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Investigation into the Mechanism of Salicylate-Associated Genotypic Antibiotic Resistance in Staphylococcus aureus

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Investigation into the Mechanism of Salicylate-Associated Genotypic Antibiotic Resistance in *Staphylococcus aureus*

by

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DEDICATION

I dedicate this to my husband, James, for being there for me throughout this degree. Your patience and belief in me are the reasons behind my success. I would not have been able to complete this degree with out you. I would also like to dedicate this to my parents for their faith in me, my sister, Noha, for her tough love and my friends, Iyat, Mariana and Niti for their moral support and for allowing me to vent over the years. Thank you all for helping me complete my degree. A special dedication to my former undergraduate, Jessica Cheer, who was a great help in this study and will truly be missed.

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ABSTRACT

Growth of *Staphylococcus aureus* with the NSAID salicylate increases phenotypic resistance (SAPAR), and the frequency at which heritable resistance occurs to various antibiotics (SAGAR). This study describes the effect of salicylate on heritable and phenotypic resistance to a set of antibiotics for laboratory and multi-drug resistant strains of *S. aureus* and investigates the link between resistance and SAGAR. Drug gradient plates were used to determine phenotypic resistance to antibiotics targeting DNA replication, transcription, translation and the cell wall in the presence or absence of salicylate. To measure heritable resistance, mutation frequencies were determined for each antibiotic in the presence and absence of salicylate. Salicylate significantly increased mutation frequency of SH1000 to ciprofloxacin 27- fold from 4.9×10^{-8} to 8.5×10^{-7}. A significant 8.5- fold increase was observed for LAC from 5.2×10^{-7} to 2.1×10^{-6}. Conversely, salicylate significantly decreased mutation frequency for SH1000 to lincomycin 0.035-fold from 3.4×10^{-7} to 1.3×10^{-7}. Deletion of the general stress sigma factor sigB encoding σ^B in SH1000 resulted in decreased heritable and phenotypic resistance, signifying the importance of σ^B in the full expression of both phenotypes. Metabolite profiling revealed downregulation of glycolysis, TCA, pentose phosphate pathway, and amino acid metabolism. The downregulation of the TCA cycle was confirmed as observed through an increase in NAD^+ at growth toxic concentrations of salicylate. Salicylate has been shown to result in ROS accumulation and disruption of proton motive force in mitochondria.

SAGAR was only detected for fluoroquinolones, which have been shown to impair TCA cycle and result in ROS accumulation. Examination of ROS under growth-toxic concentrations of salicylate did not reveal a significant increase in ROS levels. Also, the combination of ciprofloxacin and salicylate did not result in an increase in ROS levels. Despite this, addition of the antioxidant glutathione abrogated SAGAR for ciprofloxacin in SH1000 but not for SAPAR. Analysis of SAGAR with NSAIDs benzoate and acetyl salicylic acid revealed a necessity for the ortho hydroxyl group on salicylate to fully express SAGAR. These results suggest that salicylate has pleiotropic effects on *S. aureus* that include antimicrobial resistance, altered metabolic flux and accumulation of ROS as well as unidentified regulatory genes.

CHAPTER ONE: INTRODUCTION

1.1 NSAIDs and salicylate

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic and anti-inflammatory properties and are used in the treatment and control of inflammatory conditions and in the treatment of various cancers [1-5]. NSAIDs inhibit cyclooxygenases, a class of enzymes responsible for producing mediators of inflammation including prostaglandins, prostanoids, thromboxanes. NSAIDs are among the most widely prescribed drugs, with approximately 100 million prescriptions filled annually in the USA alone [6]. Acetylsalicylic acid (aspirin) was the first synthesized NSAID, and is known for its extensive analgesic, anti-pyretic and anti-inflammatory activity [2, 3]. Salicylate, the primary metabolite of aspirin, is also recognized for its strong analgesic and antipyretic properties [3, 7] and in addition, is broadly used in oral and topical medications, as a preservative in foods, and in various commercial applications [8].

1.2 The effects of salicylate on eukaryotes

Salicylate and its chemical derivatives have been used since 400 B.C for their analgesic and antipyretic properties, and more recently as antiplatelet agents for the prevention of myocardial infarction and stroke [9, 10]. Salicylate and acetyl salicylic acid (aspirin) have also been shown to prevent colon cancer [11], and to have chemoprotective properties against lung and breast cancer [12]. This is believed to result from

enhancement by salicylate of the mitochondrial permeability transition-dependent apoptosis (MPT), which acts by promoting apoptosis of transformed cells [12].

Salicylate has been shown to decrease the threshold for the onset of mitochondrial permeability transition (MPT) [13, 14]. The MPT involves the formation of a non-specific pore across the inner membrane permitting the free distribution of ions, solutes, and small-molecular-weight molecules across the membrane [15]. The collapse of the mitochondrial membrane potential and uncoupling of the electron transport chain from ATP production has been shown to promote MPT. This disruption or collapse is also associated with the loss of matrix calcium and glutathione, increased oxidation of thiols, and further depolarization of the inner mitochondrial membrane, which increases the gating potential for the MPT pore [15-19]. Salicylate can induce MPT at low concentrations, resulting in an increase in the vulnerability of rat hepatocytes to necrosis from oxidant stress [11], while high concentrations of salicylate (>3 mM) can lead to MPT and cell death. It is believed that salicylate-dependent onset of the MPT may be responsible for Reye's syndrome [11]. Reye's syndrome is a rare and severe illness in children with a mean mortality rate of 40% [20]. The etiological cause is presently unknown. However, this disease is believed to correlate with the use of salicylates and/or other anti-pyretic drugs [21-24].

1.3 The effects of salicylate on bacteria

Salicylate has been shown to induce a number of distinct morphological and physiological changes in bacteria. Importantly, when grown in the presence of sub-inhibitory concentrations of salicylate, clinically significant species of bacteria including

Escherichia coli, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and others, express increased levels of intrinsic antibiotic resistance [7, 25-27]. Importantly, these effects are induced under concentrations of salicylate that do not impair bacterial growth rates, suggesting that they are specific to salicylate. Increased resistance to antibiotics in the presence of salicylate has been attributed to the alteration in membrane-associated proteins such as porins and transporters, leading to a reduction in drug accumulation [7]. This salicylate-associated phenotypic resistance is non-heritable, and results in reduced susceptibility to mechanistically and structurally distinct antimicrobials [26-37]. Phenotypic resistance has been attributed to a weak acid effect, which is thought to be due to an increase in membrane potential and altered permeability and a decrease in internal pH, the pH gradient, and the proton motive force [38]. Foulds *et al*. [39] demonstrated a 3- to 5-fold decrease in permeation of cephalosporins through the outer membrane of *E. coli* induced with salicylate.

Salicylate has been shown to alter energy metabolism in many organisms. For example, in *E. coli,* growth with salicylate altered the expression of over 130 genes [41]. In addition, it was shown to dissipate the proton gradient across the inner membrane, chelate iron, induce heat shock as well as inhibit growth [40-41]. Similarly, in *Bacillus subtilis,* salicylate was shown to impair energy metabolism as observed through a down-regulation of ATPases, suggesting energy impairment [41]. Salicylate also induced the general stress sigma factor B (*sigB*), and *sigB* dependent genes in *B. subtilis*. In addition, salicylate decreases metabolism of purines, pyrimidines, coenzymes, as well as metabolism of carbohydrates involved in glycolysis [42]. Growth with salicylate has similar modulatory effects on metabolism in *S. aureus*. Therefore, it is of no surprise that

salicylate altered metabolism in *S. aureus,* resulting in inhibition of glycolysis as seen in the downregulation of glyceraldehyde-3-phosphate dehydrogenase (*gapA2)* and phosphoglucoisomerase (*pgi*) [43]. The findings by Riordan *et al.* [43] indicate an impairment of growth and overall alteration in central and energy metabolism. This existing data suggest that salicylate has pleiotropic effects on bacterial cells that are physiochemical and metabolic.

1.4 *Staphylococcus aureus*

Staphylococcus aureus is a low GC Gram-positive, non-spore forming bacteria that was discovered in the 1880s [44]. *S. aureus* is a facultative anaerobe [46] that can grow at temperatures between 25°C to 43°C and at pH levels of 4.8 to 9.4 [45]. *S. aureus* mainly colonizes the membranes and skin of warm-blooded animals, and infections range from benign skin lesions to life-threatening systemic illnesses such as endocarditis and osteomyelitis [45]. The Center for Disease Control and Prevention estimated in 2005 that there were 31.8 culture confirmed invasive methicillin resistant *S. aureus* (MRSA) infections in the U.S. per 100,000 individuals, which amounted to 94,360 cases [47]. The primary mode of transmission is through direct contact, usually skin-to-skin contact with a colonized or infected individual, although contact with contaminated objects or surfaces also plays a role [48-51]. *S. aureus* is capable of producing many toxins and is able to acquire resistance to many antibiotics [45]. Currently, greater than 60% of *S. aureus* isolates are resistant to methicillin and some strains have developed resistance to more than twenty antimicrobial agents [52]. Acquisition of resistance in *S. aureus* commonly results from either gene mutations leading to drug target modifications or reduction of

drug efficacy, as well as acquisition of a resistance gene(s) from other organisms by some form of horizontal genetic exchange [53]. The genome of *S. aureus* is approximately 2.8 Mbp with a GC content of 33% [45, 52, 53].

1.5 Antibiotic Resistance in *S. aureus*

Staphylococcus aureus is exceptionally resistant to a wide-range of antibiotics (for review see [44]). This resistance evolved in *S. aureus* via horizontal gene transfer, chromosomal mutation and antibiotic selection [44]. Antibiotic resistance in *S. aureus* emerged in a series of waves [44]. The first wave of resistance began in the mid 1940s where penicillin-resistant strains began to surface in hospitals shortly after its introduction [54, 55]. Introduction of methicillin in 1960 initiated the second wave of resistance. Methicillin is a narrow spectrum β-lactam antibiotic that inhibits cross-linkages between the linear peptidoglycan polymer chains that make up a major component of the Gram positive cell wall [56]. It was as early as 1961 that resistance to methicillin was detected in *S. aureus*. The *mecA* gene, encoding alternative penicillin binding protein 2 (PBP2) responsible for the methicillin resistance phenotype, was not identified until more than twenty years later. The emergence of methicillin resistant *S. aureus* (MRSA) strains led to an increase in the use of vancomycin [57, 58]. Vancomycin is a glycopeptide antibiotic used in the prophylaxis and treatment of serious infections caused by Gram positive bacteria [57, 58]. Vancomycin acts by inhibiting proper cell wall synthesis through formation of hydrogen bond interactions with the terminal D-ala-D-ala moieties of the NAG/NAM peptides [57, 58]. The first vancomycin intermediate resistant *S. aureus* (VISA) strain was reported in 1997 with MICs <16 μg/ml [59]. The

first VRSA strain was isolated in June 2002 with high-level vancomycin-resistance and an MIC=1024 μg/ml [59]. This isolate was also found to be resistant to aminoglycosides, rifampin, and tetracycline. This VRSA strain was later determined to acquire the vancomycin resistance gene through horizontal gene transfer from *Enterococcus faecalis* [57, 58]. The invasion of MRSA into the community constitutes the fourth and latest wave of resistance. It is during this wave that community associated (CA) MRSA and vancomycin resistant strains began to emerge [44]. Despite all the resistance only two antibiotics besides vancomycin, linezolid and daptomycin have been approved for therapy since the 1990s [60].

1.6 Salicylate associated phenotypic and genotypic antibiotic resistance in *S. aureus*

Phenotypic antibiotic resistance was first characterized in *E. coli.* Resistance to chloramphenicol and ampicillin was induced during incubation with the weak acids acetate or benzoate, the NSAIDs aspirin and salicylate, and other chemical repellants such as dimethyl sulfoxide (DMSO), and 1-methyl-2-pyrrolidinone, [29]. Importantly, cells were sensitive to the antibiotics when grown in the absence of these inducers, and thus resistance was described to be inducible and non-heritable. Salicylate-inducible antibiotic resistance in *E. coli* was subsequently found to be due, in part, to increased transcription of the *marRAB* operon [25]. The *marRAB* operon consists of *marR*, which encodes a negative regulator of the operon, and *marA*, a transcriptional activator. Salicylate has been found to interact with the ligand binding domain of MarR, interfering with its ability to efficiently recognize the *mar* operator, *marO*. This, in turn, leads to induction of MarA and a decrease in antibiotic accumulation by reduced production of

outer membrane porins OmpF and OmpC, and a concomitant increase in the production of the multidrug efflux pump AcrAB [61, 62]. Salicylate has also been shown to induce phenotypic antibiotic resistance in *Salmonella typhimurium.* When grown in the presence of salicylate, *S. typhimurium* developed increased resistance to chloramphenicol, and enoxacin. This was found to be due to the induction of a *S. typhimurium mar* system homologous to the *mar* operon of *E. coli* [28]. *Klebsiella pneumoniae* also exhibits increased phenotypic resistance to tetracycline, β-lactams, clindamycin and norfloxacin in the presence of salicylate, which has been reported to be due to increased transcription of *ramA*, encoding a MarA homolog [35].

Phenotypic antimicrobial resistance is also induced by salicylate in *S. aureus*. When exposed to salicylate, *S. aureus* develops increased resistance to fluoroquinolones, the steroid antibiotic fusidic acid, hard surface disinfectants and ethidium bromide [26, 33]. The mechanism for phenotypic resistance in *S. aureus* is thought to be achieved in part by the upregulation of drug efflux pumps including NorA, NorB, MdeA and SepA [7, 63-66] and through alterations in membrane permeability [33, 43, 67-71]. As in other bacteria, phenotypic resistance in *S. aureus* appears to be dependent on Mar family homologs, such as the Sar-family of proteins, MgrA, as well as the general stress resistance protein sigma factor B [66, 72-76]. Sigma factor B is one of two alternative sigma factors in *S. aureus* [77] and is essential for the general chemical and physical stress response of the organism [78]. *sigB* regulation has been found to be intertwined with the expression of SarA, which regulates the expression of a number of staphylococcal virulence factors [78-81]. Analyses by Riordan *et al*. [71] determined that altered expression of *sigB* and *sarA* is not required for the salicylate-inducible

mechanism. Riordan *et al.* [43] also observed impairment in the glycolytic pathway as seen through a decrease in glyceraldehyde-3-phosphate dehydrogenase (*gapA2*) and phosphoroglucoisomerase (*pgi*) in response to salicylate stress, which is supported by evidence in eukaryotes [8, 9, 82-84].

Salicylate has been shown to alter the level of resistance as well as the frequency at which antibiotic resistance occurs in *S. aureus* [7, 26, 33, 85]. Specifically, salicylate has been observed to increase the frequency at which *S. aureus* mutates to become resistant to fluoroquinolones and fusidic acid [7, 26, 33, 85]. The addition of salicylate significantly increased the number of ciprofloxacin and norfloxacin resistant *S. aureus* colonies compared to resistant colonies selected in the absence of salicylate; ciprofloxacin-resistant mutants arose at mutation frequencies of 1.8×10^{-9} on plates containing ciprofloxacin, compared to a mutation frequency of 1.8×10^{-7} on plates containing ciprofloxacin and salicylate [33]. Colonies selected from ciprofloxacin in the presence of salicylate containing plates had MICs > 0.8 mg/l, which was higher than colonies selected in the absence of salicylate [33]. The mutations leading to fluoroquinolone and fusidic acid resistance in these isolated were heritable, and resistance to these antibiotics occurred at unrelated loci within the *S. aureus* genome [86, 87]. The underlying mechanism of salicylate-associated genotypic resistance [26, 33] and the extent to which salicylate alters resistance to other antibiotics is currently unknown.

1.7 Hypothesis and aims of the study

This study seeks to understand the basis of salicylate-associated genotypic antibiotic resistance (SAGAR) in the deadly human pathogen, *S. aureus*. The overarching hypothesis is that the SAGAR phenotype is common to mechanistically and structurally

distinct antibiotics, and is attributable to the effect(s) of salicylate on *S. aureus* metabolism. The following aims test this hypothesis:

Aim 1. Assess the ability of salicylate to alter the frequency at which genotypic resistance to mechanistically and structurally distinct antibiotics occurs in *S. aureus.*

Aim 2. Examine the role for salicylate-associated metabolic stress in the genotypic antibiotic resistance phenotype.

CHAPTER TWO: CHARACTERIZATION OF THE SALICYLATE-ASSOCIATED GENOTYPIC ANTIBIOTIC RESISTANCE PHENOTYPE IN *S. AUREUS*

2.1 Background

The nonsteroidal anti-inflammatory drug (NSAID) salicylate has been shown to increase the frequency at which heritable (genotypic) fusidic acid and ciprofloxacin resistance occurs in *S. aureus* [7, 26, 33, 43, 69, 70]. These antibiotics have distinct cellular targets, and are structurally unique. This salicylate-associated antibiotic resistance (SAGAR) suggests that salicylate, and perhaps other NSAIDs, may have a generalized effect on mutation frequency in the cell. Yet, salicylate has not been shown to be directly mutagenic by the Ames test [85], which suggests that this increase in mutation frequency may result from the alteration of an existing physiological process which occurs in combination with specific antibiotic chemistries or antibiotic mechanistic activities. For example, bactericidal drugs such as ciprofloxacin have been shown to increase oxidation of NADH via the electron transport chain [88, 89], resulting in formation of the reactive oxygen species (ROS) superoxide [90-92]. Superoxide and other ROS damage iron-sulfur clusters, making ferrous iron available for oxidation by the Fenton reaction [88, 89]. The Fenton reaction leads to hydroxyl radical formation, which damages DNA, proteins and lipids, ultimately resulting in cell death [88, 89]. Subinhibitory concentrations of certain antibiotics, specifically compounds whose

primary mode of action is DNA damage, are known to enhance mutation rates in bacteria [93, 94]. This elevation in mutation frequency is partly a result of transcriptional changes in genes responsible for DNA repair and preservation of the integrity of the genome [93, 94]. This effect is also observed with rifampin, an antibiotic that interacts specifically with the β subunit of the bacterial RNA polymerase encoded by the *rpoB* gene [95].

Growth with salicylate also leads to a non-heritable (phenotypic) increase in resistance to many antimicrobials [7, 26, 33, 43, 69-71, 96]. As in *E. coli,* this salicylate-inducible phenotypic resistance in *S. aureus* partly results from a decrease in drug accumulation due to alterations in membrane permeability, proton motive force, and efflux [7, 25, 26, 33, 36, 37, 43, 69-71, 96]. A number of proteins have been determined to be involved in this phenotypic resistance mechanism of *S. aureus* including: multidrug efflux pumps NorA, NorB, MdeA, and SepA as well as other chromosomally encoded efflux pumps [7, 63-66, 97]; the global regulatory protein MgrA [66, 73, 76]; staphylococcal accessory regulator (SarA) [74]; and alternative sigma factor B (SigB) [70]. Mutations in the *S. aureus* genes encoding these proteins resulted in increased [70, 74, 98-100] or decreased [63-65, 80, 97, 99] susceptibility to antimicrobials. Phenotypic resistance may allow the cell prolonged exposure to low levels of antibiotic, leading to the acquisition of mutations and high level heritable (genotypic) resistance [101].

Currently, the salicylate-associated genotypic antibiotic resistance (SAGAR) phenotype has only been described for a limited number of antibiotics, or for antibiotics, which are not commonly indicated for *S. aureus* infections. In addition, the relationship between salicylate-inducible phenotypic resistance and the SAGAR phenotype is unknown. The following experiments are designed to examine the SAGAR phenotype,

and to test the hypothesis that growth of *S. aureus* with salicylate increases the frequency at which resistance to structurally and mechanistically distinct antibiotics occurs.

2.2 Methods

Bacterial strains and culture media and conditions

S. aureus strains used in this study are listed in Table 2.1. Unless otherwise noted, strains were grown aerobically at 37°C with shaking (200 RPM) in baffled Erlenmeyer flasks (5:1 volume ratio of flask:media). Cultures were generally maintained in tryptic soy broth (TSB) or TSB with 1.5% agar (TSA), and stocked at -80°C in TSB with the addition of 20% (vol/vol) glycerol.

Antibiotics and NSAIDs

All antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs) were dissolved, filter sterilized and stored according to Material Safety Data Sheet guidelines (Version 3 [102]). The antibiotics used in this study included ciprofloxacin (2.5 mg/ml in 0.1 N HCl), norfloxacin (5 mg/ml in 0.1 N HCl), fusidic acid (1 mg/ml in sterile water), vancomycin (1 mg/ml in sterile water), tetracycline (1 mg/ml in sterile water), rifampin 500 μg/ml in methanol), oxacillin (1 mg/ml in sterile water) and lincomycin (1 mg/ml in sterile water). The NSAIDs and weak acids used in this study included: sodium salicylate (0.5 M in sterile water), acetylsalicylic acid (3 mM in sterile water) and sodium benzoate (0.5 M in sterile water).

Table 2.1. Bacterial Strains and Plasmids

Strain name	Relevant Characteristics	Source/reference
S. aureus		
SH1000	*rsbU*+ derivative of 8325-4	Dr. Lindsey Shaw, USF, [103]
SaRNH-1	SH1000Δ *sigB*	Dr. Lindsey Shaw, USF, [103]
Newman	Wild-type	Dr. John Gustafson, NMSU, [104]
SaRNH-2	Newman Δ*mgrA*	Dr. John Gustafson, NMSU, [105]
LAC	USA300 CA-MRSA	Dr. Lindsey Shaw, USF, [106]
E. coli		
MG1655	Wild-type	Dr. James Riordan, USF, [107]

Table 2.2 Primers used in this study

Primer name	Sequence (5'→3')
grlA+2402	ACTTGAAGATGTTTTAGGTGAT
grlA+2961	TTAGGAAATCTTGATGGCAA
grlB+1520	CGATTAAAGCACAACAAGCAAG
grlB+1894	CATCAGTCATAATAATT CTC
gyrA+2311	AATGAACAAGGTATGACACC
gyrA+2533	TACGCGCTTCAGTATAACGC
gyrB+1400	CAGCGTTAGATGTAGCAAGC
gyrB+1650	CCGATTCCTGTACCAAATGC

All primers were designed based on this study.

Determination of mutation frequency

The protocol for mutation frequency to antibiotic resistance was adapted from Foster, 2006 [108]. Three independent cultures were grown under standard conditions in TSB overnight before sampling 0.1 ml onto TSA alone, TSA with antibiotic at ½ X, 1X, and 2X MIC, TSA with NSAID, or TSA with antibiotic and NSAID. Plates were incubated for 24 h before counting colony forming units (CFU/ml). Mutation frequency (μ) and fold-change in mutation frequency was calculated from colony counts using the following equations:

$$\text{Eq. 2.1: } \mu \text{ antibiotic} = \frac{\text{CFU (Antibiotic)}}{\text{CFU (TSA)}}$$

$$\text{Eq. 2.2: } \mu \text{ NSAID} = \frac{\text{CFU (Antibiotic + NSAID)}}{\text{CFU (NSAID)}}$$

Fold change was calculated using the following equation:

$$\text{Eq. 2.3: Fold Change} = \mu \frac{\text{Antibiotic + NSAID}}{\text{Antibiotic}}$$

Differences in the mutation frequency between control and treatment cultures were compared using a t-test (α=0.05, n≥3) (R).

Antibiotic susceptibility by the minimum inhibitory concentration assay (MIC)

MICs were determined as described [109] with slight adaptations. Overnight *S. aureus* MHB cultures were diluted to an OD_{600} = 0.01 in fresh MHB. A stock of the desired antibiotic was prepared at two times the highest concentration in MHB. Serial 2-fold dilutions of respective drugs were prepared in MHB. Of the diluted culture, 1 ml was added to each tube to achieve the final concentration with a final volume of 2 ml. A negative control containing the growth medium plus antibiotic was prepared. A positive control containing the growth medium and culture was also prepared. The set of tubes were incubated static overnight at 37°C. The MIC for each independent sample was recorded, as the lowest concentration of antibiotic at which there was no visible growth.

S. aureus chromosomal DNA extraction

A single colony of *S. aureus* SH1000 was grown under standard conditions overnight. The following day the cells were pelleted by centrifugation at 3,700 x g for 10

min. Pelleted cells were resuspended in 5 ml 1X TE buffer (pH 8.0) and centrifuged as above. Cell pellets were then resuspended in 600 µl of 1X TE buffer and transferred to a 1ml Eppendorf tube containing 0.5 cm^3 of 0.1 mm glass beads. The tube was placed in a bead beater (Mini-bead beater-16, Biospec) and pulsed at 10 sec intervals for a total of 60 seconds without a break. Homogenates were then centrifuged for 5 min at 13,200 x g. The supernatant was transferred to a sterile microtube, and 0.2 ml of 1.6% (vol/vol) sarkosyl and a total of 25 µg of proteinase K was added to the tube and incubated at 60°C for 60 min. Eight-hundred microliters of phenol/chloroform/isoamyl was added, vortexed, and centrifuged at 13,200 x g for 5 min. The upper aqueous layer was transferred to a fresh Eppendorf tube, and 0.5 ml isopropyl alcohol and 100 µl of 3 M sodium acetate were added and mixed by inversion. This was allowed to incubate at -80°C for 15-30 min. The samples were then centrifuged at 13,200 x g for 5 min, and the supernatant was carefully discarded. Five-hundred microliters of 70% ethanol was added to the pellet and centrifuged at 13,200 x g for 5 min. The supernatant was then discarded. The samples were allowed to air dry for 3-4 min at room temperature with the lid open. Two-hundred microliters of ddH_2O was used to resuspend the DNA.

Sequencing of target site modifications in antibiotic resistant mutants

Antibiotic resistant and susceptible isolates were passaged three times in the absence of antibiotic before MICs were determined. DNA from two representative isolates was extracted and PCR was used to amplify a 559-bp fragment of *grlA* using primers grlA+2402/grlA+2961, a 374-bp fragment of *grlB* using primers grlB+1520/grlB+1894, a 222-bp fragment of *gyrA* using primers gyrA+2311/gyrA+2533,

and a 250-bp fragment of *gyrB* using primers gytB+1400/gyrB+1650 (Table 2.2). *grlAB* and *gyrAB* were amplified at an annealing temperature of 55°C, and an extension time of 50 seconds for 30 cycles. Products were confirmed by ethidium bromide agarose gel electrophoresis and were purified using Qiagen purification kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Products were sequenced using standard Sanger dye chain-termination sequencing through the services of MWG Operon (Huntsville, AL) using forward and reverse primers (Table 2.2) to read both template and coding strands. Sequencing reads were compared to publically available sequences of *S. aureus* N315 (NCBI) by pairwise alignment. Target site modifications were recorded and annotated according to genomic position.

Selection for resistance to sodium salicylate

Spontaneous selection

An overnight culture of *S. aureus* strain SH1000, LAC and *E. coli* strain MG1655 (K-12) was prepared under standard conditions. Large TSA plates (100 x 200 mm) as well as TSA gradient plates were prepared at ¼, ½, 1, 2, 3, 4, 5, 6 X MIC of either salicylate or acetyl salicylic acid. Cultures (100 μl) were inoculated onto each of the plates incubated at 37°C, and putative resistant colonies were selected after 24 h. Colonies were then passaged twice in the absence of NSAID before MICs were performed to determine if the recovered isolates had a higher level of resistance to the NSAID when compared to WT.

N-methyl-N-nitro-N-nitrosoguanidine (NTG)-mutagenesis

To select for a salicylate resistant straing NTG mutagenesis was used. A single colony of *S. aureus* SH1000 was prepared under standard conditions. One milliliter of overnight culture was inoculated into 100 ml TSB and allowed to grow for 3 hours before addition of NTG (50 μg/ml final) or an equal volume of sterile water (control). Cell counts (CFU/ml) were performed immediately before addition of NTG. Cells were grown for 45 min with and without NTG before harvesting by centrifugation at 1,929 x g for 10 min. NTG has been shown to induce at least one mutation per cell under the above growth conditions, which has been shown to correlate with a 50% survival rate [110]. NTG is known to add alkyl groups to O^6 of guanine and O^4 of thymine [110]. The supernatant of NTG-treated and non-treated (control) cultures was then removed and discarded, and the cells were resuspended in 100 ml of TSB by vortexing. These cells were then centrifuged again as before and the supernatant was discarded before being resuspended in 100 ml of TSB and allowed to grow for 2 hours. Cultures were then sampled to verify the efficiency of NTG killing, and serial dilutions were plated to determine final CFU/ml for NTG-treated compared to untreated cultures. Multiple libraries of NTG mutants were stocked by taking ten 1 ml aliquots in Eppendorf tubes and centrifuging them at 1,372 x g for 3 minutes. The supernatant was discarded and the cells were resuspended in TSB with 20% (v/v) glycerol and stored at -80°C.

Stepwise selection

An overnight culture of *S. aureus* strain SH1000 was prepared under standard conditions. Cells were inoculated in fresh TSB at 1:100 containing 1.5 mg/ml salicylate.

The following day cells were plated on to TSA plates for cell counts and were also inoculated into fresh TSB at 1:100 containing 2X the initial concentration of salicylate. This process was repeated until no growth was recovered on TSA plates. MICs were determined for all colonies recovered following passage in TSB without antibiotic to determine if any of the cells acquired a higher level of resistance.

2.3 Results

Antibiotic specificity of the salicylate-associated genotypic resistance phenotype

Salicylate-associated genotypic antibiotic resistance (SAGAR) has been described for the fluoroquinolone antibiotics ciprofloxacin and norfloxacin, as well as the steroid antibiotic fusidic acid in both laboratory and MDR strains of *S. aureus*. To further assess the scope of this phenotype, the impact of salicylate on the frequency of resistance to a spectrum of antibiotics belonging to several drug classes was investigated for *S. aureus* laboratory strain SH1000, and when possible, the CA-MRSA strain LAC (Table 2.1). Of the nine antibiotics tested, salicylate was observed to only alter the frequency at which resistance to ciprofloxacin (Cip^R), norfloxacin (Nor^R) and lincomycin (Lin^R) occurred (Table 2.3). The frequency at which Cip^R occurred in SH1000 increased by 27-fold ($p=0.03$) in the presence of salicylate (Table 2.3). The frequency at which Cip^R mutants in strain LAC occurred with salicylate also increased, but only by 6.2-fold and not significantly. Growth of SH1000 with salicylate also significantly increased the mutation frequency to Nor^R by 4-fold ($p=0.01$). Unlike ciprofloxacin and norfloxacin, the frequency at which resistance to lincomycin occurred with salicylate decreased by 3-fold in SH1000, but not significantly ($p=0.05$). Mutation frequency to norfloxacin and

lincomycin resistance for strain LAC was not determined, due to its high intrinsic resistance to these antibiotics; MICs for ciprofloxacin and lincomycin in LAC were 64 μg/ml and an MIC of 25 μg/ml, respectively.

To determine the effect of adapting *S. aureus* to salicylate on the SAGAR phenotype, cultures were grown overnight with salicylate (adaptive environment) or without (un-adapted) and then tested for SAGAR on plates, which contained ciprofloxacin alone, or ciprofloxacin and salicylate. Adaptation to salicylate did not influence the frequency at which Cip^R colonies occurred compared to un-adapted cultures when selected on ciprofloxacin plates without salicylate (Fig. 2.1). Also, adaptation to salicylate had no effect on the increase in frequency to Cip^R observed when selected in the presence of salicylate. Collectively, these findings reveal that the SAGAR phenotype is antibiotic specific. Furthermore, the results suggest that salicylate is not a chemical mutagen in *S. aureus*, agreeing with previous studies in *Salmonella typhimurium* [28], and reveals that adaptation of cultures to salicylate has no apparent impact on SAGAR.

Table 2.3. Antibiotic specificity of salicylate-associated genotypic phenotype.

Antibiotic	Strain	Plated (µg/ml)	Mutation Frequency		Fold change	SD
			Antibiotic (Abx)	Abx + 500 µg Salicylate		
Ciprofloxacin	SH1000	1	4.9×10^{-8}	8.5×10^{-7}	27*	2.9×10^{-7}
	SaRNH-1	1	5.2×10^{-7}	2.1×10^{-6}	8.5*	6.9×10^{-7}
	LAC	16	8.2×10^{-4}	2.4×10^{-3}	6.2	3.6×10^{-3}
	Newman	1	3.2×10^{-6}	4.7×10^{-6}	1.5	1.2 X 10-6
	SaRNH-2	1	3.8×10^{-6}	4.3×10^{-6}	1.1	1.8×10^{-6}
Fusidic Acid	SH1000	0.5	6.1×10^{-7}	8.7×10^{-7}	1.4	6.0×10^{-7}
	SaRNH-1	2	3.6×10^{-6}	3.2×10^{-6}	0.9	2.4×10^{-6}
	LAC	4	7.7×10^{-8}	1.6×10^{-7}	2.2	1.2×10^{-7}
Lincomycin	SH1000	1	3.4×10^{-7}	1.3×10^{-7}	0.4*	1.0×10^{-7}
	SaRNH-1	1	1.2×10^{-7}	1.2×10^{-9}	0.02*	5.9×10^{-10}
Norfloxacin	SH1000	4	1.5×10^{-7}	6.6×10^{-7}	5.2*	2.6×10^{-7}
	SaRNH-1	4	6.8×10^{-8}	6.9×10^{-7}	7.2	6.7×10^{-7}
	Newman	2	2.1×10^{-6}	4.6×10^{-6}	2.8	1.6×10^{-6}
	SaRNH-2	2	2.7×10^{-6}	4.3×10^{-6}	1.6	1.3×10^{-6}
Rifampin	SH1000	1	2.9×10^{-7}	2.3×10^{-7}	0.8	1.4×10^{-7}
	SaRNH-1	1	6.2×10^{-8}	6.3×10^{-8}	1	3.1×10^{-8}
	LAC	0.1	9.8×10^{-8}	1.2×10^{-7}	1.7	8.6×10^{-8}
Tetracycline	SH1000	0.5	5.6×10^{-7}	1.0×10^{-6}	2	3.8×10^{-7}
	SaRNH-1	190	4.5×10^{-6}	7.0×10^{-6}	1.9	1.7×10^{-6}
Vancomycin	SH100	3	5.7×10^{-6}	2.3×10^{-6}	0.3	3.0×10^{-6}
	LAC	7	6.43×10^{-8}	2.6×10^{-8}	0.4	1.9×10^{-8}

SD indicates standard deviation for mutation frequency with antibiotic (abx) with salicylate. Asterisks denotes statistical significance $p < 0.05$, n=3. Strains were plated at 1 or 2X the MIC.

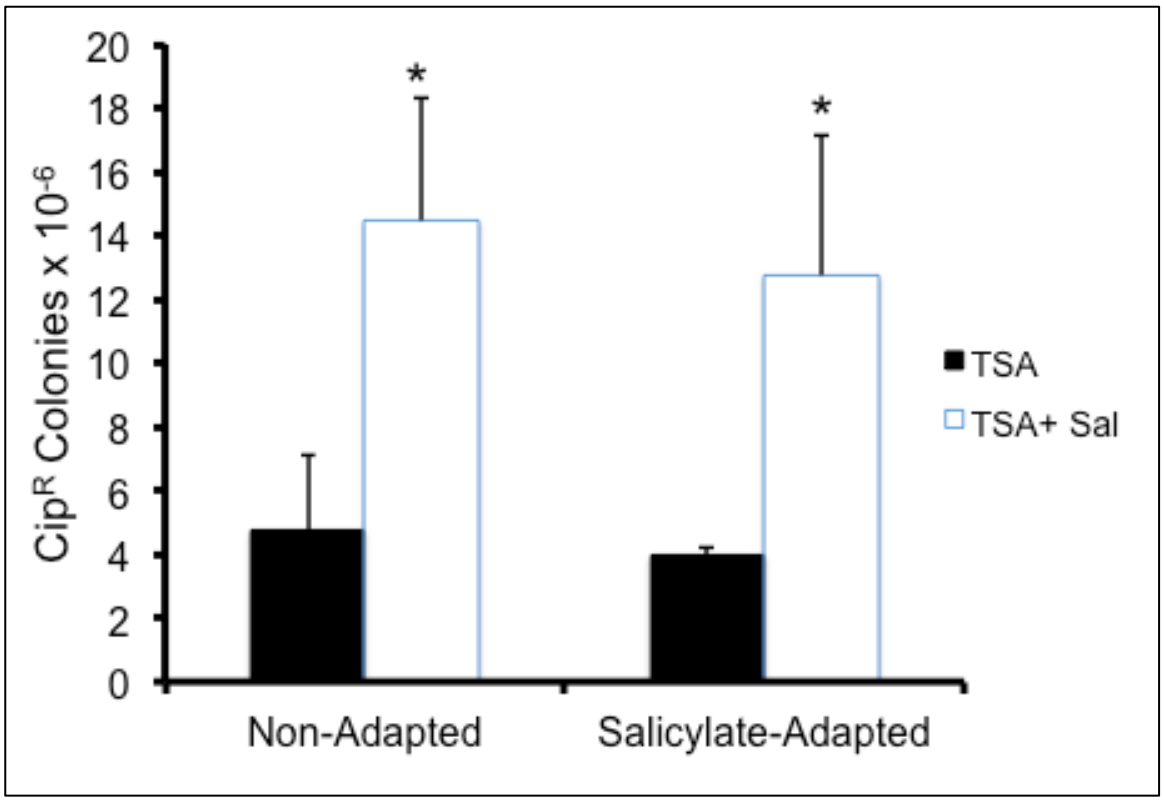

Figure 2.1. Frequency of resistance to ciprofloxacin in *S. aureus* cultures adapted to salicylate. CFU/ml of CipR colonies of SH1000 are plotted as a function of treatment: growth overnight with salicylate (adaptive environment) or without (control, non adaptive) and then plated to ciprofloxacin alone (filled bars) or ciprofloxacin and salicylate (hatched bars). Asterisks denote statistical significance by ANOVA and Tukey's HSD (p= 0.01).

Antibiotic specificity of the SAGAR phenotype

Broth microdilution minimum inhibitory concentration (MIC) assays were used to determine if selection for resistance to antibiotics with salicylate conferred the same level of resistance as selection in the absence of salicylate. MICs for antibiotic resistant isolates of strains SH1000, SaRNH-1 (a.k.a. SH1000sigB::tet), and LAC exceeded clinical and laboratory standards institute (CLSI) breakpoints for all drugs, except for lincomycin (breakpoint = 10 μg/ml) and vancomycin (breakpoint = 16 μg/ml) (Table

2.4). MICs for ciprofloxacin did not differ for resistant isolates selected with or without salicylate, but were lower for fusidic acid, lincomycin and norfloxacin resistant isolates for all strains when selected in the presence of salicylate (Table 2.4). MICs for SaRNH-1 resistant isolates did not differ between MICs for SH1000 resistant isolate strains for ciprofloxacin. Interestingly, a difference in the level of resistance between resistant isolates was observed. For example SaRNH-1 lincomycin isolates selected in the absence of salicylate had higher MICs (4 μg/ml) than SaRNH-1 lincomycin isolates selected in the presence of salicylate with an MIC of 2 μg/ml, These results suggest that the mutations, which lead to resistance to some antibiotics when selected in the presence of salicylate, may differ from those that lead to resistance in the absence of salicylate. Also, a difference in colony morphology was observed. Small colonies like variants were observed on antibiotic plates selected in the presence and absence of salicylate.

Table 2.4. Minimum inhibitory concentrations (MICs) for antibiotic resistant isolates selected with or without salicylate

Drug	Strain	Initial MIC (μg/ml)	MICs (μg/ml) Sal -		Sal +	
			LC	SCV	LC*	SCV*
Ciprofloxacin	SH1000	0.5	4	4	4	4
	SaRNH-1	1	4	4	4	1
	LAC	64	512	512	124.3	124.3
Fusidic Acid	SH1000	0.24	124.8	124.8	124.8	124.8
	SaRNH-1	0.98	NP	NP	NP	NP
	LAC	0.24	249.8	124.9	NP	NP
Lincomycin	SH1000	1	4	4	4	4
	SaRNH-1	1	4	4	2	2
Norfloxacin	SH1000	2	16	16	8	4
	SaRNH-1	4	32	16	16	8
Rifampin	SH1000	0.25	NP	NP	NP	NP
	SaRNH-1	0.5	NP	NP	NP	NP
	LAC	0.02	NP	NP	NP	NP
Tetracycline	SH1000	1	4	1	4	1
	SaRNH-1	64	NP	NP	NP	NP
Vancomycin	SH100	1	4	4	4	4
	LAC	3	NP	NP	NP	NP

All values are in micrograms per milliliter.
Large colony variant (LC); small colony variant (SC).
NP denotes not performed.
Sal denotes salicylate at 500 μg/ml

Characterization of mutations conferring resistance to fluoroquinolones

Quinolone resistance is gained through modification of *gyrAB* and *grlAB* targets as well as modification through the *norA* efflux pump promoter sequence, which are associated with clinical levels of resistance [111-114]. A difference in the level of quinolone resistance in some strains selected for in the presence or absence of salicylate was detected. It was of interest to sequence *gyrAB* and *grlAB* quinolone resistance determining region (QRDR) to determine if there was a difference between the acquired

SNPs. All mutations conferring resistance to ciprofloxacin and norfloxacin were determined to be in a 347-bp region of *grlA*, encoding the A subunit of topoisomerase IV. This resulted in a common mutation at amino acid Ser80 [113, 115], as well as less common (or unreported) mutations at Arg43 and Ala115 (Table 2.5). The location of the SNP had no apparent effect on the level of ciprofloxacin resistance, MIC = 4 μg/ml (Table 2.4). However, for norfloxacin resistant large colony (LC) isolates, the location of the SNP did not differ, but the level of resistance did: MIC of 16 μg/ml (without salicylate) and 8 μg/ml (with salicylate) (Table 2.4 and 2.5). Lincomycin, a macrolide, acts on the 50S ribosomal subunit, specifically targeting 23S rRNA [116]. Macrolides, such as erythromycin have been shown to target A2058G/U or A2059G, however, the exact mutation responsible for conferring lincomycin resistance remains to be identified [117]. Therefore, target site modifications conferring resistance to lincomycin were not determined.

Table 2.5. Sequencing results for *grlA* in fluoroquinolone resistant isolates in SH1000 compared to NCBI *S. aureus* sp N315.

SH1000	Antibiotic	Original	SNP	Genomic Location	Mutation	Original	AA Location
LC	Cipro	A	G	1,356,325	His	Arg	43
LC*		C	T	1,356,564	Phe	Ser	80
LC		C	G	1,356,671	Ala	Ala	115
LC, LC*	Nor	C	T	1,356,564	Phe	Ser	80
SCV		C	T	1,356,672	Val	Ala	115

Large colony variant (LC); small colony variant (SC).
Selection for antibiotic resistance in the presence of salicylate denoted by *.
Single nucleotide polymorphism (SNP).
Amino acid location (AA location).

Role of sigB and mgrA in the SAGAR phenotype

Alternative sigma factor B (σ^B) directs the transcription of more than one hundred genes in response to different stressors [98, 118]. Sigma factor B is necessary for full expression of salicylate-inducible phenotypic resistance, and its expression was shown to be upregulated upon salicylate exposure [71, 74]. MgrA is a helix-turn-helix DNA binding protein and, like SigB, regulates many *S. aureus* genes (approx. 355) [66] including genes shown to control autolytic activity and the expression of several virulence factors, including alpha-toxin, nuclease and protein A [66]. Importantly, MgrA negatively regulates the multiple drug efflux pumps NorA, NorB, NorC, Tet38 and AbcA, shown to be important for resistance to fluoroquinolones [75, 76], and *mgrA* transcript levels are repressed by salicylate [71]. In previous studies, an *mgrA* mutant showed resistance to ciprofloxacin, which was linked to increased expression of *norA* [66, 70]. Since σ^B and MgrA contribute to the phenotypic resistance mechanism, we aimed to ascertain the role for these regulators in the SAGAR phenotype using strains, which are isogenic for either *sigB* or *mgrA* (Table 2.1).

Removal of *sigB* in SH1000 (SH1000*sigB*::*tet*) did not significantly alter the mutation frequency in the absence of salicylate to ciprofloxacin (p=0.07), norfloxacin (p=0.43) or lincomycin (p=0.09) when compared to WT SH1000 (Table 2.3). However, removal of *sigB* significantly reduced the mutation frequency to ciprofloxacin with the addition of salicylate from 27-fold in SH1000 down to 8.5-fold in SH1000*sigB*::*tet* (p=0.02). Interestingly, the same effect was not seen with norfloxacin, where the mutation frequency in SH1000sigB::tet slightly but insignificantly increased to 7.2-fold compared to 5.2-fold in SH1000 (p=0.09). Although SAGAR was not observed for

lincomycin in SH1000, in SH1000*sigB::tet* salicylate decreased the mutation frequency to lincomycin by 0.02-fold (or a 50-fold reduction) (p=0.04). These findings emphasize a significant role for *sigB* in the salicylate associated genotypic antimicrobial resistance phenotype (SAGAR) phenotype.

For *mgrA* analysis, *S. aureus* strain Newman [110], and its *mgrA* null derivative (Table 2.1) were used to assess the potential contribution of *mgrA* to the SAGAR phenotype. In Newman, salicylate was shown to slightly increase mutation frequency to ciprofloxacin and norfloxacin (p=0.02 and p=0.01, respectively). However, the increase in ciprofloxacin mutation frequency was less than that observed for strains SH1000 or LAC (Table 2.3). Deletion of *mgrA* in the Newman background did not significantly alter mutation frequency in the presence of salicylate for ciprofloxacin (p=0.10) and norfloxacin (p=0.07) (Fig. 2.2). Although we did not observe a significant change in mutation frequency upon deletion of *mgrA* in the Newman background, we cannot rule out a potential role for *mgrA* in SH1000 as our results have indicated strain specificity for SAGAR.

Investigating the chemical signature associated with salicylate associated genotypic resistance to antibiotics

Salicylate-inducible phenotypic resistance to fluoroquinolones has been attributed to the carboxylic acid group of salicylate and acetyl salicylic acid [33], however the importance of this functional group, and others intrinsic to salicylate in SAGAR, is unknown. To identify chemical features of salicylate that contribute to SAGAR, the effect of the salicylate analogs sodium benzoate (weak acid) and acetylsalicylic acid

(NSAID) (Fig. 2.2) on mutation frequency to ciprofloxacin, norfloxacin and lincomycin resistance was determined.

Mutation frequency to ciprofloxacin resistance was significantly higher with salicylate when compared to sodium benzoate, acetylsalicylic acid (ASA), and controls ($p=0.03$) (Fig. 2.3). Benzoate and ASA differ structurally at the ortho position, the hydroxyl group of salicylate being reduced to hydrogen at this position in benzoate, whereas in ASA, this hydroxyl group is acetylated (Fig. 2.2). For lincomycin, a significant decrease in mutation frequency was observed for benzoate, salicylate, and ASA when compared to untreated cultures ($p<0.05$), but not between treatments (Fig. 2.3). Similarly, for norfloxacin, there was no significant difference in the mutation frequency of SH1000 to ciprofloxacin with salicylate when compared to benzoate, or salicylate compared to ASA, or benzoate to ASA ($p=0.07$, $p=0.06$, and $p=0.05$, respectively). Our results indicate that salicylate is needed to fully propagate the SAGAR phenotype for ciprofloxacin and norfloxacin.

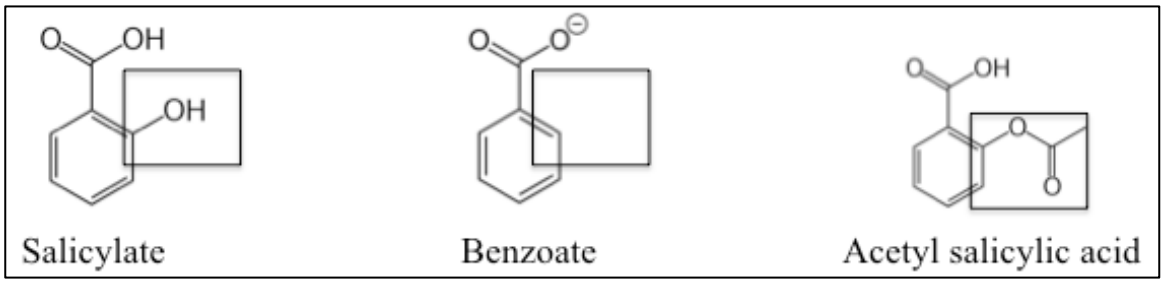

Figure 2.2. Structural differences between salicylate, benzoate and acetyl salicylic acid. Box indicates altered chemistry at the ortho-position.

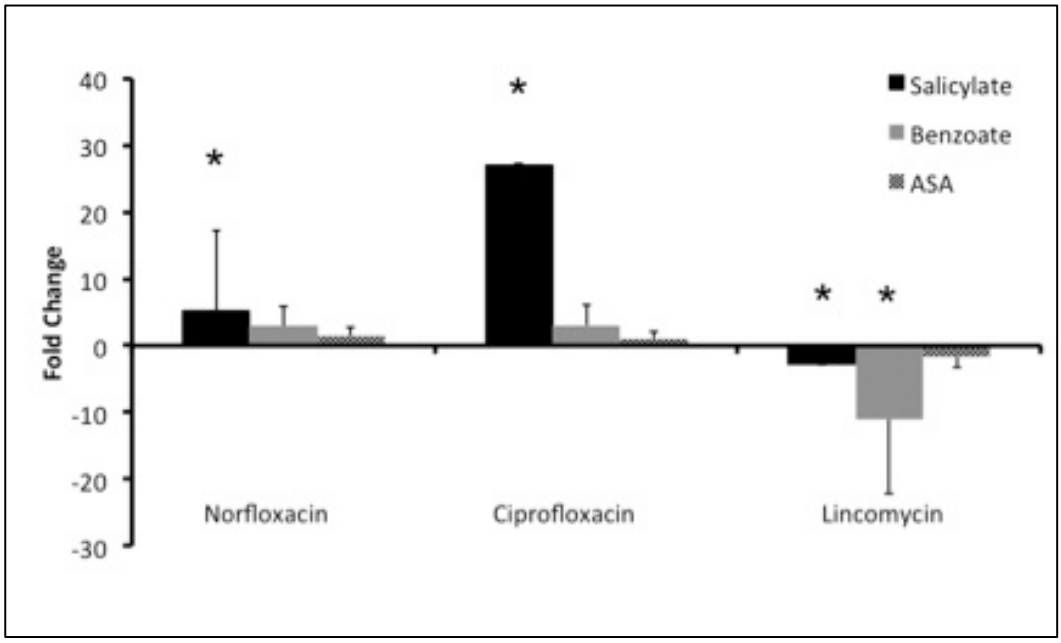

Figure 2.3. Dependence on salicylate chemical structure for SAGAR. Fold change in frequency of resistance to antibiotics determined for cultures selected in the presence of salicylate (black), benzoate (hatched), and acetyl salicylic acid (dotted). Error bars indicate standard deviation of the mean, and asterisks denote groups that differ significantly by ANOVA and Tukey's HSD (a=0.05, n=3).

2.4 Discussion

The results of this study revealed that the SAGAR phenotype for salicylate is drug specific. The salicylate associated antibiotic resistance (SAGAR) phenotype was only observed for the fluoroquinolones, ciprofloxacin and norfloxacin, in strain SH1000. The phenotype was more prominent with ciprofloxacin than with norfloxacin. This suggests that SAGAR is highly selective and sensitive to subtle changes in antibiotic structure. Ciprofloxacin and norfloxacin are structurally very similar in that both have fluorinated quinoloic acid cores; however there are differences. For example, ciprofloxacin has a cyclopropane ring at the N-1 position, while norfloxacin has an ethyl group at the same position [119]. In addition, studies by Chin *et al.* [120] found that ciprofloxacin was 4 to

32 times more active than norfloxacin. They proposed that the cyclopropane ring that ciprofloxacin possesses is able to alter the DNA gyrase activity, rendering it more efficient [120].

Ciprofloxacin resistance is a highly common occurrence in *S. aureus* strains [91, 121, 122], as such it is not indicated for infections and the clinical ramifications of SAGAR in *S. aureus* are currently benign. Quinolones, however, are the primary treatment option for urinary tract infections caused by *Escherichia coli* [123]. Despite the similarity in the quinolone resistance mechanism, this SAGAR phenotype has yet to be reported in other bacteria besides *S. aureus*. In order to determine the mechanism responsible for this SAGAR, and the significance of the structure of ciprofloxacin to the phenotype, more quinolones/fluoroquinolones need to be tested. Studying other bacterial species in which quinolones are still being used for therapeutic treatments can also further elucidate the mechanism.

Ciprofloxacin is associated with DNA damage, specifically through double stranded DNA breaks and stalled replication forks, which are processed to single-stranded DNA [88]. Looking at the global transcriptional response to ciprofloxacin treatment by Cirz *et al.* [88] revealed induction of Pol III and Y-family polymerases, which emphasizes a common strategy of reduced metabolism and funneling of resources into DNA synthesis in response to these antibiotics. It is also believed that DNA synthesis might be error prone due to the down-regulation of mismatch repair genes during exposure to ciprofloxacin [124, 125]. Therefore, it is possible that salicylate, when coupled with ciprofloxacin, exacerbates this effect, which may explain the drug specificity of SAGAR.

Through DNA sequencing of the ciprofloxacin targets, *grlAB* and *gyrAB,* our results revealed mutations only in the QRDR domain of *grlA*. No difference in the level of ciprofloxacin resistance was observed between LC and SCV colonies selected in the presence or absence of salicylate. However, for norfloxacin, a higher level of resistance was observed between LC selected in the absence of salicylate than those selected in the presence of salicylate. This indicates a mutation outside of the *grlA* QRDR in the salicylate treated colony that is accounting for this slightly decreased level of norfloxacin resistance. Determining the exact mutation responsible for this difference is not practical without resorting to whole genome sequencing [126-128].

S. aureus has been shown to result in heterogeneous expression of resistance to various antibiotics [133, 57, and 134]. For example, heterogeneous intermediate resistance to vancomycin (hVISA) is attributed to several genes related to cell regulation pathways including *vraSR, graSR saeSR,* and *agr* [129-132]. A similar heterogeneity has been observed by Price *et al.* [26] for salicylate inducible phenotypic resistance to fusidic acid. The level of fusidic acid resistance induced by salicylate was found to be dependent upon the genetic background of the strain.

It was thus no surprise when this study revealed that the SAGAR phenotype was strain specific. Expression of SAGAR varied substantially between strains in this study, as well as in previous studies for ciprofloxacin, ranging from a 8-fold to a 100-fold increase in mutation frequency [33]. This variation likely reflects the inherent genetic variation among these *S. aureus* strains and suggests that there are unknown genetic factors important for this phenotype.

One of the genetic factors important for this heritable phenotype is likely to be σB, as mutation of *sigB* significantly reduced expression of SAGAR for ciprofloxacin. σB plays a prominent role in the cell, controlling roughly 198 genes [79], many of which are involved in resistance to stressors [133]. σB has also been shown to be involved in the repression of exoproteins and toxins, and is a positive regulator of adhesion factors [79, 80, 103, 133]. More importantly, σB is believed to play a role in mediating antibiotic resistance [79]. Inactivation of *sigB* in MRSA-COL was found to increase its susceptibility to methicillin [98] while mutations within the *rsbU*-defective strain BB255, leading to SigB hyperproduction, were associated with an increase in glycopeptide resistance [134]. σB has been shown under stress, to upregulate genes responsible for maintaining cell integrity, membrane transport processes and intermediary metabolism [118]. Thus, it is possible that salicylate in σB null strains is able to permeate the cell more readily, allowing more salicylate to enter the cell, further enhancing its toxicity and mitigating SAGAR.

S. aureus BB255, a ciprofloxacin sensitive strain carrying an 11-bp deletion in *rsbU* encoding a positive regulator of σB [33, 135, 136], demonstrated a 100-fold increase in mutation frequency to ciprofloxacin with salicylate [33]. SH1000 differs from BB255 in that SH1000 is *rsbU*+, and has increased pigmentation, more vigorous growth, decreased secreted exoproteins and decreased *agr* expression. Thus, perhaps differences in SAGAR for ciprofloxacin between strains reflect differences in sigma B activity. However, the frequency of mutation to ciprofloxacin resistance for SaRNH-1 (SH1000sigB::tet) was actually less than 50% of that observed for SH1000, and less than 10% that of BB255. Despite the 11-bp deletion in the *rsbU* gene, researchers have shown salicylic acid to

activate σB in both *rsbU*+ and rsbU null strains [137]. Therefore, it is possible that σB positively influences SAGAR while *rsbU* negatively influences SAGAR. Our results indicate σB dependence for full expression of the SAGAR phenotype.

The use of salicylate analogs to investigate the importance of chemical structure in the phenotype revealed the importance of the ortho-hydroxyl group of salicylate. Sodium benzoate lacks this hydroxyl group, while acetylsalicylic acid has an acetyl group in the ortho position. Removal of the hydroxyl group mitigated SAGAR for both ciprofloxacin and norfloxacin. While for lincomycin removal of the hydroxyl group (i.e. by use of the structural analog benzoate) potentiated the effect salicylate had on reducing the frequency at which resistance occurred. Interestingly, the reactive group of salicylate for inducing oxidative stress in mitochondria has been shown to be this hydroxyl group [138], which is believed to interact with a Fe-S cluster of mitochondrial Complex I resulting in the production of ROS [14, 15, 82, 138]. A structural dependence was also observed for salicylate-inducible phenotypic resistance to ciprofloxacin [33]. However, the functional group was determined to be the carboxylic acid [33], which suggests a potential mechanistic distinction between inducible phenotypic resistance and SAGAR. The results suggest that this hydroxyl group is important for SAGAR, which could possibly lead to ROS accumulation. This ROS accumulation could result in DNA damage, which could explain the increase in mutation frequency.

Finally, our results indicate both inducible phenotypic resistance (data not shown) and SAGAR require the presence of salicylate in the media for the phenotype, which suggests a mechanistic link. It is possible that salicylate has a physiochemical effect on the cell which leads to metabolic toxicity or that salicylate is a ligand for a yet to be

determined protein which can both affect antibiotic permeability results in interference with DNA repair mechanisms.

CHAPTER THREE:

ROLE FOR METABOLIC STRESS IN THE SALICYLATE-ASSOCIATED ANTIBIOTIC RESISTANCE PHENOTYPE

3.1 Background

Metabolic alterations play an important role in resistance of bacteria to antimicrobials [139]. Changes in growth rate have been shown to influence bacterial susceptibility to antibiotics [139]. For example, metabolically moribund *E. coli* have been shown to be highly resistant to ampicillin or tetracycline, and many antibiotics have reduced activity against stationary phase cultures [140]. Also, changes in bacterial metabolism, such as those associated with dormancy or biofilm formation, are associated with reduced susceptibility to antibiotics [141]. Alterations in metabolism in response to antimicrobials have been also linked to small colony variant (SCV) formation in *S. aureus* [142]. SCVs display a decreased rate of cell wall biosynthesis, a reduction in the uptake of positively charged antimicrobials and an increase in survival in host cells [142]. These reductions are associated with a 4-fold increase in MICs to cell wall targeting antibiotics [142].

Growth with salicylate induces phenotypic resistance to antimicrobials, and increases the frequency at which genotypic resistance (SAGAR phenotype) to some antibiotics occurs in *S. aureus* [7, 26, 33, 43, 70, 71]. These phenotypes occur at

concentrations of salicylate which are subinhibitory, but which are still toxic to growth. Previous studies revealed substantial alterations in the transcript levels of genes associated with central metabolism during growth with sub-MIC levels of salicylate [43]. Specifically, salicylate reduced the expression of genes important for glycolysis/gluconeognesis such as *gapA2* (encoding GAPDH) and *pgi* (glucose-6-phosphate isomerase). In addition, salicylate increased expression of genes for gluconate metabolism via the pentose phosphate shunt. This indicates that growth with salicylate at concentrations that induce phenotypic resistance, and which are associated with SAGAR, may inhibit glycolysis and increase gluconate metabolism [43]. Furthermore, these findings suggest that *S. aureus* may alter flux through metabolic pathways to counter the toxic effects of salicylate. In support of this claim, growth with gluconate was shown to rescue the cell from the toxic effects of salicylate on growth [43]. Salicylate has been shown to also result in ROS accumulation and DNA damage in eukaryotic models [5, 7, 82]. Specifically, salicylates have damaging effects on isolated mitochondria and have been shown to result in uncoupling of oxidative phosphorylation as well as swelling [138]. It is possible that salicylate has the same DNA damaging effects which can ultimately lead to this increase in mutation frequency; i.e SAGAR. Collectively, these studies reveal that salicylate may act at several levels to negatively impact metabolism in the cell. The importance of these metabolic alterations in the presence of salicylate to antibiotic resistance in *S. aureus* is currently unknown. The following experiments were designed to examine the link between the metabolic toxicity of salicylate and the SAGAR phenotype.

3.2 Methods

Generation Time Determination

The following protocol was adapted from Neoh *et al.*[132]. An overnight culture of strain SH1000 was prepared under standard conditions before being subcultured into fresh TSB to an optical density at 600 nm OD_{600}=0.05. Optical density readings were recorded from an OD_{600}=0.05 to an OD_{600}=0.5. Cells were treated with increasing concentrations of NSAID, a weak acid, or for controls, an equal volume of water (or respective solvent used for stocking NSAIDs/weak acids). Samples or plate counts (CFU/ml) were taken every 30 min. Generation times were determined using Eq. 3.1 (below), where *g* is generation time, N_1 is optical density at which the last reading was taken, N_0 is the initial optical density taken and t_1 is the time in minutes from the initial reading ($t_{0)}$) to reach an optical density of 0.5. Data was analyzed using a t-test to identify statistically significant differences between controls and treatment.

$$\text{Eq. 3.1: } g = \frac{\log(N1N0)}{t1-t0}$$

Mutation frequency determination

The following protocol was adapted from Foster, 2006 [108]. This protocol is identical to that of Chapter 2 Methods, section 2.2, except that mutation frequency to the antibiotic was determined over a range of NSAID and weak acid concentrations. For mutation frequency determinations under anaerobic conditions, cultures were grown in anaerobic chambers with CO_2 packs (EZ container system, Becton, Dickson and company) and incubated at 37°C.

Metabolite profiling

The following protocol was adapted from Meyer *et al.* 2010 [143]. Overnight cultures (n=3) of *S. aureus* strain SH1000 were prepared under standard conditions. The following day the culture was inoculated into 100 ml fresh TSB at OD_{600}=0.05. This culture was grown at 37°C at 448 x g until an OD_{600}=0.5 was reached. At this point either 500 μg/ml or 2500 μg/ml of salicylate was added to the experimental flask or the equivalent volume of water was added to the control flask and cultured for 30 min. From each flask, 20 ml of culture was extracted and then filtered through a 0.22 μm Millipore filter. Cells were then washed twice with 10 ml cold 0.6 % (wt/vol) NaCl. The filter was then cut out and placed into a corning tube containing 10 ml of ice cold 60% (vol/vol) ethanol. The corning tubes were vortexed for 10 minutes to ensure that all the cells came off the filter. The filter was then discarded and the corning tubes were stored in the -80°C freezer for an hour. Cells were then thawed on ice, while being rigorously mixed and shaken 10 times alternately. Aliquots of 1 ml cell suspensions were transferred into an appropriate number of tubes containing 0.5 cm^3 glass beads (diameter 0.10-0.11 mm). Cells were disrupted for 2 cycles for 30 seconds at 5,179 x g in a bead-mill (Mini-bead beater, Biospec). After cell disruption the glass beads and the cell debris were separated from the supernatant by centrifugation for 5 min at 4°C and 10,000 x g. The aliquoted samples were combined, and the glass beads were washed once with 1 ml ddH_2O each. Washing entailed vortexing of the beads in the water and centrifugation for 5 min at 4°C and 10,015 x g. The washing solutions were added to the combined samples. The supernatant including the metabolites were brought to a final ethanol concentration of 10 % (vol/vol) and stored at -80°C. The metabolites were shipped on dry ice to the

University of Illinois for analysis. The metabolites were analyzed separated by liquid and gas chromatography and analyzed by mass spectrometry at the Metabolomics Center, Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign (http://www.biotech.uiuc.edu/centers/MetabolomicsCenter/index.html).

Analysis of intracellular metabolites

Metabolite peaks were identified by comparison to a spectrum generated from standards. The means and standard deviation were calculated for each treatment. An ANOVA was performed comparing 0 μg/ml, 500 μg/ml and 2500 μg/ml salicylate (a=0.05, n=3). A 2- fold change was used as a cutoff for biological significance. Those in which statistics and biological significance were satisfied were further characterized based on metabolic pathway. The online software Metaboanalyst was used to identify pathways (http://www.metaboanalyst.ca/MetaboAnalyst/faces/Home.jsp).

Reactive oxygen species assay (ROS)

ROS was measured using luminol and DCFH-DA. Overnight cultures of *S. aureus* strain SH1000 (n=3) were prepared under standard conditions. The following day the culture was diluted into fresh TSB at a 1:100 to obtain OD_{600}=0.05. The culture was allowed to grow in a shaking incubator at 37°C at 448 x g to an OD=0.5. The appropriate volumes of salicylate were added to obtain a final concentration of 500 and 2500 μg/ml. These treatments served as the experimental conditions. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) was added to a final concentration of 100 μM, which served as a positive control, and glycerol at 0.4% (vol/vol) as a negative control. An

additional treatment, which included only cells grown in TSB, served as a control for basal ROS levels. Cells were exposed to each treatment for a total of 24 hours, and readings were taken at 0.5 h, 3 h, 8 h, and 24 h. At each time point 1 ml of culture was placed into a 1.5 ml microtube and centrifuged at 2,500 x g for 15 min. The supernatant was decanted and the cells were resuspended in 500 µl of phosphate buffered saline (PBS, pH 7.1).

For DCFH-DA, the following protocol was adapted from OxiSelect ROS Kit (Cell Bio Labs, California) and performed per the manufacturer's instructions. To each sample, 2',7'-dichlorfluorescein-diacetate (DCFH-DA) was added to a final concentration of 10 µM. The samples were vortexed and incubated in the dark at 37°C for 5 minutes. From each tube, 100 µl was added in triplicates to a 96 well plate. Luminol was used for ROS detection following previously described methods (Chen *et al.* 2011). Briefly, for each treatment, 100 µl of the prepared sample was added in triplicate to a 96 well plate. To each prepared well 100 µl of 250 µM luminol was then added. A synergy2 plate reader with Gen5 software (BioTek, Vermont) was used to record fluorescence. Fluorescence was recorded at 460/40 emission and 360/40 excitation. The following protocol was performed with a sample size of 3. Optical density for each treatment at each time interval was recorded. Relative fluorescence units recorded were adjusted to OD. Statistical significance between treatments was compared using an ANOVA (a=0.05, n=3) (R ver 2.13.0).

NAD^+/NADH Assay

To conduct a standard curve for NADH/NAD quantitation, 10 μl of a 1 nmol/μl NADH standard (Biovision, California) was diluted with 990 μl NADH/NAD Extraction Buffer to generate 10 pmol/μl standard NADH. Of the diluted NADH standard, 0, 2, 4, 6, 8, 10 μl were added into labeled 96-well plate in duplicates, resulting in a dilution series of 0, 20, 40, 60, 80, 100 pmol/well. The final volume was adjusted to 50 μl with NADH/NAD extraction buffer (Biovision). Readings were taken using a plate reader at OD_{450}. The standard curve was analyzed for strength of linearity by regression analysis (R). For NADH/NAD quantitation, an overnight culture was prepared in TSB with *S. aureus* strain SH1000. The following day 1 ml of cells was washed with 500 μl cold PBS and pelleted at 448 x g for 5 min. To each sample, 400 μl of NADH/NAD extraction buffer was added followed by two cycles of freeze/thaw (20 min at -80°C, then 10 min room temperature). The cells and buffer were then vortexed for 10 sec and pelleted at 21,952 x g for 5 min. The extracted NADH/NAD supernatant was transferred into a labeled tube. This was performed before addition of salicylate or CCCP, and 3 h following addition of 1 μg ciprofloxacin, 500 μg/ml salicylate, 2500 μg/ml salicylate or an equal volume of water (control).

To detect total NAD (NAD^+ + NADH) 50 μl of the extracted samples were transferred into a 96-well plate in duplicates. An NAD cycling mix was prepared for each reaction by adding 100 μl of NAD cycling buffer and 2 μl of NAD cycling enzyme mix. One hundred microliters of the NAD cycling mix was added to each well and incubated at room temperature for 5 min. Ten microliters of NADH developer was added to each well and incubated at room temperature for 1 hr. Readings were taken using a plate

reader at OD_{450}. NADH was detected by adding 200 µl of the extracted samples into Eppendorf tubes. The samples were then heated to 60°C for 30 min. This step decomposed all NAD^+. The samples were then cooled on ice and vortexed to remove precipitates. Fifty microliters of the NAD^+ decomposed samples were transferred into 96-well plates in duplicates. One hundred microliters of the NAD cycling mix was added to each well and incubated at room temperature for 5 min. Ten microliters of NADH developer was added to each well and incubated at room temperature for 1 hr. Readings were taken using a plate reader at OD_{450}. Statistical significance was determined between treatments using an ANOVA (a=0.05, n=3) (R ver 2.13.0). The sample readings were applied to the NADH standard curve with the following equation X=(Y+0.0527)/0.3071. The amount of NAD^+ or NADH in the sample wells were calculated then divided by OD_{600} of the culture prior to extraction.

Eq3.2: NAD/NADH Ration is calculates as: $\frac{\text{NADtotal-NADH}}{\text{NADH}}$

3.3 Results

Dose-dependence of salicylate-associated genotypic antibiotic resistance

To determine the relationship between salicylate, sodium benzoate, and acetylsalicylic acid growth toxicity and the salicylate associated genotypic antibiotic resistance (SAGAR) phenotype, generation time (g) and mutation frequency (μ) were calculated for a wide range of salicylate, sodium benzoate, and acetylsalicylic acid concentrations. Salicylate was only shown to significantly impair growth at 2500 µg/ml,

increasing *g* from 29.7 to 48.8 min (p=0.002) (Fig. 3.1A). For Asa, this was slightly less at 2000 μg/ml, increasing g to 54 min (p=0.002) (Fig. 3.1C), and benzoate was the least growth toxic, requiring 5000 ug/ml to significantly inhibit growth, increasing g to 45.7 min (p=0.012) (Fig. 3.1E).

For Sal, Asa, and Ben, SAGAR was determined to be concentration-dependent, and only occurred at non-growth-toxic concentrations. For Sal, SAGAR was observed at 50-1000 ug/ml, but was absent at 2500 ug/ml (Fig. 3.1B). Likewise, for Asa and Ben SAGAR was observed at 50-500 μg/ml and 50-1000 ug/ml, but absent at 2000 μg/ml and 5000 μg/ml, respectively (Fig 3.1 D and F). Thus, the expression of the SAGAR phenotype is suppressed at growth-toxic concentrations of Sal, Asa and Ben.

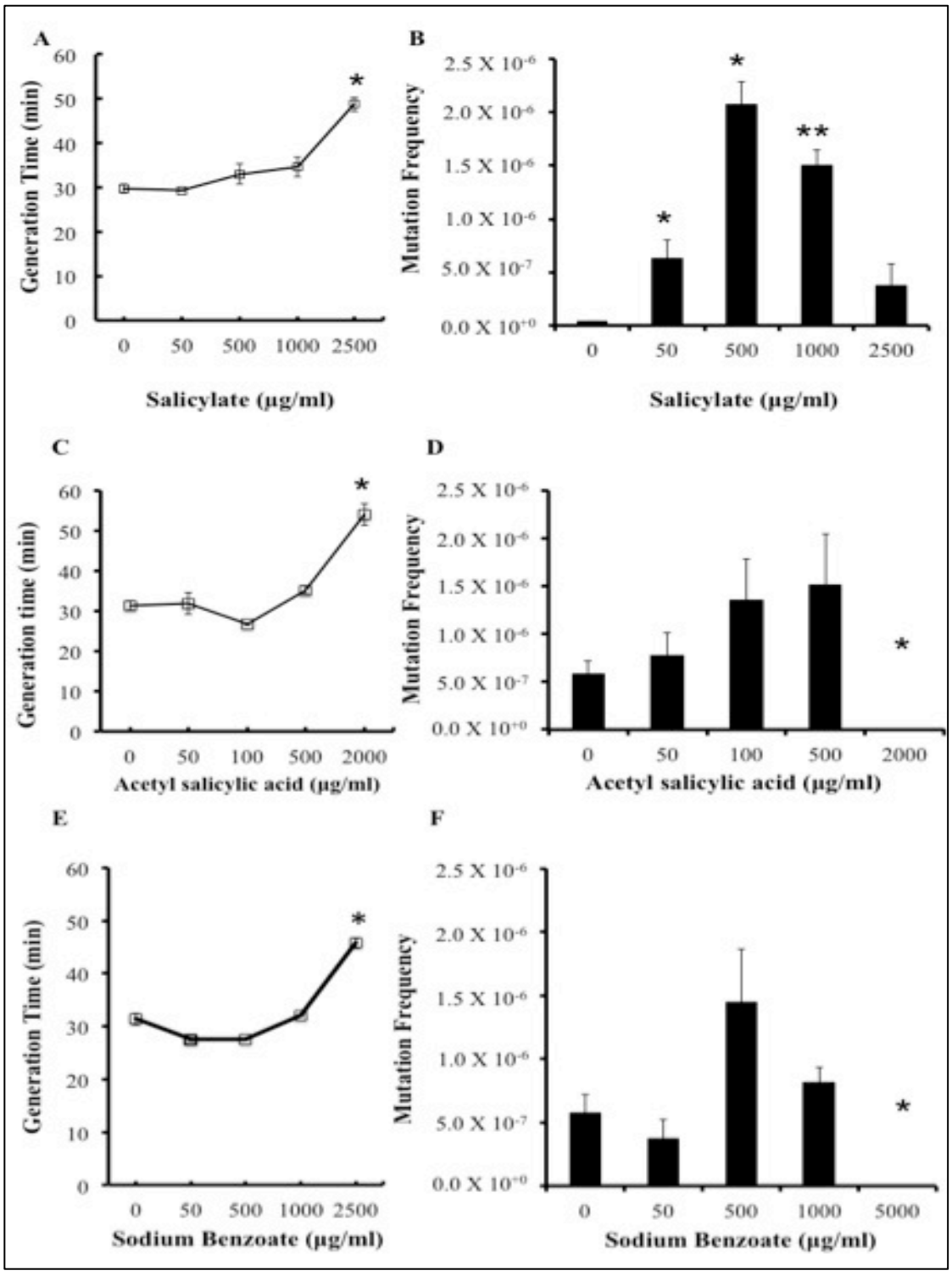

Figure 3.1. Dose dependency of salicylate, aspirin, and benzoate for increased frequency of resistance to ciprofloxacin. Generation time of SH1000 as a function of increasing concentrations of salicylate (panel A), aspirin (panel C) and benzoate (panel E). (**B**) Mutation frequency of SH1000 to ciprofloxacin resistance plotted against an increasing concentration of salicylate (panel B), aspirin (panel D) and benzoate (panel E). Asterisks denote statistical significance using a t-test (*$p<0.05$).

Metabolite profile of S. aureus grown in the presence of salicylate

To ascertain the metabolic alterations associated with salicylate exposure, metabolite profiling was performed for *S. aureus* strain SH1000 exposed to nontoxic (500 μg/ml) and growth-toxic (2500 μg/ml) concentrations of salicylate, which select for genotypic resistance, or are non-selective, respectively. Metabolite profiling revealed a total of 153 altered metabolites among the three treatments (Table 3.1). Of the 153 altered metabolites 88 were less abundant, 3 had no change, and 44 were more abundant for the 2500 μg salicylate treatment compared to the control treatment. Also, 78 metabolites were less abundant, 3 had no change, while 61 were more abundant for the 500 μg/ml salicylate treatment compared to the control. Between the 2500 μg/ml and 500 μg/ml salicylate treatments 82 metabolites were less abundant, 4 had no change and 53 were more abundant for the 2500 μg/ml treatments when compared to the 500 μg/ml treatment.

Metabolite profile revealed a decrease in TCA metabolites at the 500 μg/ml salicylate treatment, as seen through a reduction in citric acid (0.4-fold), acontic acid (0.2-fold), α-ketoglutaric acid, (0.4-fold) and fumaric acid (0.6-fold) in relation to non-salicylate treated cultures, respectively (Table 3.1). A 2.1 fold accumulation of 2-phosphoglycerate was also observed in the 500 μg/ml salicylate treatment when compared to the 2500 μg treatment ($p=0.04$) (Table 3.1). Previously a study by Riordan *et al.* 2007 [43] showed downregulation of *gapA2* encoding a glyceraldehyde-3-phosphate dehydrogenase, *pgi* encoding a glucose-6-phosphate isomerase, and SACOL1838 encoding a phosphoenolpyruvate carboxykinase. Each is indicative of reduction in glycolysis. Accumulation of 2-phosphoglycerates revealed further

downregulation of glycolysis as this gene is involved in the final step of converting glucose to pyruvate. This is also corroborated in eukaryotic systems, specifically as seen in mitochondria [84]. A 8.8-fold increase in 2-ketogluconic acid compared to non-salicylate treated cultures was observed at the 500 μg/ml treatment as well (p=0.023) (Table 3.1). The *S. aureus* gluconate operon *gntRKP* was shown to be upregulated with salicylate stress [43]. This increase in gluconate utilization gene expression may reflect an important metabolic alteration necessary to compensate for the growth inhibitory effects of salicylate. This effect on gluconate metabolism genes was also observed at the 2500 μg/ml salicylate treatment (p=0.00019), with a 6.7-fold increase in levels compared to the non-salicylate treated treatment (Table 3.1). Interestingly, an accumulation of lactic acid was observed with salicylate treated cultures, which is indicative of anaerobic fermentation (p=0.035) [145]. In addition, butanediol increased by 4-fold in the presence of salicylate (Table 3.1). Lactic acid and butanediol production are used by *S. aureus* to cope with acid-stress [146], suggesting that perhaps growth with salicylate leads to acidification of the cytoplasm. For example, to avoid further acidification of an already acidic internal environment pyruvate is metabolized via acetolactate or the diacetyl pathway to butanediol. It is believed that *S. aureus* increases pH through accumulation of ammonium and through the removal of acid groups, which results in the production of 2,3-butanediol [146]. Also, the accumulation of acid may aid in stress as lactic acid was initially synthesized from pyruvate, which can then be oxidized further by the TCA cycle [141].

Two important mechanisms for increasing pH in *S. aureus* cultures which are acid stressed is through the production of ammonia by urease and removal of acids [146]. An

increase in urea was observed in the presence of 500 μg/ml salicylate by 1.8-fold (P=0.0026) (Table 3.1). A dose dependent effect was observed between the 500 and 2500 μg/ml treatments. Specifically, a 5-fold increase in uracil was observed at the 500 μg/ml treatment when compared to the 2500 μg/ml treatment indicating acid-stress. Also, 2-phosphoglycerate was observed to be 2-fold higher at 500 μg/ml than 2500 μg/ml indicating impairment in glycolysis. Butanediol was 4-fold higher at the 2500 μg/ml treatment than the 500 μg/ml treatment again indicating acid stress.

Table 3.1. Metabolite profile for *S. aureus* grown with salicylate

Metabolite	**Salicylate concentration** (μg/ml)		**Confidence Interval (CI)**	
	Relative Abundance			
1,2-Benzenedicarboxylic acid	**2500**	**500**	**2500**	**500**
1-Ethylglucopyranoside	2.3	2.6	1.11	0.39
1-Methyl-beta-D-galactopyranoside	NA	1.1	NA	NA
1-Monooctadecanoylglycerol	0.4	0.7	NA	0.16
2(1H)-Pyrimidinone, 1-D-ribofuranosyl-4-hydroxy-5'-p	1.4	1	0.38	0.38
2,3-hydroxybutane	0.4	0.5	0.98	0.38
2,3-hydroxysuccinic acid	1.2	1.3	0.15	0.1
2,4,6-hydroxypyrimidine	1.3	1.1	0.33	0.33
2,4,6-Tri-tert.-butylbenzenethiol	0.5	1.5	0.38	0.22
2,4-hydroxybenzoic acid	1.7	1.7	0.21	0.69
2,4-hydroxybutanoic acid	3.3	2.7	0.65	0.98
2-Amino-4,6-dihydroxypyrimidine	1	0.9	0.65	0.65
2-Aminobutyric acid	1.2	1.7	0.15	0.35
2-aminoethylphosphoglycerate	NA	0.5	0.37	0.68
2-Furancarboxylic acid	0.5	NA	NA	0.15
2-hydroxybenzoic acid	0.7	0.9	0.34	NA
2-hydroxybutanoic acid	528.2	166.6	0.16	0.16
2-Hydroxyglutaric acid	0.9	1	306.07	176.77
2-Ketogluconic acid	0.6	0.8	0.31	0.12
2-methyl-2,3-hydroxypropanoic acid	6.7	8.8	0.12	0.29
2-methyl-2-hydroxybutanoic acid	0.7	0.7	10.43	7.36
2-methylbenzoic acid	1.3	1.7	0.03	0.24
2-oxo-3-hydroxypropanoic acid	1.9	1.9	0.95	1.52
2-oxophosphoglycerate	0.8	0.6	0.38	0.39
2-phosphoglycerate	0.9	1.1	0.57	0.36
3,4,5-Trihydroxypentanoic acid	0.1	0.3	0.32	0.12
3,4-Dihydroxybutanoic acid	2.4	4.7	0.03	0.11
3-methyl-3-hydroxybutanoic acid	1.2	2.1	0.2	2.72
3-phosphoglycerate	1.3	1.4	0.14	1.32
4,5-dimethyl-2,6-hydroxypyrimidine	1	1.1	0.2	0.21
4-Hydroxybutanoic acid	0.4	0.2	0.3	0.46
aconitic acid	0.9	1.3	0.29	0.15

Table 3.1 continued

Metabolite	**Salicylate concentration** (μg/ml)		**Confidence Interval (CI)**	
	Relative Abundance			
Adenosine	NA	0.3	0.13	0.12
Adenosine-5-monophosphate	0.2	0.6	NA	0.07
a-Glycerophosphate	0.6	0.8	0.12	0.09
Agmatine	0.4	1.1	0.14	0.16
a-ketoglutaric acid	0.5	0.4	0.1	0.1
Alanine	0.5	0.4	0.09	0.11
Aminomalonic acid	0.8	0.5	0.15	0.11
arabitol	0.1	0.4	0.19	0.07
Asparagine	0.9	1.2	0.18	0.21
Aspartic acid	0.3	0.3	0.04	0.28
B-alanine	1.1	1.2	0.11	0.14
Benzoic acid	1.3	1.2	0.2	0.21
Butylamine	1.3	1.4	0.45	0.07
citric acid	1.1	1	0.16	0.21
Diethyleneglycol	0.4	0.4	0.33	0.15
digalactosylglycerol	1.1	1.3	0.08	0.11
Eicosanoic acid	0.6	1	NA	NA
erythronic acid	1.6	1.3	0.26	0.39
ethanolamine	1	1.3	0.09	0.48
Ethyl phosphoric acid	0.9	1	NA	NA
Ferulic acid	0.4	0.8	0.76	0.45
fructose	1	0.7	0.14	0.44
Fructose-6-phosphate	0.2	0.3	0.25	0.55
Fumaric acid	1	2.3	0.09	0.51
Galactaric acid	0.7	0.6	0.23	0.04
galactose	0.3	0.2	0.13	0.21
Glucaric acid	0.5	0.7	0.24	1.28
Glucoheptulose	0.2	0.3	0.1	0.15
Gluconic acid	0.5	0.9	0.13	0.09
glucose	1.6	1.6	0.18	0.11
glucose-6-phosphate	0.2	0.5	0.07	0.17
Glutamic acid	0.4	0.3	0.25	0.65
Glutaric acid	0.3	0.5	0.79	0.67
galactose	0.3	0.2	0.13	0.21
Glucaric acid	0.5	0.7	0.24	1.28
Glucoheptulose	0.2	0.3	0.1	0.15

Table 3.1 continued

Metabolite	**Salicylate concentration** (mg/ml)		**Confidence Interval (CI)**	
	Relative Abundance			
Gluconic acid	0.5	0.9	0.13	0.09
glucose	1.6	1.6	0.18	0.11
glucose-6-phosphate	0.2	0.5	0.07	0.17
Glutamic acid	0.4	0.3	0.25	0.65
Glutaric acid	0.3	0.5	0.79	0.67
Glyceric acid	0.8	1	0.11	0.34
Glycerol	0.9	0.8	0.22	0.11
Glycine	1	2.3	0.16	0.36
glycolic acid	0.5	0.5	NA	NA
Glycopyranose	0.9	1.1	0.04	0.23
Guanine	0	0.2	0.32	0.22
Hexadecanoic acid	1.5	0.9	0.93	1.75
Hexanoic acid	1.2	1	0.47	0.1
Hydroxylamine	0.2	0.7	0.69	0.26
Hydroxyphosphinyloxy-acetic acid	0.7	1.1	0.87	0.36
Hydroxyproline	1.2	1.1	0.69	0.53
Inositol, chiro-	0.8	1.4	0.29	0.33
inositol, myo-	0.7	0.8	0.06	0.25
Inositol, scyllo-	0.6	0.7	0.11	0.44
isoleucine	NA	1	NA	NA
Isoxanthopterin	0.7	0.7	0.3	1
lactic acid	3.2	0.9	0.13	0.16
lactose	1.7	1.1	0.31	0.18
leucine	0.7	0.3	NA	0.68
lysine	0.6	1.2	0.09	0.3
Maleic acid	0.6	0.3	3.4	0.16
Malic acid	1.1	1.2	0.7	0.05
Malonic acid	0.4	0.6	0.25	0.14
Mannitol	1	0.8	0.19	1.47
mannose	1.7	1.8	0.29	0.2
mannose-6-phosphate	2	1.1	0.33	0.37
Melibiose	0.9	0.9	0.17	0.49
methionine	1	1.2	0.15	0.23
Methylmalonic acid	0.6	0.7	0.55	1.18
Monomethylphosphate	NA	1.1	1.34	0.05
N-Acetyl aspartic acid	1.2	1	0.38	0.45
N-Acetylglutamic acid	0.7	1.1	0.62	0.82

Table 3.1 continued

Metabolite	**Salicylate concentration** (mg/ml)		**Confidence Interval (CI)**	
	Relative Abundance			
N-Acetyl-Lysine	0.6	0.9	0.4	0.16
N-Acetyl-serine	0.7	0.6	NA	0.54
Nicotinic acid	1	1	0.3	0.54
Nonanoic acid	0.9	0.8	0.5	0.13
Octadecanoic acid	1.1	0.9	0.33	0.04
Octadecanol	1.5	1.3	0.21	0.2
ornitine	0.8	0.9	0.32	0.11
Orotic acid	0.2	0.5	0.46	0.09
oxalic acid	1	0.8	0.63	0.57
Panthotenic acid	0.5	0.5	0.99	0.45
phenylalanine	0.6	0.6	0.11	0.11
phosphoric acid	0.6	0.7	0	0.54
Pinitol	0.6	0.6	NA	0.22
Pipecolic acid	0.8	1.2	0.56	0.37
Pyroglutamic acid	1.2	1.9	0.24	0.09
pyrophosphate	0.6	0.5	0.12	0.28
Pyrrole-2-carboxylic acid	3	1.9	NA	NA
pyruvic acid	0.5	1.3	0.13	0.32
ribitol	0.7	1.1	0.48	0.04
ribose	0.8	1	0.28	0.39
ribose-5-p	1.3	2.3	0.2	1.13
Sedoheptulose	0.9	1	0.15	0.2
serine	0.8	0.8	2.26	0.59
Sorbitol	0.6	0.6	NA	0.33
sorbose	0.5	0.8	0.15	0.21
Succinic acid	0.6	1	0.6	0.52
sucrose	1	1	0.51	1.7
Threonic acid	0.3	0.2	0.17	0.41
Threonine	4.4	5.2	0.1	0.06
Thymine	0.9	1.1	0.35	0.25
Trehalose	0.5	0.5	0.55	0.11
Tryptophan	0.5	0.2	0.18	0.12
tyrosine	0.5	0.3	0.32	0.43
Uracil	0.7	0.5	0.09	0.16

Table 3.1 continued

Metabolite	**Salicylate concentration** (mg/ml)		**Confidence Interval (CI)**	
	Relative Abundance			
Uridine	1.2	0.7	NA	NA
valine	0.4	0.8	NA	NA
vanillic acid	0.7	0.8	6.49	3.17
xylitol	1	1	0.14	0.7

All values mean fold change and are relative to the control, non-salicylate treated treatment.
Not recorded (NA).

The effects of salicylate on the TCA cycle

Metabolite profiling revealed down regulation of glycolysis as well as the TCA cycle. We therefore wanted to determine the effect of salicylate on the TCA cycle. These findings correlated to a significant increase in levels of NAD^+ in cells treated with 2500 μg/ml salicylate for 3 hours with a p=0.036 (Fig. 3.2). Ciprofloxacin, a positive control, has been shown to result in an increase in NAD^+ levels [89]. No increase in NAD^+ levels was detected at the 500 μg/ml treatment of salicylate. Oxygen dependence has been linked to downregulation of the electron transport chain and the TCA genes and can be seen through a decrease in NADH levels (Fig. 3.3) as well as the buildup of lactic acid and butanediol as we have seen through the metabolite profile (Table 3.1) [145].

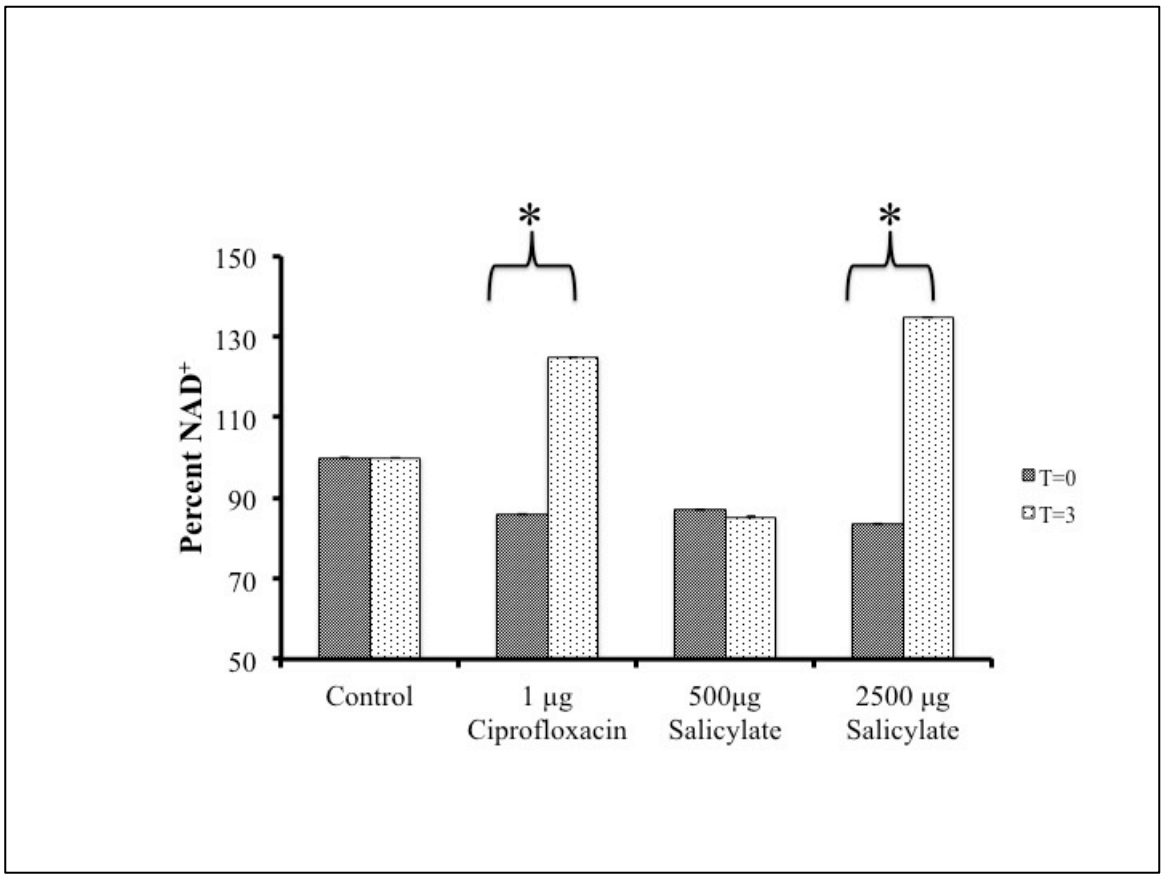

Figure 3.2. Effect of salicylate on cellular NAD^+ levels. Mean ($n \geq 3$) percent nicotinomide adeninine dinucleotide (NAD^+) plotted for ciprofloxacin and different salicylate concentrations, at time 0 (filled) and 3 h after treatment (stippled). Asterisks denotes statistical significance $p<0.05$ when compared to identical treatment at time 0.

Oxygen dependence on the SAGAR phenotype

Oxygen has been shown to play an important role in the persistence and overall growth of *S. aureus* in different conditions [145]. We were interested in determining the oxygen dependence on the genotypic heritable phenotype. As shown in Fig. 3.3, in the presence of oxygen there were 6.5-fold more cells recovered on 1 µg cipro plus 500 µg/ml salicylate plates when compared to the number of cells recovered in the absence of oxygen, 2.6×10^{-6} to 3.9×10^{-7}. This shows an inverted phenotype in the absence of oxygen, which implies a dependence on oxygen or aerobic growth for this phenotype to

be expressed. On the other hand, at 2500 μg/ml of salicylate and 1 ug ciprofloxacin, there was no difference observed between treatments. This indicates that the effect observed at 2500 μg/ml of salicylate is linked to toxicity rather than oxygen availability (Fig 3.3). Also, the SAGAR phenotype was recapitulated in that the addition of 500 μg/ml salicylate increased frequency of ciprofloxacin resistant isolates by 10.3-fold when compared to without salicylate aerobically.

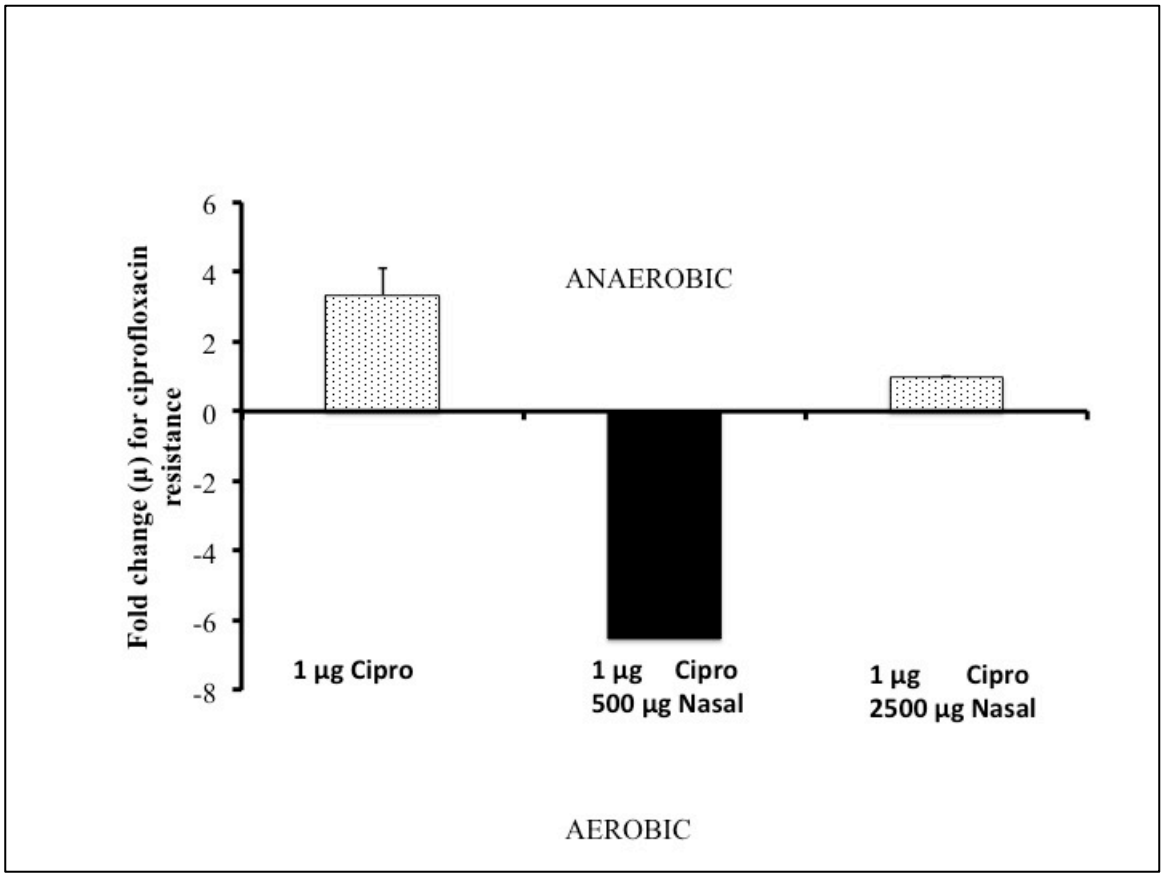

Figure 3.3. Expression of SAGAR during anaerobic growth. Mean (n=3) fold change in the frequency of mutation to CipR for anaerobic cultures relative to aerobic cultures plotted for growth on ciprofloxacin (control), and ciprofloxacin with 500 μg/ml or 2500 μg/ml salicylate.

Role for reactive oxygen in the SAGAR phenotype

Salicylate has been shown to result in reactive oxygen species (ROS) accumulation in mitochondria [82, 147]. ROS damages DNA resulting in oxidation of

guanine to 8-oxo-7, 8-dihydro-guanine, which can result in mutations [148]. To gain further insight into the role for ROS in the SAGAR phenotype, ROS levels were recorded using either DCFH-DA (Fig. 3.4 A) or luminol (Fig. 3.4 B). ROS levels from salicylate stressed cells were compared to levels recorded from 100 μM CCCP, a positive control, and the control treatment, which were SH1000 cells grown in the absence of any stressor. A statistical significance with a $p<0.05$ was only observed with the positive controls (Fig. 3.4 AB). Specifically, a 2.7-fold and 6-fold increase in ROS was observed for CCCP when compared to the control for DCFH-DA and luminol, respectively. However, no increase in ROS was observed for both DCFH-DA and luminol for 500 and 2500 μg/ml salicylate or the negative control, 04 % glycerol.

Fluoroquinolones are known to result in ROS accumulation [89]. We hypothesized that since the genotypic heritable phenotype is only observed in the presence of salicylate and an antibiotic such as ciprofloxacin, that we would detect ROS in the presence of both drugs. Therefore, combinational effects of salicylate with ciprofloxacin were tested for ROS levels (Fig. 3.4 C). ROS accumulation was observed at 1 μg/ml ciprofloxacin and also when combined with salicylate. However, ROS levels were lower in the presence of salicylate when combined with ciprofloxacin than with ciprofloxacin alone.

Recently, in a study by Paez *et al.* 2010 [92], the addition of glutathione to ciprofloxacin treated cells was able to significantly reduce the MIC to ciprofloxacin resistant *S. aureus* cells. The ability of salicylate to induce ROS was addressed with the addition of glutathione, an antioxidant. The addition of glutathione to ciprofloxacin and salicylate stressed cells, as expected, mitigated the number of CFUs/ml recovered (Fig.

3.5) indicating antioxidant properties for glutathione. However, the addition of glutathione to ciprofloxacin alone or ciprofloxacin and salicylate treatments did not decrease ROS levels (Fig. 3.4 C) indicating that glutathione was mitigating the SAGAR phenotype in a different manner. Therefore, we believe that glutathione is not reducing ROS, but rather altering the structure of salicylate, which impairs the ability of salicylate to induce its genotypic effects.

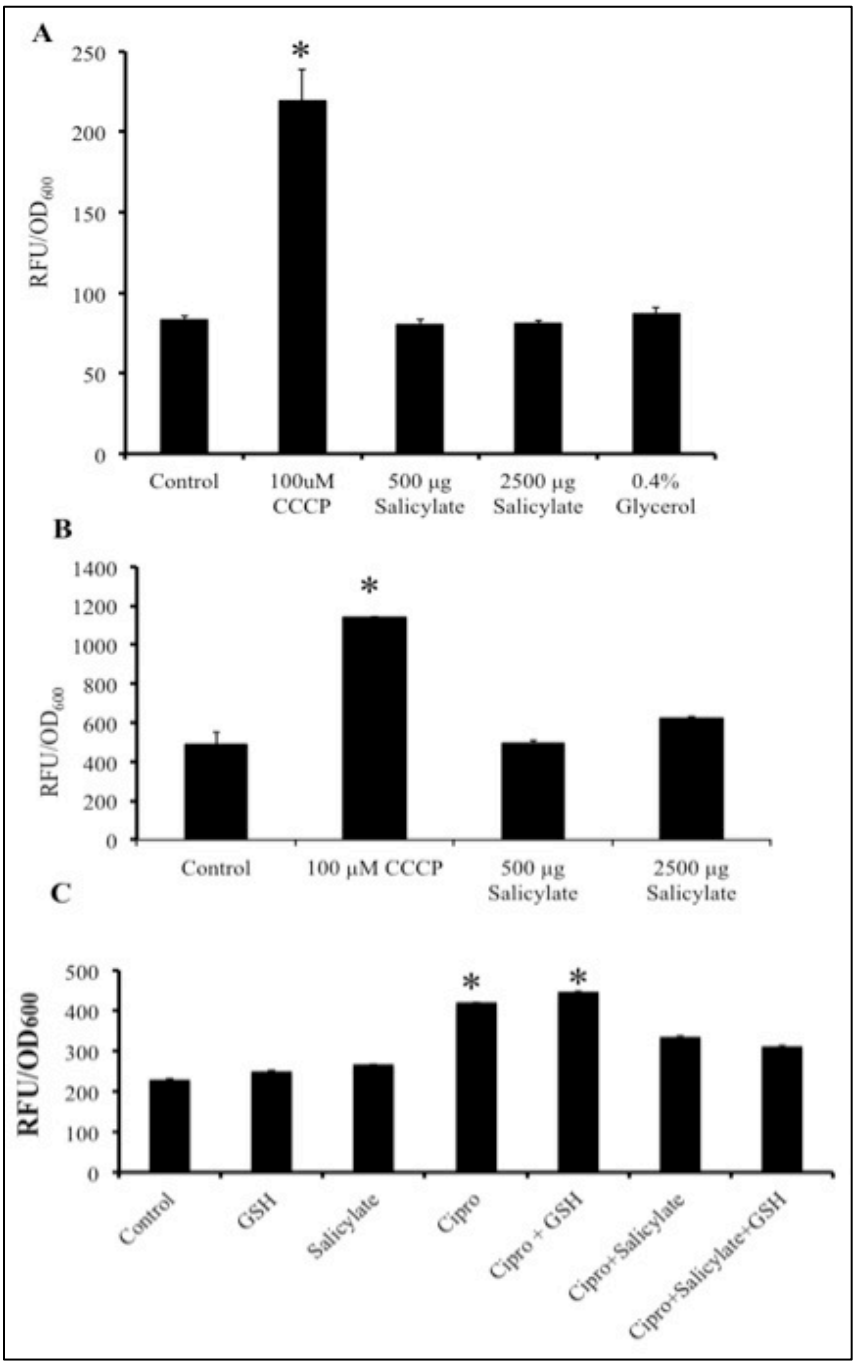

Figure 3.4. Salicylate associated ROS accumulation. ROS accumulation using: **(A)** DCFH-DA (**B)** luminol. **(C)** Combinational effects of ciprofloxacin and salicylate on accumulation of ROS using luminol. Asterisks denote statistical significance compared to control with a $p<0.05$, $n=3$. All values were recorded after 3 hours of stress under each treatment. GSH indicates 10 mM glutathione. Cipro indicates 1 µg/ml ciprofloxacin. Salicylate indicates 500 µg/ml salicylate. CCCP indicates carbonyl cyanide m-chlorophenylhydrazone.

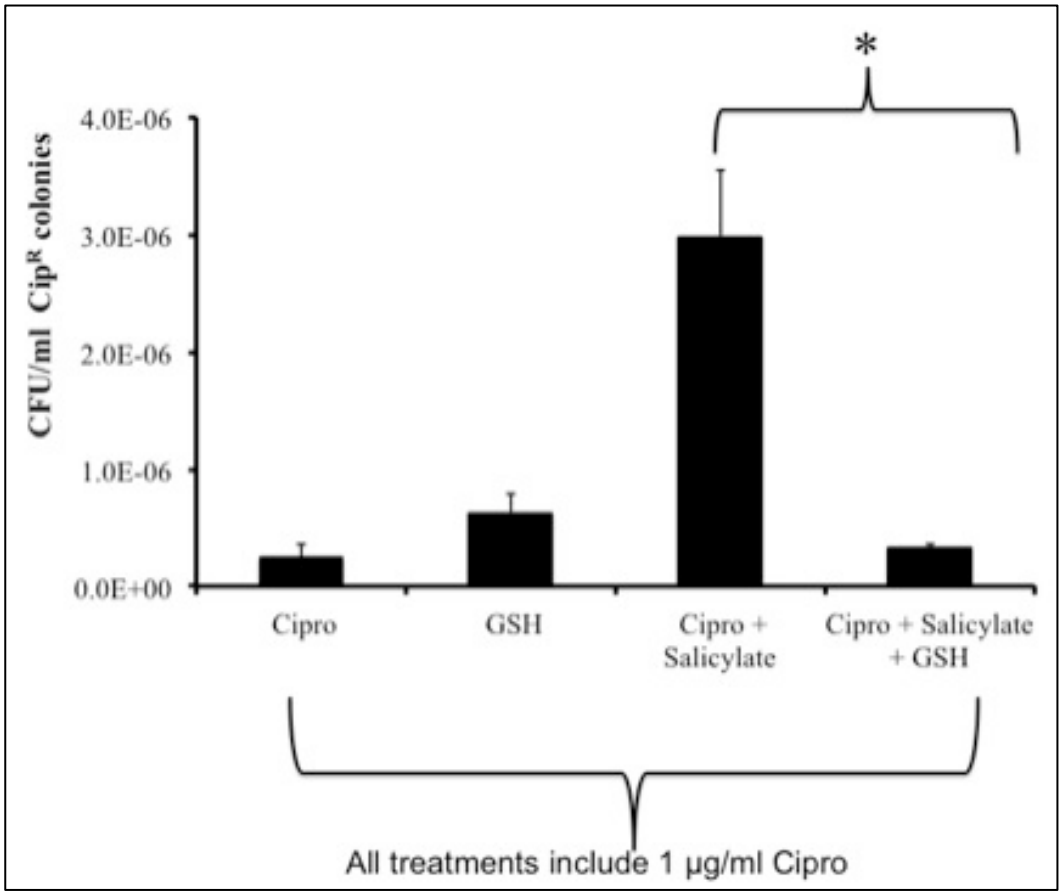

Figure 3.5. The effects of glutathione on the SAGAR phenotype. Control treatment was strain SH1000 grown strictly in TSB. SH1000 cells were plated on TSA plates containing the above treatments. CFUs recovered were recorded. Asterisks denotes statistical significance with $P<0.05$ between cipro and salicylate treatment compared to cipro, salicylate and glutathione. GSH indicates 10 mM glutathione. Cipro indicates 1 μg/ml ciprofloxacin. Salicylate indicates 500 μg/ml salicylate.

3.4 Discussion

The results of this study revealed that the SAGAR phenotype is sensitive to salicylate concentration; the phenotype is expressed at non-toxic concentrations, but is suppressed at growth-toxic concentrations. Growth toxic concentrations were associated with a metabolic switch to anaerobiosis, supported by the accumulation of lactic acid and butanediol in our metabolite profile. Accumulation of such metabolites implies that this

concentration of salicylate induced weak acid stress [138]. Mixed acid (lactate, formate, and acetate) and butanediol fermentation in *S. aureus* occur under anaerobic conditions[138]. Pyruvate from glycolysis can be reduced to either lactate by activity of lactate dehydrogenase or metabolized to acetoin and 2,3-butanediol by the activity of acetolactate synthase (BudB), α-acetolactate decarboxylase (BudA1), and acetoin reductase (SACOL0111) [138]. This process requires the oxidation NADH, which is a requisite under fermentation conditions [138]. 2,3-butanediol is involved in a variety of physiological activities such as homeostasis of pH and regulation of cellular NAD/NADH ratio in bacteria [145].

The anaerobic effect induced by growth toxic concentrations of salicylate led to question if the impairment in generation time was a causative or correlative effect. Under anaerobic conditions SAGAR for ciprofloxacin was substantially reduced suggesting a requirement of oxygen for the phenotype. Oxygen is necessary for the formation of a functional electron transport system [150]. It is possible that the lack of oxygen prevents ROS as well as DNA damage from occurring, i.e. an elevated mutation frequency, since regulation of the electron transport chain is vital to the homeostasis of *S. aureus*. This reveals that SAGAR requires an oxidative environment.

ROS damages iron-sulfur clusters making ferrous iron available for oxidation by the Fenton reaction [84, 85]. The Fenton reaction leads to hydroxyl radical formation. The hydroxyl radicals damage DNA, proteins, and lipids, which results in cell death [84, 85]. In a study by Chatterjee *et al.* inactivation of *fur*, a ferric uptake regulator homolog, decreased *butA*, which decreased 2,3-butanediol productions, as well as the TCA cycle

genes *citC*, isocitrase [147]. These findings support the idea that toxic concentrations of salicylate inhibit the TCA cycle, therefore, impairing the reduction of NAD^+.

Salicylate in mitochondria has been shown to interact with the respiratory chain resulting in hydrogen peroxide and other ROS which in turn oxidize thiol groups and glutathione [131]. This oxidative stress leads to the induction of the mitochondrial permeability transition in the presence of Ca^{2+}. This leads to further increase of oxidative damage, resulting in impairment of oxidative phosphorylation [131]. Based on these findings we hypothesized that salicylate would result in ROS accumulation in *S. aureus* cells. However, contrary to our hypothesis, induction of growth toxic and non-growth toxic concentrations of salicylate did not result in detection of ROS using either chemiluminescent DCFH-DA or luminol. Fluoroquinolones, specifically ciprofloxacin, have been shown to result in ROS production, which is one of their main bactericidal characteristics [85, 105, 106, 108, 146, 148, 149]. Considering that the mutation frequency observed is always in the presence of an antibiotic, such as ciprofloxacin, we hypothesized that the combination of salicylate and ciprofloxacin would result in a significant increase in ROS. We however, did not observe ROS accumulation upon the combination of both drugs. Also, the addition of glutathione, an antioxidant, did not alter the levels of ROS produced for both ciprofloxacin and salicylate, which leads to conclude that salicylate does not result in detectable ROS accumulation in *S. aureus*. However, the addition of glutathione to ciprofloxacin and salicylate treatments did substantially mitigate the SAGAR phenotype. This suggests that glutathione may not be acting like an antioxidant, but rather could be altering the structure of salicylate. Alteration in the

structure of salicylate, as seen in Chapter 2 results section 2.3, also mitigates the SAGAR phenotype.

A significant increase in mutation frequency is observed at non-growth toxic concentrations of salicylate (500 μg). Despite the lack of ROS accumulation, metabolite profiling revealed a decrease in glycolysis and TCA cycle as seen in decreased levels of citric acid, acontic acid, α-ketoglutaric acid, and fumaric acid, which was not mirrored in the increase in NAD^+ levels at 500 μg/ml of salicylate. However, at growth toxic concentrations of salicylate (2500 μg/ml), a significant increase in NAD^+ levels was observed, indicating either an increase in the oxidation of NADH or rather impairment in TCA cycle, which is responsible for reducing NAD^+. It is possible that the disruption of the TCA cycle in addition to the growth impairment effects of salicylate at 2500 μg/ml in combination are responsible for the toxicity and in result are responsible for mitigation of the SAGAR phenotype.

Another effect observed with toxic concentrations of salicylate by Riordan *et al.* [42] is a significant decrease in transcription of glycolytic genes *gapA2* and *pgi*, which are important genes in this process. Also, a significant increase in the gluconate operon (*gntkPR*) was observed upon salicylate stress [42]. Interestingly, Riordan *et al*. [42] observed exacerbation and reduction in growth inhibitory effects of salicylate upon glucose and gluconate addition, respectively. We cared to further explore this observation with a wide range of fermentable and non-fermentable sugar sources in a sugar free media, CASY (data not shown). We did not see any sugar that significantly impaired growth toxic effects of salicylate when compared to treatments of salicylate in the absence of a sugar source. We did however; observe that glucose exacerbated the

inhibitory effects of growth toxic concentrations of salicylate (data not shown). We hypothesized that this effect was due to a decrease in pH, which altered membrane permeability and in result enhancing the toxicity of salicylate. To test this hypothesis we used a buffer, MOPS to determine if it was in fact an acid effect (data not shown). As hypothesized, lowering the pH decreased the concentration of salicylate needed to inhibit growth. This finding leads us to believe that it is in fact the acidification of media that results in alteration of membrane permeability. This finding was observed in *Serratia marcescens* [89]. The effect of salicylate has been attributed to a weak acid effect that possibly leads to an increase in the membrane potential [89].

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