition of NO·, these strains have a similar growth rate (**Figure 1A**). However, following the addition of NO·, one of these strains, Δ*recG*, exhibits a significant growth defect (**Figure 1B,C**).

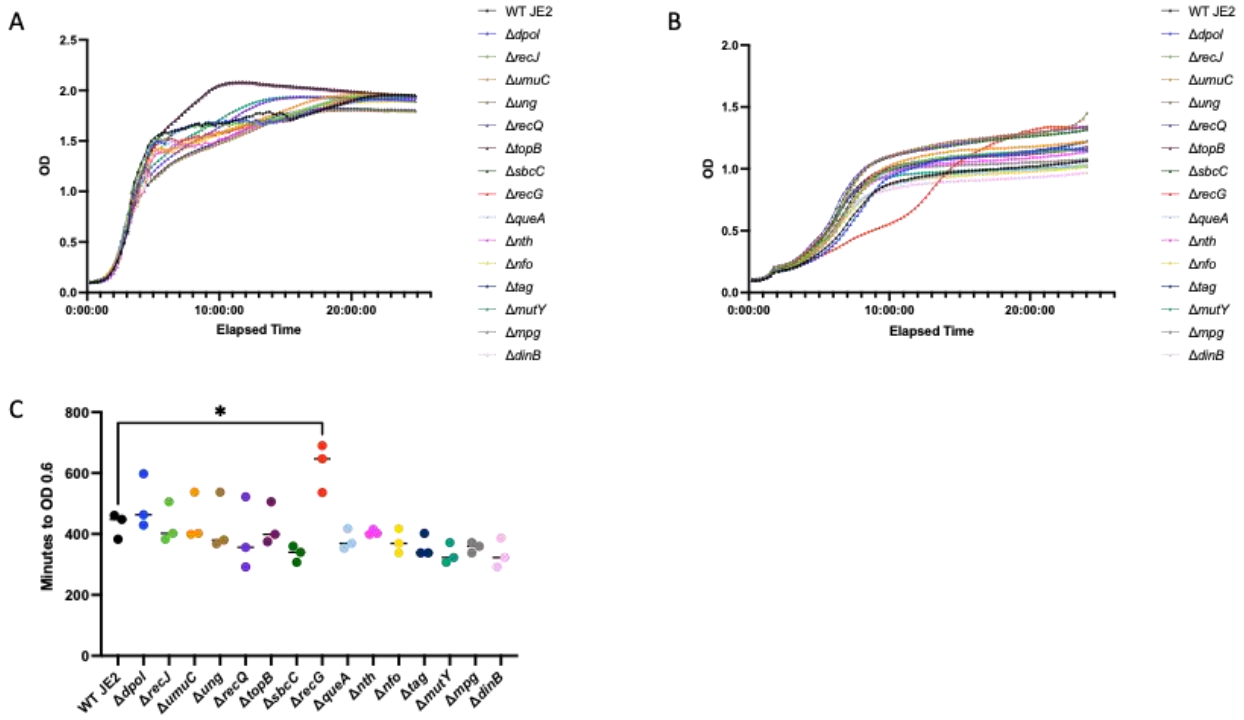

Figure 1. Growth curves suggest RecG may contribute to *S. aureus* ability to confer fitness under NO· stress.

Representative growth curves are shown of *S. aureus* WT JE2 and 15 DNA repair transposon mutants grown in TSB either aerobically (A) or with 10mM DETA/NO added at OD 0.2 (B) (n=3). The amount of time it took each mutant strain to reach an OD of 0.6 with 10mM DETA/NO added at OD 0.2 (C) (n=3). Data were analyzed via one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons test for correction (*, P<0.05).

3.2 Identification of DNA repair mechanisms that contribute to *S. aureus* ability to modulate mutagenesis

We also wanted to determine if there were any DNA repair mechanisms involved in targeting NO·-mediated mutagenesis. To do so, we performed mutation rate assays with the same 15 DNA repair mutant strains alongside a WT control. We found Δ*recG*, Δ*nth*, Δ*nfo*, Δ*ung*, and Δ*mutY* transposon mutants display a significantly increased mutation rate compared to WT JE2 without NO· exposure (**Figure 2A**). Additionally, the mutation rate was further increased in the Δ*mutY* mutant in the presence of NO· (**Figure 2B**).

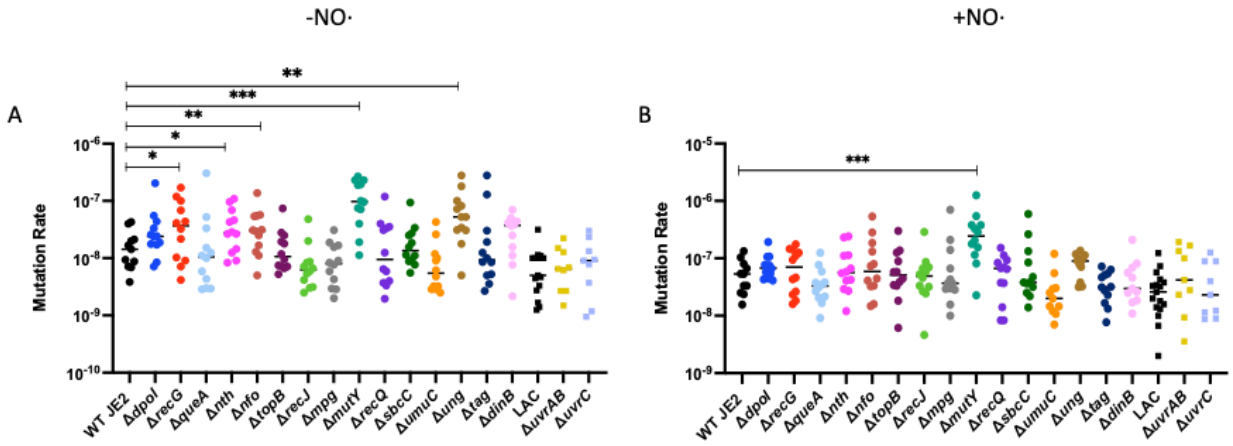

Figure 2. Elevated mutation rates suggest MutY may play a role in modulating mutagenicity in the presence of NO· in *S. aureus*.

Mutability of *S. aureus* WT JE2 and 15 DNA repair transposon mutants shown either unexposed (A) or exposed (B) to a disc of 500mM DETA/NO (n=12). Data were analyzed via Wilcoxon Rank-Sum Test for nonparametric analyses (*, P<0.05, **, P<0.005, ***, P<0.0005).

3.3 Mutation accumulation suggests deamination and/or oxidation of *S. aureus* DNA under NO· stress

We performed a mutation accumulation assay to determine if there were any specific mutations accumulated under NO· stress compared to an unexposed group. Upon performing breseq analysis on the genomic sequences isolated on day 40, we found that out of the 472 mutations, 263 were in the exposed group and 209 were in the unexposed group (**Table 3**). Additionally, the mutations most frequently induced under NO· stress compared to the unexposed group were C:G-->T:A, G:C-->A:T, C:G-->A:T, and T:A-->C:G, which are all products of DNA deamination and oxidation.

Table 2. Mutation Accumulation

Mutation	Group - NO·	Group + NO·	Total
(A)7->6	1	0	1
(A)7->8	2	0	2
(ATT)7->6	1	1	2
(T)7->6	1	0	1
(T)7->8	4	1	5
(TACAGAAACAAA) 2->1	0	1	1
2 bp->AC	0	1	1
2 bp->AG	0	1	1
2 bp->TT	0	1	1
Δ3,125 bp	2	0	2
Δ4 bp	1	0	1
Δ54 bp	0	1	1
Δ54,659 bp	1	0	1
Δ69 bp x 2	1	1	2
A->C	1	5	6
A->G	11	9	20
A->T	5	8	13

C->A	6	23	29
C->G	23	22	45
C->T	39	68	107
G->A	75	88	163
G->C	2	4	6
G->T	22	9	31
T->A	4	5	9
T->C	4	12	16
T->G	3	2	5
	209	263	472

3.4 Role of mismatch repair pathway in modulating mutagenesis in *S. aureus*

It has been previously shown, by inactivating Δ*mutSL,* that a defect in the mismatch repair system in *S. aureus* results in an increased mutation rate. However, we now know that *S. aureus* has 3 MutS homologues (**Figure 3**). Thus, we wanted to determine if another homologue or a combination of homologues were responsible for the elevated mutation rate. Moreover, we wanted to determine if this were further increased under NO· exposure. To do so, we performed mutation rate assays with single and combinatorial inactivated MMR mutant strains. We found that Δ*mutS,* Δ*mutL,* Δ*mutS1*Δ*mutS2,* Δ*mutS1*Δ*mutX,* and Δ*mutS1*Δ*mutS2*Δ*mutX* exhibited a significant increase in mutation rate compared to WT LAC (**Figure 4A**). We did not see an increase in mutation rate in Δ*mutS2,* Δ*mutX,* or Δ*mutS2*Δmut*X,* suggesting this increased mutation rate phenotype is likely due to MutS1 activity. Furthermore, following the addition of NO·, we see an additional increase in the mutation rate of Δ*mutS,* Δ*mutL,* Δ*mutS1*Δ*mutS2,* Δ*mutS1*Δ*mutX,* and Δ*mutS1*Δ*mutS2*Δ*mutX* strains compared to WT LAC, though, the mutation rate of the double and

triple mutants do not extend higher than Δ*mutS1*, suggesting this is not a combinatorial effect but likely due to the activity of MutS1 alone (**Figure 4B**).

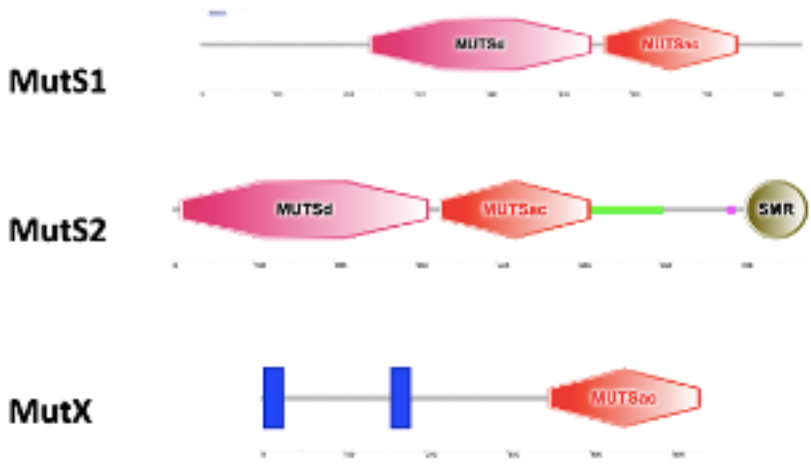

Figure 3. Mismatch repair domains in *S. aureus.*

Schematic of the 3 MutS homologues in *S. aureus* and their domains. Mismatch repair domain annotated by MUTSd, ATPase domain annotated by MUTSac, small MutS-related domain annotated by SMR, and transmembrane domain signified by the blue rectangles.

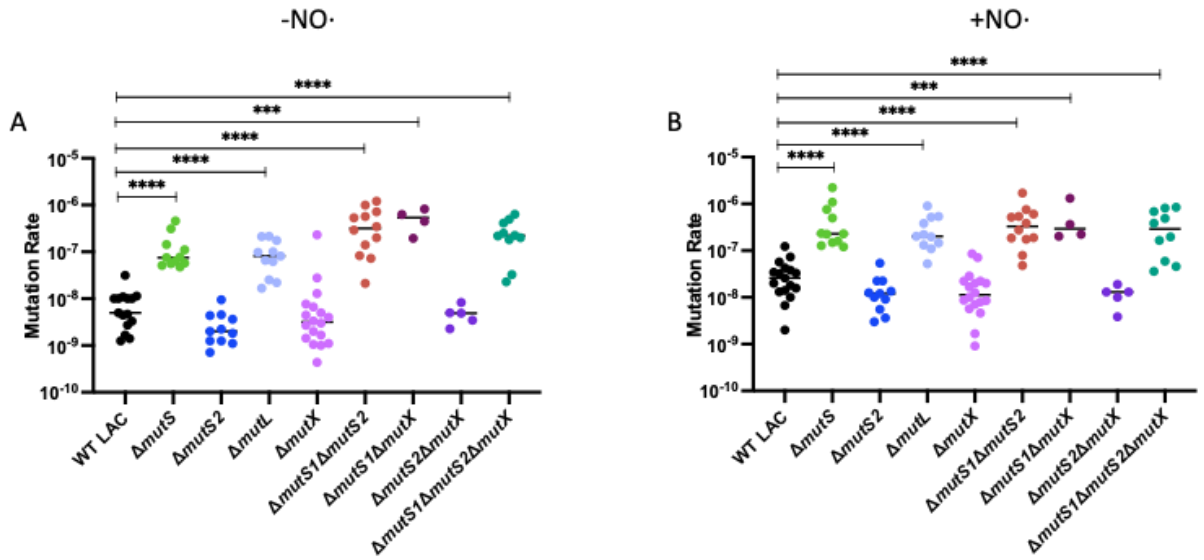

Figure 4. Both single and combinatorial mismatch repair mutants result in increased mutagenesis in the presence of NO·.

Mutability of *S. aureus* WT LAC and MMR mutant strains either unexposed (A) or exposed (B) to a disc of 500mM DETA/NO. Data were analyzed via Wilcoxon Rank Sum Test for nonparametric analyses (***, P<0.0005, ****, P<0.0001).

3.5 Role of mismatch repair pathway in preventing illegitimate homologous recombination in *S. aureus*

Given that there are 3 *mutS* paralogs in *S. aureus*, we wanted to determine if one or more combinatorial mutants would result in elevated recombination of divergent sequences similar to that observed with 100% identical sequence. Thus, we designed an assay to observe the frequency of integration relative to sequence divergence in various MMR mutant background strains. More specifically, we observed the integration rate of a plasmid containing an *mqo2* gene fragment from various Staphylococcal species with diverging sequence similarities (**Table 3**). Overall, our results show a significant increase in integration rate in Δ*mutS1*, Δ*mutL*, Δ*mutS1*Δ*mutS2*, Δ*mutS*1Δ*mutX*, and Δ*mutS1*Δ*mutS2*Δ*mutX* compared to plasmid-containing WT LAC, which suggests a role of MutS1 (**Figure 5,6**). Interestingly, though, this effect cannot be observed until ~70% sequence similarity (**Figure 5A-C**). Accordingly, we only saw a significant effect on integration rate in the MMR mutant strains containing the *S. saprophyticus mqo2* plasmid (**Figure 6**).

Table 3. Percent sequence similarity of the *mqo2* gene across Staphylococcal species

	S. aureus mqo2	***S. simiae mqo2***	***S. epidermidis mqo2***	***S. haemolyticus mqo2***	***S. saprophyticus mqo2***
S. aureus mqo2		89.85%	82.23%	79.54%	70.85%

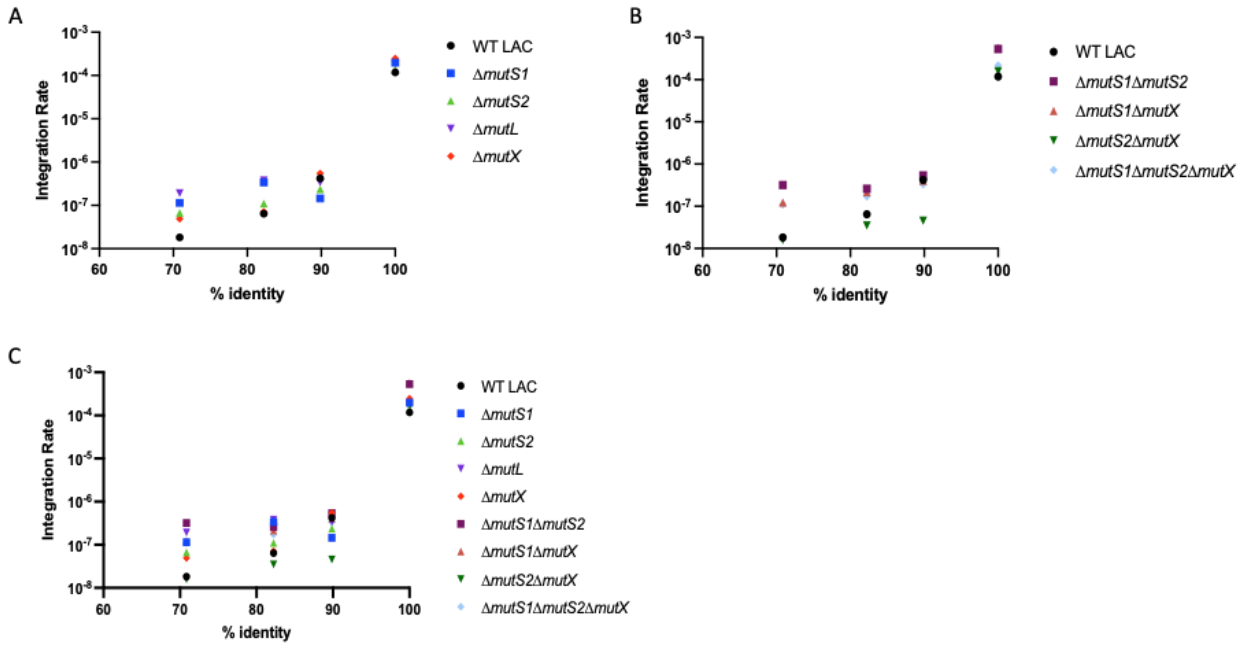

Figure 5. Integration rate across diverging sequences in one or more combinatorial mismatch repair mutant.

Mean rates of recombination across diverging sequences in WT LAC and single MMR mutant strains (A) or WT LAC and combinatorial MMR mutant strains (B) or altogether (C).

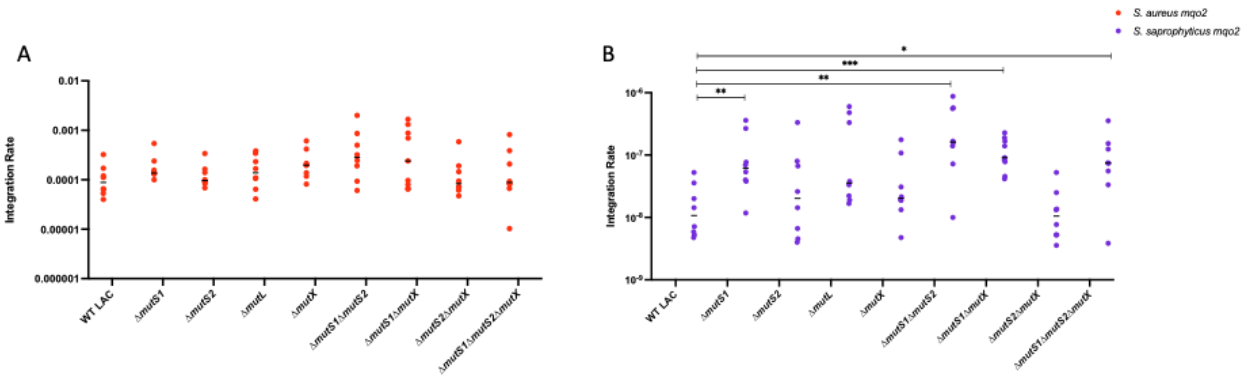

Figure 6. Elevated integration rate of *S. saprophyticus* lqo in both single and combinatorial mismatch repair mutants suggests role of MutS1.

Individual rate of recombination of either (A) *S. aureus lqo* or (B) *S. saprophyticus lqo* (70.85% sequence similarity). Data were analyzed by Wilcoxon Rank-Sum Test for non-parametric analyses (*, P<0.05, **, P<0.005, ***, P<0.0005).

4.0 Discussion

4.1 Identification of DNA repair mechanism associated with replicative fitness

In response to a typical *S. aureus* infection, activated host innate immune cells produce reactive nitrogen species that target various parts of the cell. Since we know NO· and its derivatives target *S. aureus* DNA and can subsequently lead to DNA damage, we wanted to determine if there were any DNA repair mechanisms that contribute to replicative fitness under NO· stress. We performed growth curves with a WT JE2 strain alongside several DNA repair transposon insertion mutant strains and compared the optical density over an elapsed time. We observed growth both in the presence of NO· and without the presence of NO·. Under NO· stress, the Δ*recG* mutant displayed a significant growth defect, suggesting this pathway may aid *S. aureus* ability to confer fitness in a NO· stressed environment. RecG is an ATP dependent helicase with 3'-5' activity and typically works alongside RecJ, which is a single-stranded DNA specific exonuclease with 5'-3' activity (15). Both play a critical role in homologous recombination and DNA repair, more specifically they play a role in catalyzing branch migration during replication fork restart (22). In the event of DNA double strand breaks (DSBs) and subsequently a replication fork collapse, homologous recombination is initiated. Typically, the ends of a DSB are processed by a 5'-3' helicase-nuclease complex, RexAB, which leaves behind a 3'-ssDNA overhang. This allows a recombinase, RecA, to bind to the 3'-ssDNA overhang while it searches for a homologous sequence to initiate strand invasion. Following strand invasion, DNA synthesis can occur whereby the 3' end is extended via DNA polymerase III simultaneously alongside branch migration, carried out by the 3'-5' helicase, RecG. Ultimately, the Holliday junction resolution is carried out by the

resolvase protein, RecU. Previously, it has been shown NO· causes replication fork collapses, which can result in DNA double strand breaks, leading to mutagenic DNA rearrangement in *S. aureus* (23). In this study, our results suggest a putative role of RecG in maintaining replicative fitness under NO· stress.

4.2 Identification of DNA repair mechanism that contributes to *S. aureus* control of NO·-induced mutagenesis

Although we have identified a process involved in maintaining replicative fitness under NO· stress, we also wanted to know if there were any DNA repair mechanisms contributing to *S. aureus* ability to mitigate mutagenesis in this NO·-concentrated environment. We observed mutagenicity across several DNA repair transposon insertion mutants compared to WT JE2 both in the presence of NO· and without the presence of NO·. Overall, we observed increased mutation rates in Δ*recG,* Δ*nth,* Δ*nfo,* Δ*mutY,* and Δ*ung* compared to WT without the presence of NO·, suggesting inactivation of these genes results in the acquisition of spontaneous mutations. As I previously mentioned, RecG is involved in replication fork restart following double strand breaks. Without RecG activity we would expect to see an increase in mutation rate since its absence would likely result in the incomplete resolution of DNA Holliday junctions and thus, an accumulation of mutagenic DNA. Nth, Nfo, MutY, and Ung are all members of the base excision repair (BER) pathway involved in single-stranded DNA damage repair (17, 24). The main function of the BER pathway is to repair non-bulky single base DNA lesions such as oxidized or deaminated DNA bases, alkylated or abasic sites, and dUTP incorporation during DNA replication. The

misincorporation of dUTP during DNA replication likely occurs one of two ways: either due to deamination of dCTP or incorporation of dUTP before the intermediate can be catalyzed to dTTP, since the replicative polymerase cannot distinguish between them. Nth and Nfo are endonuclease III and endonuclease IV, respectively. They both play a role in resolving apurinic or apyrimidic (AP) sites left by DNA glycosylase activity when repairing damaged DNA during BER (27, 28). In inactivating these genes, we would expect to see the increase in mutability that we observe in our results since insufficient removal of AP site would subsequently result in an interruption of this repair process. Ung and MutY are both DNA glycosylases involved in BER (29, 30). Ung excises uracil residues from DNA, which can arise as a result of misincorporation of dUMP residues or as a result of cytosine deamination. MutY is an adenosine DNA glycosylase, which hydrolyzes free adenine bases from 8-oxo-guanine:A mismatches. An inactivated *mutY* or *ung* mutant strain would likely lack the capability to initiate the BER process, resulting in accumulation of single-base lesions in DNA left unresolved, which likely explains our results. Interestingly, following the addition of NO· in our mutation rate assay, we observed Δ*mutY* displays a further enhanced mutation rate. Since we know NO· causes deamination and/or oxidation of DNA bases and both Ung and MutY target oxidized and/or deaminated DNA bases, the hypermutable phenotype seen with the inactivated Δ*mutY* strain is likely a result from the accumulation of unresolved deaminated DNA bases. Overall, our results suggest a role of the BER pathway, more specifically, the DNA glycosylase MutY in targeting NO·-mediated mutagenesis in *S. aureus*.

4.3 Mutation accumulation analysis suggests NO· stress leads to DNA deamination

Although we suspected NO· exposure likely results in deamination and/or oxidation of DNA bases in *S. aureus,* we performed a mutation accumulation (MA) experiment to observe via sequencing analysis which mutations most frequently occur under NO· stress. The MA experiment is ideal to estimate rates of spontaneous mutations that occur over the duration of the experiment and in our case, we were able to compare the accumulation of mutations under NO· exposure compared to an unexposed group. Additionally, we performed our experiment without selection, ensuring the elimination of selection bias. Overall, we observed the mutations that most frequently induced by NO· were all products of DNA oxidation and deamination: C-->T, G-->A, C-->A, and T-->C. The mutations that occurred most frequently under NO· stress were C-->T and G-->A transitions. These mutations likely occurred due to deamination of cytosine or guanine, respectively (8, 31). A C-->T transition can arise in two mechanisms. Firstly, in a C:G pair, under NO· exposure, the cytosine is deaminated to uracil, which results in a U:G pair followed by synthesis to a U:A pair and upon further replication, results in a T:A pair. Another mechanism of C-->T transition could result from a deaminated guanine in a C:G pair to xanthine, resulting in C:X pair. Since xanthine typically pairs with thymine, upon further replication the C:X would result in a T:X pair, and finally repaired to T:A. A G-->A transition would likely follow a similar mechanism of DNA deamination where a G:C pair under NO· exposure results in deamination of a guanine base to xanthine, which results in X:C, followed by replication to X:T and finally A:T. Otherwise in a G:C pair, the cytosine is deaminated to uracil under NO· exposure, resulting in a G:U mismatch, which upon further replication results in an A:U and finally an A:T pairing. The C-->A transversion occurred less frequently; however, is interesting due to the bias of occurrence

in the NO· exposed group. A common DNA base lesion formed during replication stress is 8-oxoG, which can mismatch to adenine (33). What most likely occurs is a C:G pair oxidized to C:8-oxoG, followed by replication to a A:8-oxoG mispair. Upon replication, A:oxo-G is likely repaired to A:T, resulting in a C-->A transversion. Finally, the mutation that least likely occurred is the T-->C transition. This transition likely arises due to the deamination of adenine to hypoxanthine so a T:A pair turns into T:HX. HX typically binds to cytosine, resulting in a HX:C pair, which following another round of replication results in C:G pair. Ultimately, our results show NO· exposure results in the accumulation of DNA transitions over transversions. Furthermore, the overall takeaway from the MA analysis is the exposure of WT *S. aureus* to NO· results in accumulation of DNA damage, more specifically, deaminated and oxidized DNA. *S. aureus* likely relies on the BER pathway to repair these accumulated lesions, ultimately aiding the overall ability of this pathogen to modulate mutagenesis in a NO· concentrated environment.

4.4 Mismatch repair pathway plays a role in modulating mutagenesis

It has been previously shown in both *B. subtilis* and *S. aureus* that a defect in MMR results in an increase in overall mutation rate (15, 19). However, this phenotype has been shown by inactivating Δ*mutS,* Δ*mutL,* or Δ*mutS*Δ*mutL*. Interestingly, this group showed inactivated Δ*mutL* led to a mutation frequency that was 100-fold higher than an inactivated Δ*mutS.* Since we now know *S. aureus* encodes 3 MutS homologues, we wanted to determine if any single or combinatorial homologue, alongside MutL, was responsible for this enhanced display of hypermutation. Our results confirmed that an inactivation of Δ*mutS* and Δ*mutL* lead to increased

mutation frequency; however, we did not observe an enhanced frequency in Δ*mutL* over Δ*mutS.* Also, all combinatorial mutants containing an inactivated Δ*mutS1* displayed a similar increase in mutation but combinatorial mutants containing other inactivated MutS paralogues: Δ*mutS2* and Δ*mutX,* maintained a mutation rate similar to the WT strain, suggesting MutS1 is the homologue contributing to the control of mutagenesis in *S. aureus*. We also wanted to determine the effect of NO· on mutation rate in these MMR mutants. We saw an increase in mutation rate following NO· exposure in the single mutant strains compared to the rates of the unexposed group. However, the mutation rates of the combinatorial mutants weren't further enhanced under NO· stress. This suggests that although the mutation rates in the combinatorial mutants are higher than WT under NO· stress, the mutations that occur due to polymerase error were likely more frequent than those induced by NO·. Overall, our experiment has confirmed the control of mutagenesis in *S. aureus* is likely due to MutS1 activity and no other combinatorial homologues play a role. Furthermore, this highlights the importance of the MMR system in modulating hypermutability in *S. aureus.*

4.5 Mismatch repair pathway plays a role in preventing illegitimate homologous recombination

The MMR system has been shown in several bacterial species to inhibit RecA-mediated occurrences of illegitimate recombination between divergent sequences (34, 35). A previous group has shown that a deficiency in MMR, by inactivating Δ*mutS* or Δ*mutL,* in *S. aureus* does not result in enhanced recombination frequencies with divergent sequences (19). However, as I previously mentioned, *S. aureus* contains 3 MutS homologues that we hypothesized may play a role in

preventing illegitimate recombination. We observed an increase in integration rate of the *S. saprophyticus mqo2* plasmid (70.85% sequence similarity) in mutant strains lacking MutS1: Δ*mutS1*, Δ*mutS1*Δ*mutS2*, Δ*mutS1*Δ*mutX*, and Δ*mutS1*Δ*mutS2*Δ*mutX*. This suggests a role of MutS1 in blocking occurrences of illegitimate recombination, though, to a modest extent. Interestingly, we were not able to observe this trend until we reached up to ~30% sequence divergence, which correlates with results found in *B. subtilis* where they observed an effect of MMR during natural chromosomal transformation of ~17-23% sequence divergence (36). Overall, these results suggest a putative link between hypermutability and hyperrecombination in *S. aureus*, where a defect in the MMR system not only leads to an increase in mutation rate but also an increase in integration of divergent, exogenous DNA. This also suggests a role of MMR in horizontal gene transfer efficiency, in which a defect in the MMR system may allow for interspecies exchange of genetic material of up to ~30% divergence. In conclusion, these findings elucidate a potential correlation between the ability of *S. aureus* to integrate divergent, exogenous DNA via homologous recombination and *S. aureus* acquisition of virulence and antibiotic resistance factors, in turn, enhancing the overall pathogenesis.

Appendix A List of Strains and Plasmids Used in the Experiment

Appendix Table 1. Strains and plasmids used in this study

Strains	Genotype	Source/Reference
S. aureus LAC	USA300 Methicillin-resistant clinical isolate; laboratory strain	Laboratory strain
S. simiae	CCM 7213	Laboratory strain
S. epidermidis	RP62A	Laboratory strain
S. haemolyticus	JCSC1435	Laboratory strain
S. saprophyticus	ATCC 15305	Laboratory strain
AR1709	*S. aureus* LAC Δ*mutS::*erm^R	This study
AR1701	*S. aureus* LAC Δ*mutS::*tet^R	This study
AR1710	*S. aureus* LAC Δ*mutS2::*erm^R	This study
AR1704	*S. aureus* LAC Δ*mutS2::*spc^R	This study
AR1711	*S. aureus* LAC Δ*mutL::*erm^R	This study
AR1712	*S. aureus* LAC Δ*mutX::*erm^R	This study
AR1705	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*erm^R	This study
AR1706	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutX::*erm^R	This study
AR1707	*S. aureus* LAC Δ*mut2::spc*^{R} Δ*mutX::*erm^R	This study
AR1708	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*spc^R Δ*mutX::erm*^{R}	This study
AR1713	*S. aureus* LAC + KH01 *(S. aureus mqo2)*	This study
AR1714	*S. aureus* LAC + KH02 *(S. simiae mqo2)*	This study
AR1715	*S. aureus* LAC + KH03 *(S. epidermidis mqo2)*	This study
AR1716	*S. aureus* LAC + KH04 *(S. saprophyticus mqo2)*	This study
AR1717	*S. aureus* LAC Δ*mutS::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1718	*S. aureus* LAC Δ*mutS::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1719	*S. aureus* LAC Δ*mutS::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1720	*S. aureus* LAC Δ*mutS::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1721	*S. aureus* LAC Δ*mutS2::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1722	*S. aureus* LAC Δ*mutS2::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1723	*S. aureus* LAC Δ*mutS2::*erm^R + KH03 *(S. epidermidis mqo2)*	This study

AR1724	*S. aureus* LAC Δ*mutS2::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1725	*S. aureus* LAC Δ*mutL::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1726	*S. aureus* LAC Δ*mutL::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1727	*S. aureus* LAC Δ*mutL::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1728	*S. aureus* LAC Δ*mutL::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1729	*S. aureus* LAC Δ*mutX::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1730	*S. aureus* LAC Δ*mutX::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1731	*S. aureus* LAC Δ*mutX::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1732	*S. aureus* LAC Δ*mutX::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1733	*S. aureus* LAC Δ*mutS*::tet^R Δ*mutS2*::erm^R + KH01 *(S. aureus mqo2)*	This study
AR1734	*S. aureus* LAC Δ*mutS*::tet^R Δ*mutS2*::erm^R + KH02 *(S. simiae mqo2)*	This study
AR1735	*S. aureus* LAC Δ*mutS*::tet^R Δ*mutS2*::erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1736	*S. aureus* LAC Δ*mutS*::tet^R Δ*mutS2*::erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1737	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutX::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1738	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutX::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1739	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutX::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1740	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutX::*erm^R + KH04 (S. saprophyticus mqo2)	This study
AR1741	*S. aureus* LAC Δ*mut2::*spc^R Δ*mutX::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1742	*S. aureus* LAC Δ*mut2::*spc^R Δ*mutX::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1743	*S. aureus* LAC Δ*mut2::*spc^R Δ*mutX::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1744	*S. aureus* LAC Δ*mut2::*spc^R Δ*mutX::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
AR1745	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*spc^R Δ*mutX::*erm^R + KH01 *(S. aureus mqo2)*	This study
AR1746	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*spc^R Δ*mutX::*erm^R + KH02 *(S. simiae mqo2)*	This study
AR1747	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*spc^R Δ*mutX::*erm^R + KH03 *(S. epidermidis mqo2)*	This study
AR1748	*S. aureus* LAC Δ*mutS::*tet^R Δ*mutS2::*spc^R Δ*mutX::*erm^R + KH04 *(S. saprophyticus mqo2)*	This study
NE11	*S. aureus* JE2 Δ*recJ*::erm^R	(37)

NE22	*S. aureus* JE2 Δ*dpol*::erm^R	(37)
NE152	*S. aureus* JE2 Δ*topB*::erm^R	(37)
NE445	*S. aureus* JE2 Δ*umuC*::erm^R	(37)
NE761	*S. aureus* JE2 Δ*nth*::erm^R	(37)
NE888	*S. aureus* JE2 Δ*ung*::erm^R	(37)
NE972	*S. aureus* JE2 Δ*recQ*::erm^R	(37)
NE1028	*S. aureus* JE2 Δ*nfo*::erm^R	(37)
NE1040	*S. aureus* JE2 Δ*mutY*::erm^R	(37)
NE1344	*S. aureus* JE2 Δ*recG*::erm^R	(37)
NE1379	*S. aureus* JE2 Δ*queA*::erm^R	(37)
NE1451	*S. aureus* JE2 Δ*sbcC*::erm^R	(37)
NE1613	*S. aureus* JE2 Δ*mpg:*:erm^R	(37)
NE1825	*S. aureus* JE2 Δ*tag*::erm^R	(37)
NE1866	*S. aureus* JE2 Δ*dinB*::erm^R	(37)
Plasmids	**Description**	**Source/Reference**
pBT2ts	*E. coli/S. aureus* shuttle vector	(38)
pBTK	1.4 kb *aph-A3* allele cloned into XmaI of pBT2ts	(39)
pBTS	1.3kb *aad9* allele cloned into Xmal of pBT2ts	(39)
pBTT	1.7kb *tetK* allele cloned into Xmal of pBT2ts	(40)
KH01	*S. aureus mqo2* cloned into BamHI site of pBTK	This study
KH02	*S. simiae mqo2* cloned into BamHI site of pBTK	This study
KH03	*S. epidermidis mqo2* cloned into BamHI site of pBTK	This study
KH04	*S. saprophyticus mqo2* cloned into BamHI site of pBTK	This study

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