

STAPHYLOCOCCUS AUREUS
BACTERIAL RESISTANCE TO
SILVER NANOPARTICLE:
THE EMERGENCE AND
THE MECHANISMS OF RESISTANCE

by

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A thesis submitted in fulfilment of the requirements for the award of
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University of Technology Sydney
July 2020

Certificate of Original Authorship

I, Elizabeth Valentin, declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy in the ithree Institute, Faculty of Science at the University of Technology Sydney.

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To Roy

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Abstract

The widespread use of silver nanoparticle (NAg) in consumer products has prompted major concerns for bacterial resistance phenomena. Scholarly works have reported the natural ability of bacteria to develop resistance to the antibacterial nanoparticle. While the adaptation mechanisms have been studied in Gram-negative bacteria, it remains largely unexplored in Gram-positive bacteria. This work seeks to investigate the phenomena of NAg resistance in *Staphylococcus aureus*, a Gram-positive bacterium, to unravel the development and mechanisms of resistance in one of the WHO-listed priority pathogens.

Herein, the work found that *S. aureus* has the ability to evolve stable resistance traits, reducing the potency of NAg, upon prolonged exposure. Higher NAg concentrations are required to inhibit the bacterium growth when compared to the wild-type strain. A whole genome analysis of the resistant strain revealed mutation in gene that encodes the purine operon repressor protein. The mutation was thought to be responsible, at least in part, for the physiological adaptation of *S. aureus*, that is, the defence responses that manifest even in the absence of the nanoparticle. The role of this mutation was supported by transcriptomic analysis, whereby, increased expression of the whole purine synthesis operon were detected in the resistant strain. This, along with the observed changes in the expression of other metabolic genes (the pyrimidine, nitrogen, and capsular polysaccharide), highlight a metabolic adaptation to the likely disruptions in bacterial fitness.

The transcriptomic analysis also gives insights into the roles of specific cellular pathways, most likely responsible for the resistance characteristics. Our study found increased expression of genes that encode transporter proteins, ROS (reactive oxygen species) scavenger enzymes in the resistant strain and genes that are associated with the assembly of the iron-sulphur (Fe-S) clusters, one of the main cellular targets of NAg. For the latter, the upregulation was detected in the clusters' main synthesis genes (*suf* system) as well as in the iron and cysteine acquisition genes. Collectively, the adaptation mechanisms involve attempt by the bacterium to neutralize the over-production of ROS, while simultaneously recovering the damaged Fe-S clusters and facilitating silver efflux.

In summary, the knowledge generated herein provides insights into the emergence and molecular basis of NAg resistance in a bacterium that has been perceived to have no, or very low resistance tendency to NAg. The fundamental knowledge will allow

development of technologies to overcome the resistance phenomena and to inform strategies for an effective long term use of the nanoparticle.

List of publications

Journal article

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Conference articles

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Abbreviations

μ	Micro (10^{-6})
Ag ⁺	Ionic silver
Ag-r	Ag ⁺ -resistant strain
ANSTO	Australia's Nuclear Science and Technology Organisation
ASV	Anodic stripping voltammetry
ATP	Adenosine monophosphate
ATR	Attenuated total reflection
<i>B.</i>	Bacillus
BET	Brunauer–Emmett–Teller
BOD	Biological oxygen demand
BPEI	Branched polyethyleneimine
Ca-MHB	Cation-adjusted Mueller Hinton broth
CLSI	The Clinical and Laboratory Standards Institute
CRISPRi	Clustered regularly interspaced short palindromic repeats interference
CSR	the Centre for Staphylococcal Research
d	Diameter
dCas9	dead Cas9
DEGs	Differentially expressed genes
DNA	Deoxyribonucleic acid
dTEM	Diameter based on transmission electron microscopy
<i>E.</i>	Escherichia
EDAX	Energy dispersive X-ray spectroscopy
EXAFS	Extended X-ray fine absorption structure
Fe-S cluster	Iron-sulphur cluster
FSP	Flame spray pyrolysis

FT-IR	Fourier transform infrared
GSH	Glutathione
GSSG	Oxidized glutathione
h	Hour
HGT	Horizontal gene transfer
ICP-MS	Inductively coupled plasma spectrometry-mass spectrometry
IMP	Integral membrane protein
IMP	Inosine monophosphate
LB	Luria Bertani broth
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MBC	Minimum bactericidal concentration
MHA	Cation-adjusted Mueller Hinton agar
MIC	Minimum inhibitory concentration
min	Minute
MRSA	Methicillin-resistant <i>S. aureus</i>
NAg	Silver nanoparticle
NAG	N-acetylglucosamine
NAg-r	NAg-resistant strain
NAM	N-acetylmuramic acid
NCBI	National Centre of Biotechnology Information
OD	Optical density
Omp	Outer membrane protein
ORF	Open reading frame
<i>P.</i>	<i>Pseudomonas</i>
PAM	Protospacer adjacent motive
PBP	Penicillin binding proteins

PCA	Principal component analysis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PGRE	Pomegranate rind extract
PVP	Polyvinylpyrrolidone
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNA-seq	RNA-sequencing
ROS	Reactive oxygen species
RPM	Rotation per minute
S.	Staphylococcus
SCELSE	Singapore Centre for Environmental Life Science Engineering
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SERS	Surface-enhanced Raman spectroscopy
sgRNA	single guide RNA
SNV	Single nucleotide variants
TCA cycle	Citric acid cycle
TEM	Transmission electron microscopy
TRFLP	Terminal restriction fragment length polymorphism
UMP	Uridine monophosphate
UV	Ultra violet
WHO	World Health Organization
WT	Wild-type strain
wt%	Weight percentage
WTA	Wall teichoic acid
XRD	X-ray diffraction

Chapter 1

Introduction

Antibiotic resistance, defined as the ability of a microorganism to tolerate the drug's antibacterial activity, is a global threat to public health. Antibiotic resistance has caused more than 35,000 deaths in the United States alone every year and is projected to escalate if the issue remains unsolved^{1,2}. Some of the major factors that cause resistance to antibiotics, as identified by the World Health Organization (WHO), are the overuse or over prescription of antibiotics in patients and livestock, as well as the lack of new drug development³. For the latter, there has been an evident gap in the discovery of new antibiotics from 1960s until 2000s, known as the discovery void⁴. The rapid rise and spread of resistance has rendered new drug development deemed unprofitable market by the pharmaceutical companies.

Silver nanoparticle (NAg) is a 1-to-100 nm size metal or metal oxide silver particle that has been considered as a promising alternative to antibiotics. NAg has proven efficacy on a broad range of microorganisms, *e.g.* yeasts⁵, viruses⁶, Gram-positive, Gram-negative, and even multidrug resistant bacteria^{7–9}. For this reason, NAg has been used in medical devices, such as in wound dressings and internal catheters to fight off infections. Advances in nanotechnology, however, has enabled incorporation of the nanoparticle as antimicrobial agent into increasing arrays of everyday consumer products, including in personal care products, clothing, household appliances, food packaging and supplement, and many others¹⁰. This widespread and indiscriminate use of NAg has raised an escalating concern, that, as with the case of antibiotic overuse, bacteria can also develop resistance to the alternative nanoparticle antimicrobial.

Despite the common perception that it would be rather difficult for bacteria to develop resistance due to the nanoparticle's complex, multi-targets toxicity mechanisms¹¹, increasing studies on Gram-positive^{12,13} and Gram-negative bacteria^{14–16} have reported the otherwise. The studies on Gram-negative bacteria^{14–16} have observed genomic mutations or epigenetic cellular change that may contribute to the defence mechanism(s) against the toxicity. Up to this stage, however, there is still lack of

research efforts for the study of NAg mechanisms of resistance in Gram-positive bacteria, particularly, in the pathogenic clinically-relevant bacteria.

In view of the current overuse of NAg, this thesis aimed to investigate the development of NAg resistance in the Gram-positive bacterium *Staphylococcus aureus*, a WHO-listed priority pathogen that greatly affect human health¹⁷. The bacterium is lacking in any of the known determinants for silver resistance, therefore, this study of resistance development will shed a light on the defence mechanisms to NAg toxicity, as whether they are conserved among different species of bacteria or are species-specific. The investigations were conducted in parallel to Ag⁺ studies. This is to identify any unique resistance mechanisms of NAg, as opposed to Ag⁺, as well as to identify any shared common resistance mechanisms between the different forms of silver.

The aims were delegated into following objectives:

1. To investigate the emergence of NAg and Ag⁺ resistance in *S. aureus*.
2. To elucidate the toxicological responses of *S. aureus* to NAg and Ag⁺.
3. To elucidate the molecular basis of NAg and Ag⁺ resistance in *S. aureus*.

This thesis has 7 chapters. The first chapter is an introductory chapter describing the underlying issue that motivates this study, as well as the study's aims. A literature review follows in Chapter 2, comprehensively discussing the NAg speciation and antibacterial mechanisms along with the influencing physical and biological factors as well as the current advances in the study of NAg resistances, to ultimately identify the knowledge gap in the field. Next, in Chapter 3, this study reports the discovery of NAg and Ag⁺ resistance in *S. aureus*. This is followed by the identification of mutations in the resistant *S. aureus* genomes that are likely to play a role in the defence mechanisms, presented in Chapter 4. Chapter 5 describes the toxicological responses of *S. aureus* to NAg and in Chapter 6, the study explains the molecular basis of the defence mechanisms of the resistant *S. aureus* to NAg and Ag⁺. The thesis is concluded in Chapter 7 with a general and overall discussion of the scientific findings and recommendations for future work.

Chapter 2

Literature Review

Silver Nanoparticle (NAg): Generation of Bioavailable Silver Species, Modes of Toxicity, and Bacterial Resistance

2.1. Summary

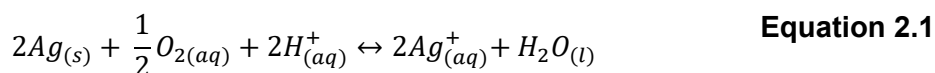
This literature review discussed the toxicity of silver nanoparticle (NAg) on bacteria that is governed by the release of ionic silver (Ag^+), the particle's physicochemical properties such as size, shape, and functionalized particle's surface, as well as the Ag^+ interaction with chemical species in its surrounding. These aspects translates to multifaceted, complex, and comprehensive NAg antibacterial mechanisms, starting from disruption to the cell surface, alteration of DNA and protein structure and function once the silver particles and ions are internalized, and the generation of intracellular reactive oxygen species (ROS) to create further cellular damage throughout. The identification of Ag^+ resistance mechanisms in bacteria *via* chromosomally- and plasmid-encoded outer membrane proteins and efflux pump proteins was also discussed. This review was concluded with recent evidences of NAg resistance reported in environmental and pathogenic bacteria. The knowledge gathered here serves as a platform in this study to unravel the emergence of NAg resistance and the molecular basis of NAg resistance mechanisms, particularly in the bacteria with no, or very low, prevalence of the previously-identified Ag^+ resistance mechanisms.

2.2. Generation of Bioavailable Silver Species from NAg Particles

2.2.1. Mechanisms of Ag^+ ion release

It has been widely accepted that NAg undergoes a so-called **oxidative dissolution** process upon contact with aqueous environment and releases Ag^+ ions. One of the most important evidence for such phenomena is given by Sotiriou *et al.* through a systematic particle size-dependent silver nanoparticle (NAg) leaching studies carried

out on Ag/SiO₂ (1 to 50 wt% Ag, $d = 4$ to 9 nm)¹⁸. The study deduced that Ag⁺ ions are released from NAg *via* dissolution of one to two Ag₂O surface monolayers as a function of particle size. This oxide layer is thought to result from the oxidation of Ag metal upon exposure to oxygen. Ag₂O has higher solubility constant in water (solubility in water = 2.22×10^{-7} g/L at 25°C, $K_{sp} = 4 \times 10^{-11}$) than Ag metal that is not soluble in water, rendering it more readily dissolves than the metal¹⁹, ultimately releasing the Ag⁺ ions into the aqueous systems. The role of oxygen on Ag dissolution has been validated by further studies. Using anaerobic chamber, Xiu *et al.* studied the leaching behaviour of NAg coated with polyethylene glycol (NAg-PEG, $d = 5$ and 11 nm) in NaHCO₃ buffer solution and observed only < 1 µg/L Ag⁺ leaching (with 2000 mg/L NAg particles) even after 120 h incubation, in comparison to the detection of > 1000 µg/L Ag⁺ in the presence of dissolved oxygen²⁰. Similar findings were also reported by Liu and Hurt with the leaching behaviour of citrate-stabilized particles (NAg-citrate, $d = 4.8$ nm) in water under oxygen-rich and -depleted conditions, the latter *via* pumping of Ar gas²¹. Apart from oxygen, research inquiries have also indicated the role of protons in the release of Ag⁺ from NAg particles. Liu and Hurt with their NAg-citrate particles observed increasing extent of leaching with decreasing pH (pH 4 to 9) under oxygen-rich conditions (9.1 mg/L dissolved O₂ in water). In fact, the work also found presence of H₂O₂ in the oxygen-rich NAg leaching systems (at pH 5.7), which is thought to be at least one of the intermediates generated during the NAg oxidative dissolution. The study was further stretched to also investigate the potential capability of the H₂O₂ as an additional oxidant apart from oxygen, to stimulate the Ag leaching process. Indeed, a 4.5-fold higher Ag⁺ concentration was detected in systems with added H₂O₂ to those with the presence of only dissolved oxygen. The following reaction (see **Equation 2.1**) has been proposed for the NAg oxidative dissolution in water containing no other oxidants or reductants.



Upon their release from NAg particles, Ag⁺ ions are thermodynamically favourable to interact with chemical moieties, such as halides, sulphides and even amino acids and peptides, typically present in the natural environments and in body fluids. The interactions of Ag⁺ with these silver-precipitating and silver-complexing moieties, as described in the following sections, will result in the generation of a vast arrays of silver species with varying degree and mode of toxicity on microbes.

2.2.2. Effect of NAg physicochemical characteristics on Ag⁺ release and antibacterial activity

Many studies have reported the effect of the NAg physicochemical particulate characteristics on its leaching behaviour and in turn, antibacterial activity. Sotiriou *et al.* observed more severe growth inhibition on *Escherichia coli* upon exposure of the Gram-negative bacteria to smaller NAg particles (1 to 98 wt% Ag deposits on SiO₂ particulate support) and linked this to the increasing extent of silver leaching²². At ≤ 8 nm Ag particle size (≤ 25 wt% Ag deposits), the work detected at least 30% silver leaching in water at equilibrium, which corresponded to 50 to 99% growth inhibition relative to those of the SiO₂-only control. At the higher 9 to 16 nm NAg sizes, less than 30% leaching was detected, with essentially no inhibition effects on the bacterial proliferation. The study revealed a significant decrease in the NAg's specific surface area from 40 m²/g to close to 0 with the increasing 4 to 16.6 nm particle size, which appeared to have caused, at least in part, the less extent of silver leaching and the corresponding passivated toxicity with increasing particle size²². The larger surface area on an equivalent mass basis of smaller NAg particulates is thought to give rise to higher proportion of reactive sites available for the oxidative dissolution²³. Research inquiries have also suggested lower redox potential of smaller NAg than those of larger particles, rendering the former more susceptible to oxidative dissolution²⁴. Other work has also reported similar trends, in this case on the Gram-positive *Staphylococcus aureus* bacteria. Martínez-Castañón *et al.* exposed the bacteria to gallic acid-functionalized NAg (d = 7, 29 and 89 nm) and found the least minimum inhibitory concentration with the smallest particle size under comparable experimental conditions²⁵.

Research inquiries on the antibacterial activity of various shaped NAg also yield interesting findings. Systematic investigation by Pal *et al.* using different shapes citrate-stabilized NAg, more specifically, rods (l = 133 nm, d = 16 nm), spheres (d = 39 nm) and truncated triangular nanoplates (edge length = 40 nm), reported significant differences in the particles' growth inhibiting effects on *E. coli*²⁶. A complete growth inhibition of the bacteria was observed at 1 μ g dosage for the triangular NAg (studies on agar plates), in comparison to 50 μ g and >100 μ g for the spherical and rod-shaped particles, respectively. As revealed by an X-ray diffraction analysis, this was most likely to result from the major presence of the high atomic density and therefore, more reactive (111) facets in the triangular NAg crystal lattices, compared to the spherical and rod-shaped particles. Note that the latter NAg shapes have predominant presence of the lower atomic density (100) facets in their crystal lattices²⁷. In agreement with Pal

et al., Sadeghi *et al.* found that the NAg platelets (polyvinylpyrrolidone (PVP)-stabilized) exhibited the largest inhibition zones (agar studies) on *E. coli* and *S. aureus*, compared to those of rod-shaped and spherical-shaped particles²⁸. The study found that the NAg platelets (10 mg/L) exhibited complete inhibition on *E. coli* and *S. aureus* growth 2 – 3 h earlier than the other two shapes. The different activity is thought to result from differences in surface area. As revealed via BET (Brunauer–Emmett–Teller) analysis, the NAg platelets had the largest surface area (121 m²/g) followed by the rods (38.8 m²/g) and spherical NAg (17.8 m²/g). In addition to the atomic density and surface area, a recent study reported that the antibacterial activity of various NAg shapes (plates, spheres, rods, and cubes) also correlates with the particles' leaching behaviour²⁹. Following 29 days of NAg incubation in ultrapure water (25°C, pH 4.8), NAg platelets (d = 20 – 60 nm) were associated with the highest extent of Ag⁺ leaching (~70% relative to total silver), followed by the spheres (~55 wt%; d = 120 – 180 nm), rods (~38 wt%; l = 1 – 20 µm, d = 50 – 100 nm), and cubes (~30 wt%; l = 140 – 180 nm). Indeed, the platelets with the largest surface area (4.4x10³ nm²/particle), eradicated *S. aureus* at the lowest concentration (~25 µg/mL) compared to the other shapes.

Next, the presence of surface functional groups on NAg has also been reported to affect the particles' toxicity on bacteria. Badawy *et al.* compared the effects of spherical bare (or unmodified) NAg to those functionalized with citrate, PVP, and branched polyethyleneimine (BPEI) (d = 10 – 18 nm) on the Gram-positive *Bacillus sp.* proliferation³⁰. Based on cell viability and BOD (biological oxygen demand) assays, the work observed the highest antibacterial activity with NAg-BPEI, followed by the NAg-PVP, bare NAg and lastly, NAg-citrate particles, most likely accountable to the differences in the particles' net surface charges. The surface of bacteria, both Gram-positive and Gram-negative, are negatively-charged under physiological conditions^{31,32} and therefore, NAg particles with more negative net surface charges, in this case (in order), the NAg-PVP, bare NAg and NAg-citrate, would be associated with a stronger repulsive effect against the bacteria, reducing the particle-bacteria interaction and subsequently, the particles' toxicity. Indeed, this postulation was confirmed by Abbaszadegan *et al.*, who observed the largest inhibition zone of the positively-charged NAg on both Gram-positive (*S. aureus*, *Streptococcus mutans*, and *S. pyogenes*) and Gram-negative bacteria (*E. coli* and *Proteus vulgaris*) as compared to the neutral and negatively-charged nanoparticles³³. Studies have also demonstrated that the presence of surface functional groups hinders the leaching of soluble Ag from the nanoparticles. Li *et al.* compared the Ag leaching profiles of the bare, PVP- and citrate-capped NAg (d

(hydrodynamic) = 79, 25, and 28 nm, respectively)³⁴. The work found that the bare particle released the highest Ag⁺ concentration in deionized water, followed by the PVP- and citrate-capped particles. A UV-vis spectra analysis revealed a 5-nm shift of the bare particle relative to the functionalized particles, suggesting the presence of an oxide layer on the surface of the former. The presence of polyvinylpyrrolidone (PVP) or citrate, as the study elaborated, may reduce the oxide layer to Ag⁰, in turn, reducing the extent of Ag dissolution. These findings are also consistent with other study²³.

Research inquiries have also deduced the inter-correlation of these characteristics on the toxicity effects. Ayala-Nunez *et al.* for example, reported lower antibacterial activity of smaller NAg (d = 30-40 nm) on methicillin-resistant *S. aureus* (MRSA) in comparison to the larger particle (d = 100 nm)⁷. Contradicting most studies on the size effect, it is deduced that there was an overlapping effect from the presence of surface functional groups on the particles as the smaller NAg was functionalized with PVP, while the larger one was bare. Similar results was also observed by Badawy *et al.*³⁰. Taken together, it is clear that the NAg's leaching behaviour that affects its antibacterial activity is governed by its physicochemical properties altogether, which include size, shape, and presence of surface functional groups.

2.2.3. Bioavailable Ag: Interactions of silver with chemical moieties

Halide ions (Cl⁻, Br⁻, I⁻) are **silver-precipitating and -complexing agents** and their presence have been known to affect the leaching behaviour of NAg as well as its bacterial toxicity³⁵. One of the most important evidence for the effects of halides was by Levard *et al.* with their systematic assessment of NAg leaching (NAg-PVP, d = 32.9 nm) in the presence of varying Cl/Ag (5 - 26750) molar ratios and subsequently, the corresponding nanoparticle's growth inhibiting activity on *E. coli*³⁶. In the absence of chloride, the work detected ~0.2%/h dissolution rate of NAg in water and a complete inhibition on the bacterial growth. In the presence of chloride with Cl/Ag ratios of 5 to 2675, the work detected lower dissolution rate of NAg as those of with zero presence of chloride, hence, the *E. coli* exposure to the former system did not inhibit the bacterial growth, in fact, the effect was essentially benign. Ultimately, at the highest studied Cl/Ag ratio of 26750, the NAg dissolution rate was found to be at ~0.8%/h, which was associated with complete inhibition of the *E. coli* growth.

Apart from the effects on NAg leaching, studies have also indicated a halide concentration-dependent Ag speciation behaviour that may affect the nanoparticle toxicity, in this case the presence of chloride. The work by Silver has proposed that the halide concentrations will govern the types of the Ag-halide moieties formed, each with

unique level of toxicity or in other word, 'bioavailability'³⁷. Moderate chloride levels will precipitate the released Ag^+ ions as the insoluble and microbiologically inert AgCl , while high chloride levels will 'bring back' the silver as the water soluble and microbiologically active Ag-chloride anionic complexes $\text{AgCl}_x^{(x-1)-}$. The latter is an often overlooked silver chemistry when assessing for NAg toxicity. At low chloride levels, Ag^+ ions will remain as the bioavailable free ions. This halide concentration-dependent Ag speciation behaviour was supported by Levard *et al.*'s thermodynamic modelling that suggested the formation of soluble and insoluble Ag-chloride species as a function of the Cl/Ag ratio³⁶. Again, the soluble $\text{AgCl}_x^{(x-1)-}$ species is thought to become more dominant at higher Cl/Ag ratios, which was associated with the observed complete *E. coli* growth inhibition and recalling the benign effect of the exposure to NAg at lower Cl/Ag ratios, the insoluble AgCl appears to dominate at lower Cl/Ag ratios. Similar chemistry is thought to also apply for the Ag-bromide speciation ($\text{AgBr}_{(s)}$, $\text{AgBr}_x^{(x-1)-}{}_{(aq)}$) and Ag-iodide speciation ($\text{AgI}_{(s)}$, $\text{AgI}_x^{(x-1)-}{}_{(aq)}$), although at lower concentrations due to the lower solubility of AgBr and AgI compared to AgCl (K_{sp} AgCl = 1.56×10^{-10} , AgBr = 7.7×10^{-13} , AgI = 1.5×10^{-16} mol/L). Gupta *et al.* systemically assessed the growth of *E. coli* (pMG101) upon exposure to AgNO_3 (0 to 600 μM Ag^+), in the absence and presence of NaCl (0 to 513 mM Cl^-), NaBr (24 to 292 mM Br^-), or NaI (33 to 200 mM I^-)³⁸. At low presence of halides (0 mM Cl^- , 24 mM Br^- , and 33 mM I^-), and therefore, low halides/Ag ratios, the formation of the inert silver-halide precipitates was thought to result in the bacteria still proliferating at the 600 μM Ag^+ dosage. Increasing the halide concentrations (513 mM Cl^- , 292 mM Br^- , 200 mM I^-) saw the returning of the growth inhibiting effects at much less than the 600 μM Ag^+ dosage. Indeed, this restored antibacterial activity was also observed upon increasing the halide/Ag ratios at the lower Ag^+ concentrations. Taken together, the findings therefore support the notion that halide concentration affects NAg dissolution and speciation of the released Ag^+ ions and in turn, the antibacterial activity.

Other commonly present chemical species with which the leached Ag^+ most likely to interact is the sulphide ions and its presence has also been found to affect the bacterial toxicity of NAg. Reinsch *et al.* pre-exposed PVP-capped NAg (d = 25-30 nm) to Na_2S solution at 0 to 2 HS^-/Ag (mol/mol) ratio³⁹. Exposure of *E. coli* to the pre-exposed NAg at increasing HS^-/Ag ratio saw diminishing growth inhibiting activity of the nanoparticles, most likely to result from the observed decreasing concentration of the leached soluble Ag (< 2 $\mu\text{g}/\text{L}$ at 2 HS^-/Ag ratio). Indeed, EXAFS analysis (extended X-ray fine absorption structure) of the NAg prior to and after incubation with Na_2S revealed increasing formation of Ag_2S with increasing HS^-/Ag ratio. At 2 HS^-/Ag ratio for

example, 97% of the Ag were in the form of Ag₂S. TEM (transmission electron microscopy) analysis by Levard *et al.* revealed the formation of sulphide bridges between the NAg-PVP particles (d = 38 nm) following sulphidation reaction with Na₂S (10⁻³ M for 24 h), which appears to enhance the aggregation of the particulates⁴⁰. Other work reported the formation of Ag₂S shell around the core wire and spherical PVP-NAg following sulphidation of the particles with H₂S under O₂ depleted condition⁴¹. It is likely that this NAg particle aggregation/coating with Ag₂S contributed, at least in part, to the decreasing extent of Ag leaching and in turn, the particle's antibacterial activity upon sulphidation.

The released Ag⁺ ions from NAg could also bind to organo-sulphur moieties, including the major bloodstream proteins human serum albumin (HSA, typical 50 g/L bloodstream concentration)⁴² and metallothionein⁴³⁻⁴⁵. A soft acid, Ag⁺ has high affinity to thiol (-SH) group and formation constants of 10⁵ and 10⁴ have been reported for Ag⁺ binding sites on HSA, with the thiol-containing amino acid cysteine (as well as the S-methyl thioether-containing amino acid methionine and disulphide bridge) as the major functional groups involved⁴⁶. Ag⁺ has also been shown to strongly bind to the cysteine moiety of the tripeptide major antioxidant reduced glutathione (GSH) (Glu-Cys-Gly, typical 1 mM bloodstream concentration, $K_{\text{formation}} = 12.3$ for Ag-GSH)^{47,48}. Like those observed with the presence of sulphide ions, research inquiries have indeed reported the seemingly negative effects of thiol-containing moieties on the antibacterial activity of silver. Mulley *et al.* observed a benign effect of Ag⁺ (AgNO₃) on the growth of *S. aureus* and *P. aeruginosa* in the presence of GSH at ≥ 1 GSH/Ag (mol/mol) ratios, in comparison to the complete inhibition detected at < 1 ratios⁴⁹. This passivated silver toxicity is likely to result from the toxic Ag⁺ ions being locked in Ag-GSH complexes, with Liu *et al.* suggesting the formation of Ag-GSH complexes stoichiometry at high GSH/Ag ratios, while the stoichiometry approximates Ag₂-GSH at low ratios⁴³.

Phosphate is also one of the major ligands to interact with silver with many studies reporting the passivation of NAg (and Ag⁺ ions) antibacterial activity in its presence. Xiu *et al.* for example, observed a 50% less cell death upon exposure of *E. coli* to Ag⁺ (2.94 μ M, in the form of AgNO₃) in the presence of PO₄³⁻ (0.98 μ M), when compared to that of the Ag⁺-only exposure⁵⁰. The reduced toxicity is most likely due to the formation of Ag-phosphate precipitate ($K_{\text{sp}} \text{ Ag}_3\text{PO}_4 = 1 \times 10^{-17.6}$), which appears to be microbiologically inert⁵⁰.

Liu *et al.* investigated the effect of selenium (Se), commonly present in human serum and plasma as cofactor to antioxidant, on NAg toxicity⁴³. A reduced selenium solution (5 mM) prepared in DI water prior to adding silver foil (99.9% Ag, 4x4 mm, 0.127 mm

thickness) was observed to alter the foil's colour to black, implying the formation of Ag_2Se as validated by XRD (X-ray diffraction) analysis. Via atomic absorption spectrometry, dissolved Ag of citrate-stabilized NAg ($d = 4.8 \text{ nm}$) in acetate buffer media was measured in the presence of ascorbic acid, methionine, serine, GSSG (oxidized glutathione), and sodium citrate following 1 incubation day in dark condition. Relative to the initial dissolved Ag^+ (2 mg/L), the compounds provided at least 85% reduction of Ag^+ at increasing concentration each (1×10^{-4} to 1 mM). Only ascorbic acid, up to 0.1 mM , showed increasing dissolved Ag^+ to 1.8 mg/L , yet the Ag^+ release was hindered rapidly afterwards. These above-mentioned ligands have been reported to affect the release Ag^+ ions in the solution, indicating the possibility of reducing NAg antibacterial capacity; however, their immediate effect to bacteria might need to be more elucidated for future references.

Up to this stage, this review has discussed important research efforts on the NAg antibacterial activity, taking into account the nanoparticle leaching behaviour, as well as the chemical transformation of the released Ag^+ into arrays of different silver species, each with unique bioavailability (**Figure 2.1**). Together, these aspects are essentials in affecting NAg toxicity towards bacteria.

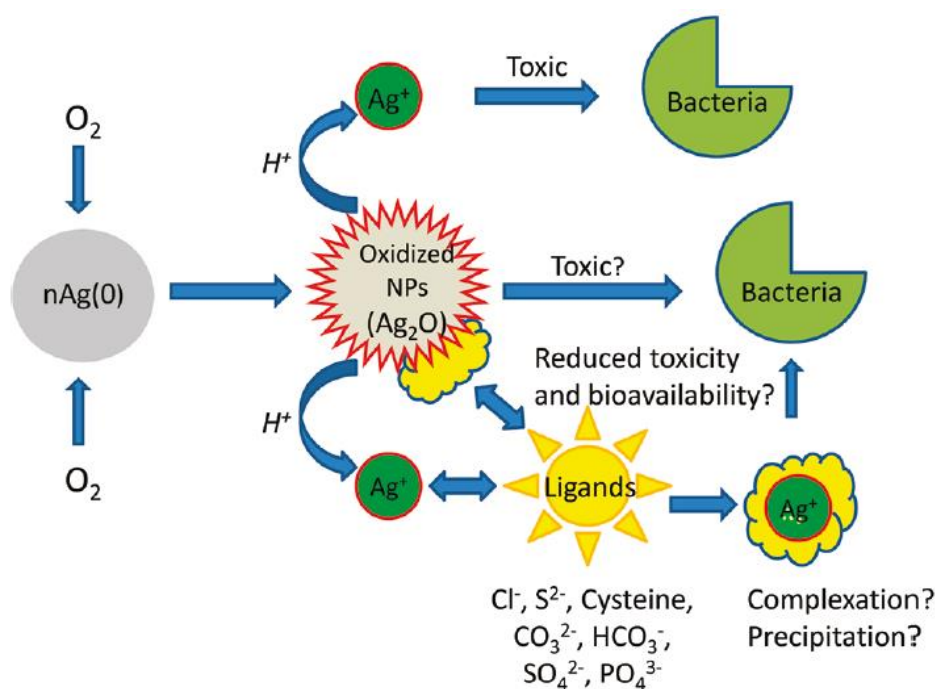


Figure 2.1. Representative scheme of Ag^+ ions release from NAg and their interaction to surroundings⁵⁰. (Reproduced with permission from American Chemical Society)

2.3. Modes of NAg Antibacterial Activity

2.3.1. Interactions of NAg with cell envelope

It has been reported by numerous research efforts that NAg targets the cell surface, causing multiple toxicity effects originating from the physical and quite recently found, oxidative damages on the cell envelope moieties. This interaction raise an important queries: does differences in the membrane structure affect the particles' antibacterial activity? In brief, the cell surface of Gram-positive bacteria have an approximate thickness of 15 – 80 nm (see **Figure 2.2**). The outermost layer is made of peptidoglycan (about 90%) with presence of lipoteichoic acids and an (inner) cytoplasmic phospholipid bilayer membrane⁵¹. The outermost layer of Gram-negative bacteria, on the other hand, are composed of the phospholipid membrane with presence of lipopolysaccharide protruding to the external environment (see **Figure 2.2**). The peptidoglycan layer in the Gram-negative bacteria are significantly thinner (~2 nm) and is present in the periplasmic space, a space between the outer and inner membrane⁵¹.

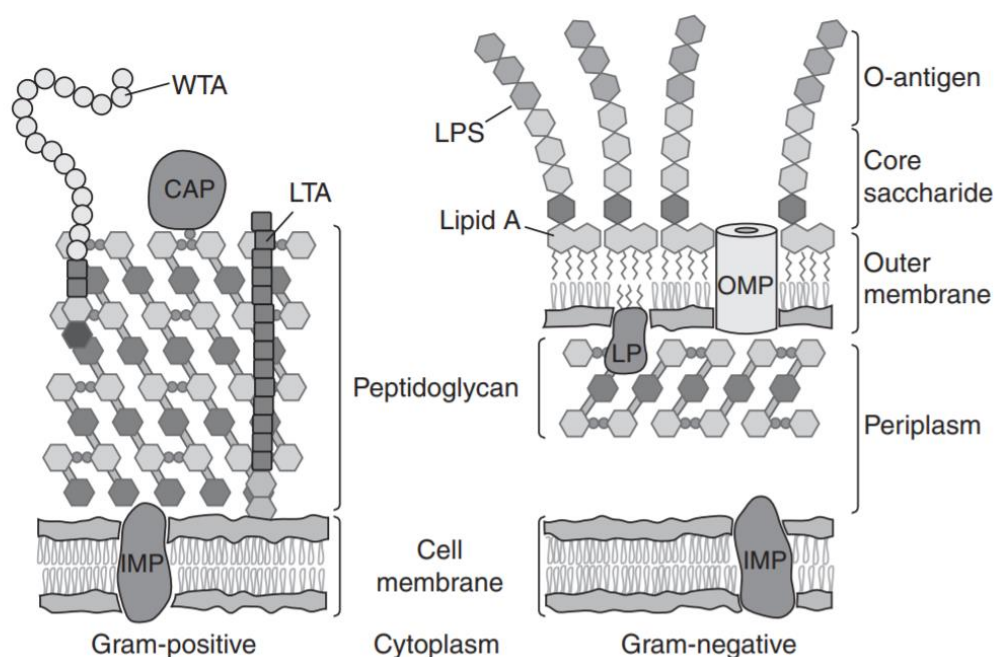


Figure 2.2. Schematic diagram of Gram-positive and Gram-negative cell surface⁵². CAP: covalently attached protein; WTA: wall teichoic acid; LTA: lipoteichoic acid; LPS: lipopolysaccharide; OMP: outer membrane protein; IMP: integral membrane protein. (Reproduced with permission from Cold Spring Harbor Laboratory Press)

This significant structural differences however, do not seem to translate into clear cut discrepancies in the extent of NAg antibacterial activity. Martinez *et al.* observed higher toxicity of gallic acid-stabilized NAg on the Gram-negative *E. coli* (minimum inhibitory concentration; MIC = 12 µg/mL) than on the Gram-positive *S. aureus* (MIC = 34 µg/mL)

at the largest particle size studied ($d = 89$ nm), while comparable MICs ($6\text{--}7.5$ $\mu\text{g/mL}$) were reported for the two bacteria at the smallest particle size studied ($d = 7$ nm)²⁵. Further studies by Hwang *et al.* also observed only little span of MICs ($0.25\text{--}2$ $\mu\text{g/mL}$) for Gram-positive and Gram-negative bacteria following their exposure to small particle size ($d = 3$ nm)⁵³. The antibacterial activity seems to be significantly affected by the particles' characteristics, in this case, their size, rather than the type of bacteria. Nevertheless, studies have reported the formation of holes on cell surface, the so-called 'cell pits', in both Gram-positive and Gram-negative bacteria as a result of exposure to NAg. TEM imaging by Mirzajani *et al.* found ruptures on the Gram-positive *S. aureus* cell envelope following exposure of the bacteria to NAg (citrate-stabilized, $d = 18.3$ nm)⁵⁴. NAg is thought to break the β -1,4 glycosidic bond in the peptidoglycan that links the two sugar derivatives, N-acetylmuramic acid and N-acetylglucosamine, as evidenced by the increase of muramic acid concentration in the supernatant upon NAg exposure (citrate-stabilized NAg, $d = 18.3$ nm) (see **Figure 2.3**). For Gram-negative bacteria, Li *et al.* noticed the formation of cell pits at multiple sites on *E. coli* cell envelope following exposure to NAg ($d = 5$ nm)⁹. Similar morphological changes were also reported by Sondi and Salopek-Sondi in NAg-treated *E. coli* samples ($d = 12$ nm)⁵⁵. This formation of cell pits may involve NAg-induced alteration of cell membrane composition, as indicated by Ramalingan *et al.*³¹. In the study, analysis of FT-IR (fourier transform infrared) spectra of *E. coli* and also the Gram-negative *P. aeruginosa* before and after NAg treatment, revealed significant depletion of the membrane phospholipids. The analysis also noticed slight reduction in the abundance of proteins and carbohydrates, known to be present on the outer membrane of Gram-negative bacteria.

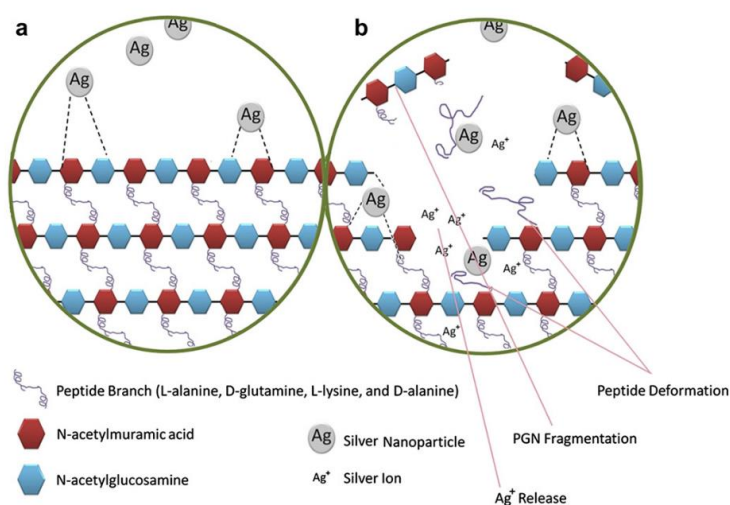


Figure 2.3. Proposed mechanism of A) NAg interaction and B) cell wall pit formation in bacterial cell wall⁵⁴. (Reproduced with permission from Elsevier)

This formation of cell pits is thought to subsequently result in the release of cellular constituents, a phenomenon frequently observed with the NAg-bacterial exposure. Using elemental mapping analysis (in TEM), Morones *et al.* found presence of NAg particulates ($d = 16$ nm) trapped in the cell membrane of *E. coli* and *P. aeruginosa*, which was associated with the observed release of cellular debris due to membrane damages⁵⁶. This leaking of cellular components was also observed in *S. aureus*^{8,54}. In agreement, Li *et al.* and Kim *et al.* detected an elevated presence of sugars and proteins in the supernatant, most likely of intracellular origins, following exposure of *E. coli* and *S. aureus* to NAg ($d = 5$ nm for the *E. coli* study)^{9,57}. Another study reported a depletion of intracellular K^+ levels in NAg-treated *E. coli* (citrate-stabilized, $d = 9.3$ nm), which was correlated to disruption of membrane integrity⁵⁸. K^+ ion is present in abundance (0.3 - 0.5 μ M) in bacteria⁵⁹ and has been known for its role in maintaining cell homeostasis. Following exposure to NAg, the study further observed influx into *E. coli* of an otherwise impermeable toxic compound sodium dodecyl sulphate (SDS), immediately inhibiting the cell proliferation.

Studies have shown that the NAg-bacteria interactions are driven, at least in part, by electrostatic attraction involving the physiological net negative charge of most bacterial surface^{9,58} and that of the particles' surface, the latter as mentioned earlier, is governed by the presence of surface functional groups. Badawy *et al.* reported the highest bactericidal activity with the most positively-charged particles studied, the BPEI-functionalized NAg (ζ -potential = $+40$ V) on *Bacillus* sp., while the most negatively-charged citrate-NAg (ζ -potential = -38 V) exhibited the least activity (d all particles = $10 - 18$ nm)³⁰. Other study with the disk-diffusion method of antibacterial assessment also reported comparable trend on the effects of particles' net surface charge on the growth of *S. aureus*, *S. mutans*, *S. pyogenes*, *E. coli*, and *P. vulgaris*³³. Interestingly, studies have also reported adverse NAg-bacteria interactions even with the net negatively-charged particles. Ramalingam *et al.* observed ruptures along with formation of large pores on the surface of *E. coli* and *P. aeruginosa* following exposure of the bacteria to NAg with presence of negatively-charged proteins on its surface (ζ -potential = -17 V, $d = 7.1$ nm)³¹. It can be deduced from the studies however, that net negatively-charged NAg particles would require higher dosage to exert comparable antibacterial activity to those of the net positively-charged counterparts.

2.3.2. Cellular internalization of NAg and its internal targets

Apart from interactions with the cell surface, there is also evidence of NAg uptake by bacteria. Upon exposure of *E. coli* to NAg ($d = 16$ nm), Morones *et al.* observed cellular internalization of the particles using TEM with further elemental mapping analysis

revealing their uniform distribution in the bacteria⁵⁶. Similar particle internalization were shown by Xu *et al.* in *P. aeruginosa*⁶⁰. In addition to TEM analysis that also displayed particle internalization, Sondi and Salopek-Sondi used energy dispersive X-ray spectroscopy (EDAX) to further demonstrate particle internalization⁵⁵. The analysis displayed Ag spectrum inside *E. coli* culture exposed to 50 $\mu\text{g}/\text{cm}^3$ NAg ($d = 12 \text{ nm}$) whereas no Ag spectrum identified in non-treated *E. coli*.

Once internalized, the particles are hypothesised to undergo further leaching, hence the so-called Trojan horse-type effect, releasing Ag^+ ions intracellularly⁶¹. Although the hypothesis has only been proven in mammalian cells⁶², studies conducted in bacteria have already shown the intracellular presence of Ag^+ . One of the examples is reported by Bondarenko *et al.* using bioluminescence that is induced by bioavailable Ag^+ ions in genetically-engineered *E. coli*⁶³. They showed that when *E. coli* was exposed to NAg ($d = 15 \text{ nm}$, casein-functionalized) for 4 h, intracellular Ag^+ concentration was 3-4 more than that of the ultracentrifuged supernatant of NAg suspensions, that translates into higher bacterial toxicity and highlighting the necessary presence of the nanoparticle⁶³. Other study that measured Ag concentrations inside NAg-treated *P. aeruginosa* ($d = 5 \text{ nm}$, PVP-coated) using ICP-MS (inductively coupled plasma spectrometry-mass spectrometry) also reported similar results⁶⁴.

The high affinity of Ag^+ ions to amine (NH_x) and thiol ($-\text{SH}$) electron donor groups of amino acids, such as histidine ($-\text{NH}^+$), arginine ($-\text{NH}_2^+$), lysine ($-\text{NH}_3^+$), as well as cysteine ($-\text{SH}$) and methionine (sulphur in thioether), is most likely a factor to the known NAg targeting on enzymes and structural proteins in bacteria, which in many cases destabilize and in turn, inactivate the proteins. Using the iodinitrotetrazolium (INT) assay, Li *et al.* reported inhibition of the respiratory chain dehydrogenase following exposure of *S. aureus* and similarly, of *E. coli* to NAg ($d = 5 \text{ nm}$)^{8,9}. Yan *et al.* found that Ag^+ indeed bound to bacterial proteins in *P. aeruginosa* following NAg treatment ($d = 5 \text{ nm}$, PVP-coated, $1.2 \mu\text{g}/\text{mL}$)⁶⁴. Using a combination of gel electrophoresis (GE) and ICP-MS, the study reported Ag^+ -binding to GroL and TufA proteins that functions in DNA and protein synthesis, respectively. The same proteins were identified in Ag^+ -exposed bacteria upon treatment ($0.6 \mu\text{g}/\text{mL}$), suggesting Ag^+ might be one of the toxicity sources for NAg. In thiol-based compounds, Liao *et al.* also demonstrated using Ellman's assay that equiv. 1M and 2M Ag^+ could bind to thioredoxin reductase and thioredoxin – both work simultaneously as a two-component antioxidant system in *S. aureus*, respectively⁶⁵. Further, they showed that the binding not only reduced the enzymes' activity but also enlarged the enzymes' size, implying the disruption in both enzyme's function and structure.

The presence of NAg in bacteria could also lead to the formation of particle-protein corona, protein layers surrounding the particle. Wigginton *et al.* used SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) to identify a number of proteins from *E. coli* that binds specifically to bare or carbonate-coated NAg ($d = \sim 30$ nm)⁶⁶. They observed that among those proteins, tryptophanase (TNase) have an especially high affinity to both bare and carbonate-coated NAg. Further, they found that TNase activity decreased significantly when bound to NAg. Using MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight) mass spectrometry, they observed difference of peptide abundance or presence in mass spectra for bare and carbonate-coated NAg suggesting preferential binding of some protein fragments based on surface coating. One high binding protein fragment contained a residue (Arg103) that is part of the active site. The formation of Ag adducts were identified for some fragments and found to be the characteristic of strong binding to NAg rather than association of the fragments with Ag^{+66} .

Next, the NAg was observed to target DNA in bacteria that consequently lead to the loss of its structure and function. A TEM study of 6 h NAg-treated ($d = 5$ nm) *S. aureus* displayed DNA condensation, which was demonstrated by the occurrence of high density region in the centre of the cell (see **Figure 2.4B**) as compared to the untreated cell (**Figure 2.4A**)⁸. Similarly, Baldi *et al.* observed clustered electron-dense region, not only in the centre but also scattered throughout cytoplasm following 3 h NAg exposure to *E. coli*⁶⁷, suggesting an interaction between DNA and silver. Via 3D fluorescence spectroscopy analysis, they confirmed the presence of bacterial DNA fragmentation based on the occurrence of two new peaks between wavelength 400 – 700 nm that changes the fluorescence intensity of the DNA extracted from the treated *E. coli* as compared to the untreated. The DNA was also shown to release Ag^{+} ions following scanning electrochemical microscopy (SECM) and anodic stripping voltammetry (ASV) measurement. In the study with Ag^{+} (supplied as AgNO_3), Feng *et al.* observed the presence of twisted and condensed string-like structure in the centre of both *E. coli* and *S. aureus* cells upon treating them with 10 $\mu\text{g/mL}$ of AgNO_3 solution⁶⁸. Using EDAX, this study suggested that the structure belongs to the condensed DNA, as high amount of phosphorus was identified.

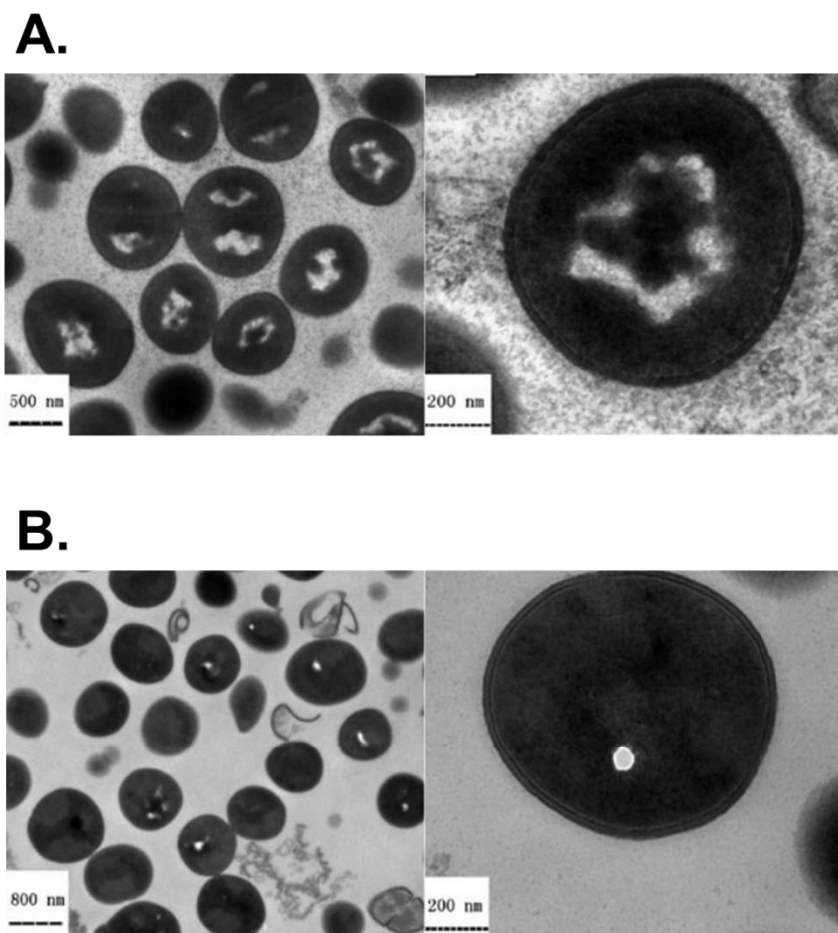


Figure 2.4. The TEM micrographs of *S. aureus* internal structure⁸. A) Untreated cell and B) NAg-treated cell, note the DNA condensation. (Adapted with permission from Springer Nature)

NAg has been indicated to affect metabolic process, in this case the purine metabolism in *E. coli*, evident by the depletion of guanosine as well as the rise of adenosine and hypoxanthine peaks analysed by surface-enhanced Raman spectroscopy (SERS) following exposure of the bacteria to citrate-stabilized NAg $d = 18$ and 80 nm)⁶⁹. The bacterial exposure to NAg also reduced the amide peak, an indicative of inhibition on protein synthesis. Using proteomic or transcriptomic approach, many studies have reported various metabolic processes affected by the presence of NAg. One example is the study on *E. coli* upon exposure to 25 $\mu\text{g/mL}$ NAg ($d = 10 - 15$ nm) that revealed alteration of tyrosine phosphorylation profile⁷⁰. This profile is of important in various cellular processes, e.g. bacterial growth and signal transduction⁷¹. Using microarray and Real-Time PCR (polymerase chain reaction), McQuillan and Shaw observed significant upregulation in the expression of genes encoding cysteine biosynthesis, iron-sulphur cluster synthesis, and transport proteins after exposure to NAg ($d = 142$ nm, 40 $\mu\text{g/mL}$) to *E. coli*⁷². Interestingly, the same genes were also highly expressed when *E. coli* was treated with Ag^+ (0.4 $\mu\text{g/mL}$, supplied as AgNO_3)⁷². In other related study with ionic silver, Yamanaka *et al.* reported loss of intracellular proteins

expression, including the 30S ribosomal protein S2 (the smaller ribosomal subunit functions as the 'mRNA' reader for protein synthesis), as well as proteins involved in the TCA cycle (succinyl coenzyme A synthetase, which catalyses the reversible conversion of succinyl coenzyme A to succinate), and glycolysis (fructose-bisphosphate adolase, catalyses fructose 1,6-bisphosphate to dihydroxyacetone and glyceraldehyde 3-phosphate), following 3 h exposure of *E. coli* to 900 ppb Ag^+ ions⁷³.

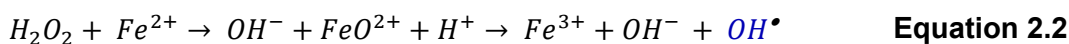
Based on these above-mentioned studies, studies have shown multiple actions of NAg antibacterial targets, starting from particle interaction with cell outer surface to internalization and interaction with various bacterial intracellular compounds. Many studies also prove that NAg antibacterial activity shares its antibacterial target with Ag^+ , therefore, reasonable to acknowledge Ag^+ ions toxicity as a part of NAg's.

2.3.3. Generation of intracellular reactive oxygen species (ROS)

Numerous studies have reported elevated generation of reactive oxygen species (ROS) in bacteria, including the superoxide anion ($\text{O}_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($\text{OH}^{\bullet}$), and its association to cell death during NAg exposure. One of the most recent evidence was by Ramalingam *et al.* that used the cell permeable ROS-reporter dye 2',7'-dichlorodihydrofluorescein diacetate ($\text{H}_2\text{-DCFDA}$), which is oxidized when in the presence of oxygen radicals to the fluorescent dichlorofluorescein (DCF) upon excitation (at 503 nm)³¹. The work detected an at least 70% higher level of ROS relative to the cell-only controls in *E. coli* and *P. aeruginosa* within 4 h exposure of the bacteria to NAg (protein-functionalized, $d = 7$ nm, at minimum bactericidal concentration (MBC) 4.5 and 2.7 $\mu\text{g/mL}$ dosage in the respective bacteria). The work also found depletion of the antioxidant molecule glutathione at these particular nanoparticle concentrations, indicating increased susceptibility of the bacteria to oxidative stress that leads to the observed cell death. In other studies, the role of ROS in NAg antibacterial activity was demonstrated with the addition of N-acetylcysteine (NAC), an antioxidant, to *E. coli*-NAg exposure systems ($d = 3$ nm). The extent of cell killing was significantly lowered upon addition of the anti-oxidant than that of without⁷⁴.

It is important to note that ROS, such as H_2O_2 and $\text{O}_2^{\bullet-}$, are naturally present in aerobic bacteria as by-products of respiration⁷⁵. Bacteria are equipped with anti-oxidant mechanisms to maintain these oxygen radicals at safe threshold. Firstly, the $\text{O}_2^{\bullet-}$ -activated *soxRS* regulon will transcribe the ROS-scavenging proteins; *sodA* (superoxide dismutase), *acnA* (aconitase A), and *nfo* (endo-nuclease IV) to convert $\text{O}_2^{\bullet-}$ to H_2O_2 . Further, *gorA* (glutathione reductase) and *kdtG* (catalase), transcribed by H_2O_2 -activated *oxyR* regulon, convert H_2O_2 to H_2O , protecting the bacteria from ROS

damages^{76,77}. Uncontrolled production of ROS can overwhelm the anti-oxidant capacity, causing disruptions in biomolecules such as DNA, lipids, and protein⁷⁸. The NAg-induced generation of cellular oxygen radicals are proposed by many studies to result from the Fenton reaction (see **Equation 2.2**).



ROS-mediated toxicity has also been indicated with ionic silver, for example in *E. coli* and *S. aureus*, with increased expression of *soxS* gene that encodes the superoxide radical sensor⁷⁹. A study conducted by Holt and Bard suggested the disruption of cell respiratory chain due to possibility of Ag^+ ions binding to NADH in complex I protein, shown by the inability of the protein to take up ferricyanide, an alternative electron acceptor⁸⁰. In other study using a fluorescent dye, 3-(*p*-hydroxyphenyl) fluorescein (HPF), Gordon *et al.* detected increasing cellular ROS presence with *S. epidermidis* upon exposure to Ag^+ (supplied as $AgNO_3$)⁸¹. The cellular ROS levels indeed significantly decreased following addition of thiourea, an iron quencher, an indication of Fenton reaction presence.

Fenton reaction is a Fe-catalysed reaction that oxidating Fe^{2+} to Fe^{3+} while reducing H_2O_2 to hydroxide (OH^-) and hydroxyl radical ($OH^\bullet$), the most reactive oxygen radical⁷⁵. The increase presence of Fenton-active Fe^{2+} ion may be facilitated by the attack on iron-sulphur cluster (e.g. 4Fe-4S) directly by the Ag compound or indirectly by the presence of $O_2^\bullet$ ^{-75,82}. In addition, the Fenton-generated hydroxyl radical has been hypothesised to also attack the iron-sulphur clusters to release the Fenton-active Fe^{2+} ⁷⁸.

Quite recent transcriptomic study in NAg- ($d = 142$ nm) and Ag^+ -exposed *E. coli* observed an at least 2-fold increased expression of some genes in *isc* and *suf* genes system⁷². The former is responsible for the assembly and scaffolding the protein whereas the latter facilitates the desulphurization of cysteine, producing S^0 ⁸³. The results indicate the bacterium's attempts to compensate the damaged clusters⁷².

Apart from triggering a ROS overproduction, it is thought that the presence of NAg or silver compound also interfere with the antioxidant defence mechanisms. Studies have reported enzyme inactivation in the presence of Ag, most likely as a result of the high affinity of Ag^+ ions to thiol ($-SH$) compound. For example, a 3-fold reduction in aconitase enzyme activity was detected in *S. epidermidis* when treated with ionic silver (supplied as 0.06 mM $AgNO_3$) compared to untreated cells⁸¹. This is in agreement with the anti-oxidant GSH depletion as described by Ramalingam *et al.*

To summarize, based on the abovementioned studies, NAg has been shown to work by creating morphological damages, interacting with essential intracellular compounds, and generating ROS that could not be intracellularly passivated due to NAg interference with the intracellular ROS defence mechanisms.

2.4. Bacterial Defence to NAg Antibacterial Activity

2.4.1. Endogenous and Exogenous Resistance Determinants to Ag^+ ions

One of the first endogenous Ag^+ resistance mechanisms to be identified is the porin system. Located in the outer membrane of Gram-negative bacteria, the OmpF and OmpC porins are postulated to act as channels for the passive diffusion of ions and molecules. Li *et al.* subjected *E. coli* strains (Ag^+ MIC = 8 $\mu\text{g/mL}$) to prolonged AgNO_3 exposure and found that, unlike the wild-type strains, the corresponding resistant strains (~124-fold MIC increase) did not express OmpF and OmpC porins, indicating the bacteria's intention to hinder intracellular accumulation of Ag^+ ⁸⁴. The expression of *ompF* and *ompC* are regulated by EnvZ, a cytoplasmic membrane protein that is thought to function as Ag^+ sensor, together with OmpR as a response regulator (see **Figure 2.5**)⁸⁵, widely studied as an osmoregulator in Gram-negative bacteria, particularly *E. coli*⁸⁶. As two-component sensor/responder, EnvZ uses ATP to undergo autophosphorylation and transfer the phosphate group to OmpR, hereby activate it, and subsequently stimulate *ompF* or *ompC* expression^{86,87}.

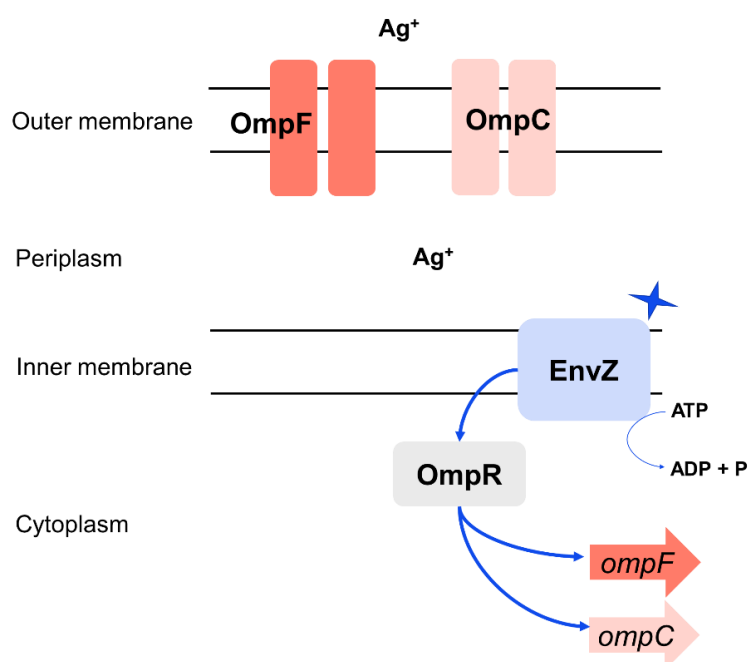


Figure 2.5. Schematic representative of OmpF and OmpC porins in the presence of Ag^+ .

Other endogenous Ag^+ resistance determinants is the *cus-RSCFBA* gene cluster (see **Figure 2.6**, previously known as the *ylcA-ybcZ* and *ylcBCD-ybdE* gene clusters), with numerous studies reporting their role in facilitating the efflux of intracellular Ag^+ in bacteria, despite the initial postulation of the gene cluster as part of only the copper resistance mechanisms⁸⁸. The two-component CusRS sensor/responder protein is thought to act as the first line of Ag^+ defence. The detection of Ag^+ by the cytoplasmic membrane histidine kinase CusS will prompt the CusR, a cytoplasmic transcriptional regulator, to initiate the transcription of the *cus* system. Gudipaty *et al.* reported a 5-fold Ag^+ MIC decrease for *E. coli* ΔcusS (deletion of *cusS* gene in the bacteria) in comparison to the wild-type strain, which was associated with reduced *cusC* expression in the ΔcusS mutant to that of the wild-type strain⁸⁵. Note that subsequent introduction of *cusS* back to the ΔcusS strain did not resume the *cusC* expression to its original level. The three-component CusCBA cation/proton antiporter is thought to function as efflux pump, pumping periplasmic Ag^+ out of the bacteria in exchange of H^+ into the bacteria. Finally, the periplasmic protein CusF is proposed to function as a chaperone, capturing the periplasmic Ag^+ and passing them to the CusCBA antiporter. Franke *et al.* further observed an at least 2.5-fold higher expression of *cusC* in *E. coli* (the bacteria contained vectors with *cusC*) following exposure to 5 and 10 μM Ag^+ (from AgNO_3), relative to that of in the absence of Ag^+ ⁸⁹. Relative to the wild-type strain, the Ag^+ MIC for *E. coli* ΔcusA was 1.5-fold lower.

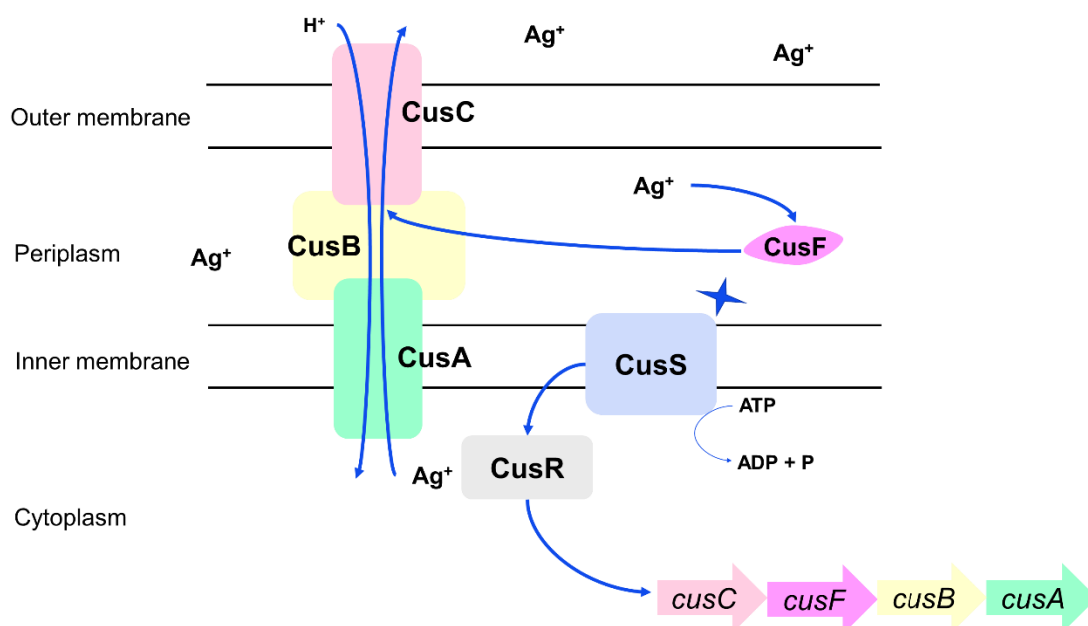


Figure 2.6. Schematic representative of Cus system in the presence of Ag^+ .

Further research inquiries have deduced **inter-correlated actions of the porins (*omp*) and the *cus* systems** in Ag^+ resistance. Upon prolonged exposure of *E. coli* to Ag^+ (supplied as AgNO_3), Randall *et al.* found a point mutation on the *ompR* (G596A

mutation) and *cusS* (G1130A mutation) genes in the resistant bacteria, with MIC >256 mg/L. Introducing the *ompR* and *cusS* point mutation, one at a time in the wild-type *E. coli*, did not result in Ag⁺ resistance with MIC ~4 mg/L, similar to the wild-type bacteria⁸⁸. Interestingly, when both the *ompR* and *cusS* mutations were introduced at the same time, indeed, the bacteria confer resistance to Ag⁺ (MIC >256 mg/L).

Randall *et al.* noticed that the mutation acquired in *ompR* resulted in the loss of OmpR, deduced by the similar growth response by both the *E. coli ompR*_{G596A} and *E. coli ΔompR* in the presence of 2 mg/L Ag⁺⁸⁸. Loss of OmpR infers the aftereffect: loss of the function to initiate the transcription of OmpF and OmpC in the bacterial system, hence preventing the Ag⁺ influx via these two protein channels. Next, via qRT-PCR (quantitative reverse transcription-PCR), the study also observed a 6-fold expression of *cusABC* in the *E. coli ompR*_{G596A} *cusS*_{G1130A} compared to the parents strain, indicating an elevated work of the *cus* operon in the presence of no OmpF/C⁸⁸. In agreement to that, Lok *et al.* have also observed that in the presence of 100 μM AgNO₃, Ag-resistant *E. coli ΔompF ΔompC* expressed CusF and CusB, whereas the expression could not be identified in the Ag-susceptible strain⁹⁰. Taken together, it is hypothesised that the loss of Omp function prevents silver influx and intracellular accumulation, while simultaneously the Cus system transports out the already-intracellularly-present silver. This implies the collaborative work between the endogenous Ag⁺ mechanisms of resistance to reduce the susceptibility of Gram-negative bacteria to silver.

The exogenous genetic determinants for Ag⁺ resistance were first identified in the Gram-negative *Salmonella typhimurium* plasmid (pMG101) isolated from a burn unit at Massachusetts General Hospital, responsible for septicaemia death, forcing the closure of the unit⁹¹. Based on homologies with other metal-resistant determinants, the plasmid-borne resistance genes were designated as the *sil-CFBAGPRSE* (see **Figure 2.7**) cluster with deduced functional features as follow^{88,92}. SilCBA is a presumed three-component membrane protein that pumps periplasmic Ag⁺ ions out from the cell in exchange of protons. The Ag⁺ efflux system comprises of SilA, an inner membrane cation/proton antiporter, SilB, a fusion protein that connects the inner and outer membranes of Gram-negative bacteria and SilC, an outer membrane protein (with homologs in the cadmium, zinc and cobalt *CzcCBA* resistance system in *Alcaligenes sp.*, a Gram-negative bacteria genus). The periplasmic protein SilE is a 143-amino-acid Ag⁺-specific binding protein (with 47% identical amino acids to the proposed metal-binding protein of the *E. coli* plasmid copper resistance system PcoE). The delivery of the periplasmic Ag⁺ to the cation/proton pumps SilCBA is thought to be facilitated by a 96-amino acid polypeptide, designated as SilF. Note that this rather small ORF (open

reading frame that can be translated into a protein) is present between *silC* and *silB*. The ORF, although is 45% identical to a 110-amino acid ORF in *E. coli* chromosome, is not found in other homologous CBA systems. Next, *SilP* is an 824-amino acid P-type ATPase inner membrane protein, which is deduced to transport cytoplasmic Ag^+ to the periplasm. Another small ORF was found between *silA* and *silP*, to potentially encode a 105-amino acid polypeptide. However, up to this stage, no known homologs have been found for the ORF. Finally, *SilRS*, a presumed two-component sensor/responder pair comprised of an inner membrane histidine kinase *SilS* to function as Ag^+ sensor (with 29% identical amino acids to *E. coli* *PcoS*) and to subsequently transmitting signals to the cytoplasmic transcriptional regulatory responder *SilR* (with 55% identical amino acids to *E. coli* *PcoR*) to activate the transcription of the *sil* cluster.

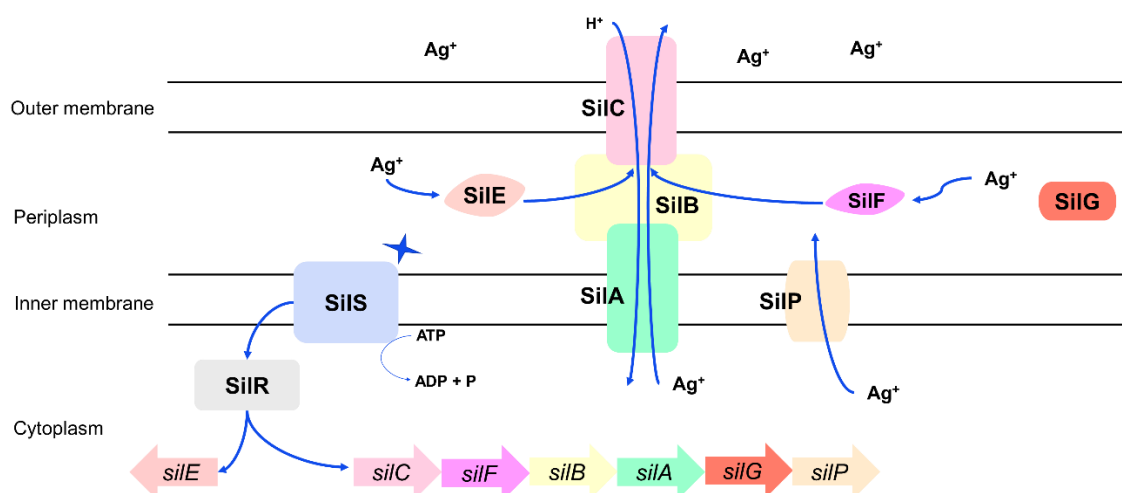


Figure 2.7. Schematic representative of *Sil* system in the presence of Ag^+

Validating the Ag^+ resistance effects, exposure of *E. coli* that contained the pMG101 plasmid (by conjugation) to high Ag^+ ions concentration (supplied as 600 μM AgNO_3) did not result in growth inhibition, with comparable extent of growth as that of without Ag^+ . The wild-type strain (no pMG101 plasmid), on the other hand, could not cope with the toxicity at $>100 \mu\text{M}$ AgNO_3 ³⁸. To investigate the role of the *sil* genes, Randall *et al.* deleted *silC*, *silB*, *silA*, and *silE*, individually, from the above described *E. coli* (pMG101) and found that the MIC of AgNO_3 on the bacterium was reduced from $>256 \text{ mg/L}$ to only 4 – 8 mg/L in all the mutants⁸⁸. Interestingly, the study further observed that deletion of *silF*, *silG* and *silP*, individually, or even together (*silG* and *silP*), did not change the MIC, indicating no loss of the resistance effects.

Although the *Cus* system (endogenous) and the *Sil* system (exogenous) shared similar function in a two-component sensor/responder protein (*CusRS* and *SilRS*), a three-component cation/proton membrane protein (*CusCBA* and *SilCBA*) and a chaperone protein (*CusF* and *SilF*), these previous studies imply that *sil* cluster is deemed to

potentially function alone, without the necessity of having other complementary system, as the loss of resistance was obtained without disrupting Cus or Omp system. In addition, *sil* cluster is transferrable to other bacteria as it is located in bacterial mobile genetic elements (MGE). Indeed, the prevalence of this cluster has been shown quite recently in the Gram-negative (*A. baumannii* and *P. aeruginosa*), and even the Gram-positive bacteria (*S. aureus*)^{11,93}. *Sil* genes have also been found in other plasmid, either in a complete set (MIP233, R478, pAPEC-O1-R and R27 plasmids in *E. coli*) or partially (MIP235, R476b, pWR23 plasmids in *E. coli*)⁹⁴.

Up to this stage, studies have shown the possible defence mechanism to Ag⁺ ions consists of outer membrane porins and Ag⁺ efflux system in Gram-negative bacteria, preventing the accumulation of silver in the cell. It is quite likely that the bacterial defence to Ag⁺ ions take places to combat one of substantial disruption Ag⁺ ions created, which is the electron imbalance. Considering the similar properties that Ag⁺ and NAg share, these defence mechanism can potentially form a part of the resistance determinants to NAg.

2.4.2. Bacterial resistance to NAg

Over the last five years, increasing number of research inquiries have reported the natural ability of microbes to adapt to NAg. In 2011, Khan *et al.* reported *B. pumilus*, isolated from a sewage in India and confirmed via 16S rRNA analysis, grow naturally at high NAg concentrations (10 – 200 mg/L, d = 10 – 40 nm) as if no toxicity was present⁹⁵. Nevertheless, the study observed less exopolysaccharide production upon treatment with NAg. Using FT-IR spectra, the study observed a match between the exopolysaccharide from lyophilized particle inoculated with *B. pumilus* for 4 h and the exopolysaccharide extracted from the supernatant of *B. pumilus*-only culture, suggesting that the bacterial exopolysaccharide coat the NAg, prevent Ag⁺ ions release thus passivating the toxicity. Indeed, further study showed that coated NAg was found to have no effect on inhibiting the growth of *E. coli*, *S. aureus*, and *M. luteus*⁹⁵.

A more recent research study was reported by Das *et al.* who exposed microbial communities from natural waters to one-off dosing of carboxy-capped NAg at 0.01 to 1 mg Ag/L (d = 1 – 10 nm) for 5 days⁹⁶. Except for the highest NAg concentration, the study observed that the microorganism started to regain their metabolism and respiration after 2 days of NAg exposure, as determined by their ability to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) dye, reaching almost similar metabolism of the no-treatment group. In addition, NAg exposure also alters the community composition. Via terminal restriction fragment length polymorphism (TRFLP) analysis

throughout the exposure of 0.1 and 1 mg Ag/L for 5 days, four community groups were identified: a) intolerant (lost the ability to survive), b) recover (initially defeated but gained the ability to proliferate overtime), c) tolerant (maintained natural growth during the treatment course) and d) stimulated (initially absent but gradually occurred upon NAg exposure). For the three latter microbial communities, there could be a possibility of a phenotypic resistant trait due to a natural adaptation to NAg.

Natural waters, sewages, and other water reservoir in the environment are most likely exposed to NAg. As the most commercialized metal nanoparticle, the incorporation of NAg in a wide range of daily goods increases the possibility of silver particle release to the environment. Following the release, the environment could dilute the bioavailable silver and create a low (non-lethal) condition for microorganism to grow and adapt to the otherwise toxic concentration.

However, these thought-to-be resistant characteristics reported previously may be caused by the depletion of bioavailable Ag^+ , shown by the exopolysaccharide coating⁹⁵ that hinders the particle oxidative dissolution (see Section 2.2.1) or insufficient toxicity occurred from one-off dosing for a prolonged exposure study⁹⁶. Therefore, to ensure sufficient bioavailable silver toxicity, further studies have been conducted by a sequential dosing method, in which exposing the microorganisms to new fresh antibacterial dosage daily for a period of time.

The first study on the development of bacterial resistance towards NAg *via* repeated induced adaptation was reported by Gunawan *et al.*, who treated *Bacillus* sp. daily with freshly prepared NAg (2 – 3 nm Ag(0) or Ag_2O deposit on 30 nm TiO_2 support) at 3 and 7 mg Ag/L for 13 days¹². The phenotypically-resistant *Bacillus* sp. were shown to be capable of proliferating significantly at an otherwise lethal NAg concentration (7 and 10 mg Ag/L) and even exhibiting growth enhancement (3-fold biomass yield) to that of the wild type in the absence of NAg (**Figure 2.8**). The study identified not only the prevalence of *silB* and *silP* in the plasmid of the native strain but also multiple single nucleotide mutations (SNPs) in the bacteria genome (unpublished data). This work, as well as a recent work in *S. aureus*¹³, has shown that bacteria can adapt to NAg toxicity by means of cell survivability responses, not by the elimination of silver toxicity, and pointing out the chance of obtaining an acquired resistance trait *via* genomic adaptation.

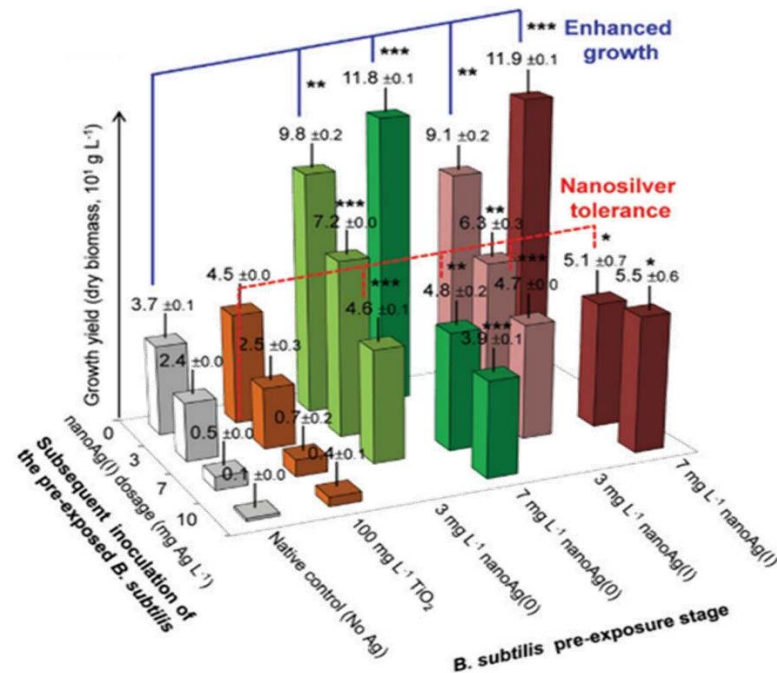


Figure 2.8. Growth of the resistant *B. subtilis* NA_g (0 – 10 mg/L) following the pre-exposure (3 or 7 mg/L NA_g) when compared to the control¹². (Reproduced with permission from John Wiley and Sons)

Further elucidation on comparative genomics of NA_g-resistant strains was reported by Graves *et al.* following a sequential NA_g exposure for approximately 29 days to *E. coli* (citrate-coated particle, $d = 10 \text{ nm}$, 50, 100, 125 $\mu\text{g/L}$)¹⁶. Indeed, the study observed the growth of the NA_g-exposed *E. coli* at toxic NA_g concentrations (250 – 750 $\mu\text{g/L}$, $\text{OD}_{620} = 0.3 - 0.4$, compared to OD_{620} of NA_g-susceptible *E. coli* = 0.05 – 0.2) after 24 h inoculation, indicating the emergence of NA_g resistance in *E. coli*. Whole genomic comparative study between the resistant and susceptible strain showed three non-synonymous chromosomal point mutations on *cusS*, *purL*, and *rpoB* which are responsible for encoding Ag⁺ ions efflux protein, purine biosynthesis protein, and RNA polymerase, respectively. In addition to that, three insert-deletion (indels) mutations were also identified in *ompR*, genes encoding for the regulation of outer membrane protein in Gram-negative bacteria.

Contradictory with what has been reported previously, Panacek *et al.* found no genomic changes in the resistant strains of *E. coli* albeit the emergence of NA_g resistance ($d = 28 \text{ nm}$)¹⁴. Following 6 and 11 cultivation steps, the tested bacteria, *E. coli* and *P. aeruginosa*, were able to grow at 4 times their inhibition concentration (MIC of the susceptible strains = 3.38 and 1.69 $\mu\text{g/mL}$, respectively) and further treatment up to 20 cultivation steps enabled the bacteria to proliferate at 54 $\mu\text{g/mL}$. Note that 1 cultivation step consists of NA_g-exposure, free-NA_g cultivations in media for 24 h each in a subsequent event. Detailed transmission electron microscopy (TEM) imaging

revealed particles' aggregation in the presence of phenotypically-resistant *E. coli* (**Figure 2.9**) and was thought to cause by the rich presence of flagellin, a protein used for the formation of bacterial flagella, identified *via* proteomic mass spectrometry and tandem-mass spectrometry. Indeed, the inoculation of NAg-resistant *E. coli* in the presence of NAg (0 – 54 µg/mL) and pomegranate rind extract (PGRE), a flagellin inhibitor, at its subinhibitory concentration (0.6% w/w) proved the loss of *E. coli* resistance to NAg as the MIC dropped back to its susceptible value.

Taken together, these findings present the progressive evidences of the NAg resistance in our midst, an often overlooked phenomenon due to the superiority of NAg antibacterial activity. With massive NAg commercialization in the past years¹⁰, the findings point out the rising possibility of the society repeating the similar antibiotic-resistance cycle, which is an unfortunate issue and lethal to healthcare systems. Further study, undoubtedly, needs to be conducted to understand the still-elusive NAg mode of actions and the protections created by the bacteria towards this toxic antibacterial agent, thus in turn, public protections can be initiated and reinforced.

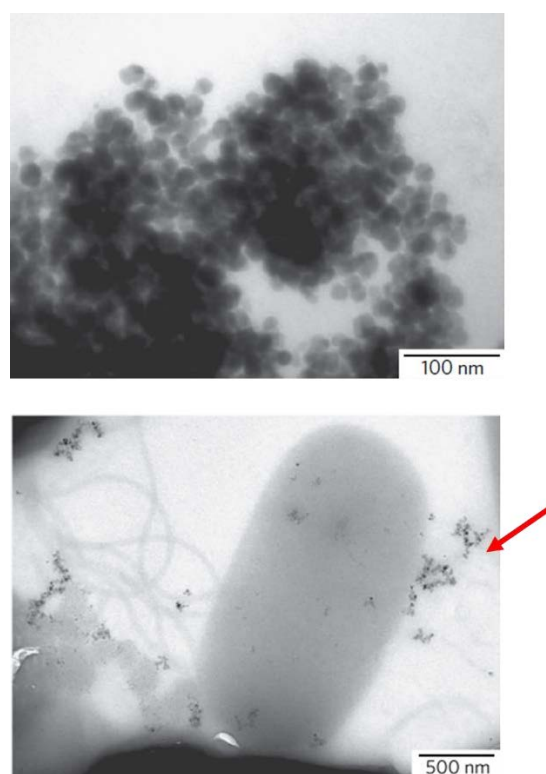


Figure 2.9. Aggregation of NAg (red arrow) by *E. coli* following 24 h inoculation, shown with TEM¹⁴. (Adapted with permission from Springer Nature)

2.5. Knowledge Gaps

In this literature review, studies have shown the capability of NAg multifaceted toxicity on bacteria, thanks to the presence of the solid particle and the behaviour of particle

leaching of Ag^+ ions. The latter is governed by the physicochemical characteristic and presence of silver-complexing and silver-precipitating ligands amidst the particles' surrounding. The attack on the whole cell, from the outer surface, the interaction with intracellular compound e.g. DNA, protein, antioxidant, to over-production of ROS, has made NAg a great alternative to antibiotic, particularly, with the threat of antibiotic resistance bacteria and antibiotic discovery void for the last 30 years. The complexity of the NAg toxicity on bacteria also lead to the thought of low probability of NAg resistance. Nevertheless, in the past years, also when high incorporation of NAg in daily products takes place, the emergence of NAg resistance has been reported in environmental and pathogenic bacteria.

This literature review also discusses bacterial defences to Ag^+ by the chromosomally-encoded *omp* and *cus* cluster, and plasmid-encoded *sil* cluster that works by preventing silver accumulation in the cell and silver efflux. With shared NAg and Ag^+ mechanism of actions, these resistance determinants are believed to contribute in providing bacterial resistance to NAg. Nevertheless, previous studies have reported different mechanisms that confer resistant to NAg, such as mutation and epigenetic changes, despite the fact that these Ag^+ resistance determinant genes are inherited in the reported NAg-resistant Gram-negative bacteria, *E. coli* and *P. aeruginosa*. Up to this stage, unlike in Gram-negative bacteria, no silver resistance mechanisms, either endogenous or exogenous, have been reported in Gram-positive bacteria. These imply that there is still a need to better understand the NAg resistance mechanisms in bacteria. In addition, to this date, mechanisms of NAg resistance in Gram-positive bacteria has not been reported.

To address the knowledge gaps, firstly, this study subjected *Staphylococcus aureus*, the most prominent Gram-positive bacteria causing diseases, to a prolonged exposure to daily-dose of NAg, followed by a confirmation of susceptibility changes in the resistant strains. Further, this study sought to investigate *S. aureus* unique mechanisms of defence to NAg by performing whole genome study to identify the possibility of heritable mutation changes in comparison with the parental strain. Next, to have a better and deeper understanding of the mechanism, whole transcriptomic study was also utilized comparing the resistant strains and parental strain with or without the presence of NAg. This thesis also included the study of *S. aureus* resistance development to Ag^+ and, followed by the mechanism of defence and its comparison to that of NAg.

The nanoparticle used in this study (6.6 wt% Ag ($d_{\text{TEM}} = \sim 2$ nm) homogenously dispersed on 30 nm TiO_2 support) was synthesised *via* flame spray pyrolysis (FSP)

method to generate particles with a more homogenous particle distribution and composition⁹⁷. The use of support, in this case TiO₂, is to represent NAg applications in products. The nanoparticles are typically coated or embedded in support materials for commercial use, e.g. in wound dressings and water filters. The use of Ag/TiO₂ as the model nanoparticle in this study is to also enable comparisons of bacterial toxicity and resistance responses studied by our group^{12,98,99}.

It is hypothesised that *S. aureus* will mutate its physiological genes to develop defence against NAg toxicity. The bacterium is lacking of the known silver resistance determinants (for silver ion) commonly present in Gram-negative bacteria (*omp*, *cus*, and *sil* system, see Section 2.4.1). The physiological changes are likely those that associate with NAg antibacterial targets, including, among others, the cell wall synthesis and DNA/protein repair mechanisms as well as anti-oxidants to overcome cellular oxidative stress (see Section 2.3). With a generally similar cell wall structure *i.e.* thick peptidoglycan layer, (albeit molecular variation¹⁰⁰) and no, or low prevalence of the silver resistance determinants, the mechanisms of resistance can be relevant to other Gram-positive bacteria.

The generated knowledge will shed a light on the toxicological responses and in turn, the adaptation mechanisms of bacteria to one of the most commercialized antimicrobial nanoparticle. The knowledge is believed to have far-reaching contributions in the design of new-generation antibacterial NAg that are less prone to resistance development, as well as, equally important, in the strategies for better risks *versus* benefits assessments of NAg applications, to ultimately ensure the successful long-term use of the alternative antimicrobial.

Chapter 3

The Emergence of Resistance to NAg and Ag⁺ in pathogenic *S. aureus*

3.1. Summary

The ability of microbiologically-active silver species to eradicate a wide-range of bacteria, including pathogenic and multi-drug resistant strains, has been well documented (Chapter 2) although the exact mechanisms of silver toxicity have still not been fully elucidated. The indiscriminate use of antibiotics has seen the rapid spread of resistance. In response to this, silver, an alternative compound to control infection, has been extensively incorporated in vast arrays of everyday products to substitute antibiotics. This, therefore, raises a critical question: is the ability to become resistant to silver widespread amongst genetically diverse bacteria? The study in Chapter 3 demonstrated that clinically-relevant WHO listed priority pathogen bacteria, *Staphylococcus aureus*, with no natural presence of the silver defence mechanisms, was able to grow in an otherwise toxic NAg exposure system following a 50-days exposure. The minimum inhibitory concentration (MIC) of NAg on the passaged strain increased by 3-7-fold compared to the wild-type susceptible counterpart. Similar response was observed in Ag⁺-passaged *S. aureus*, with the MIC of Ag⁺ increasing by ~3-fold than that of the wild-type strain. The resistant strains displayed NAg and Ag⁺ cross-resistances but not to clinically relevant antibiotics tested (ciprofloxacin, vancomycin, and cefotaxime), suggesting the antibacterial mechanism of silver in *S. aureus* differs to that of these antibiotics. This study highlights the ability of a priority pathogen which has been perceived to have no, or very low, capacity for silver resistance, to develop stable resistance characteristics to NAg and Ag⁺.

3.2. Introduction

Staphylococcus aureus is a Gram-positive bacterium, commonly inhabits our skin and nasal cavities and does not cause harm in healthy individuals¹⁰¹. However, as an opportunistic pathogen, *S. aureus* typically infects immunocompromised individuals, open wounds or disrupts commensal microbiome in organs and tissues^{101,102}. In fact, *S. aureus* has been recognized as the leading cause of lethal nosocomial (hospital-related) infections, such as bacteraemia and pneumonia^{102,103} and has the ability to

develop resistance to multiple antibiotics e.g. methicillin-resistant *S. aureus* (MRSA) that cause high mortality¹⁰³ and the emergence of *S. aureus* resistance to vancomycin (VRSA), the last line of defence against the bacteria¹⁰⁴. Therefore, *S. aureus* has been recognized as one of the most prominent Gram-positive bacteria affecting human health, being listed as 'high' level priority pathogen by the World Health Organisation^{17,105}.

Silver nanoparticle (NAg) is ultrafine 1-to-100-nm-size particles of silver (Ag) with superior antimicrobial activities towards a broad spectrum of bacteria, including multi-drug resistant bacteria^{7,22}. Thus, NAg has been proposed as an alternative to antibiotics, particularly due to the continued rise of antibiotic resistance as well as the lack of new antibiotic discovery in the last 30 years⁴. NAg has been incorporated in medical devices (e.g. wound dressing, catheters, and prosthetic implants), numerous consumer products (e.g. clothing, food packaging, baby toys, and fridges)¹⁰, rendering NAg as the most commercialized nanoparticle-based antibacterial agent.

Overuse of NAg in these commercialized daily products has raised an escalating concern on the potential emergence of bacterial resistance. The first observation of NAg resistance was reported by Gunawan *et al.*, in *Bacillus subtilis*, an environmental Gram-positive bacterium¹². The work found that the bacterium developed resistance as a result of prolonged exposure to a sub-inhibitory NAg concentration. Recently, NAg adaptive responses have also been seen in clinically-relevant pathogenic Gram-negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa*^{14,15}. Considering the overuse of NAg that might be in contact with the ubiquitous presence of pathogenic bacteria, this study sought to understand if the resistance to silver is also widespread to other clinically-relevant pathogenic bacteria.

The mechanism of silver resistance has been linked to the presence of the chromosomally encoded Omp/EnvZ and Cus systems, involved in osmoregulation and efflux, as well as the plasmid-encoded silver efflux Sil system, originally identified in Ag⁺-resistant *E. coli* and *S. typhimurium*^{12,106–108} (see Chapter 2). These determinants have also been identified bioinformatically in *P. aeruginosa* (BLAST, this study), therefore, could act as an asset for *P. aeruginosa* to develop silver resistant. A study has also reported the importance of pyocyanin, an electron carrier and acceptor, to facilitate Ag⁺ resistance in *P. aeruginosa*¹⁰⁹. The study observed that pyocyanin mediated Ag⁺ resistance by Ag⁺ reduction to Ag⁰ (metallic Ag) and consequently, reduced the bioavailable Ag⁺ that is toxic to the bacteria. Further, a transient non-genetic adaptation mechanism was detected in *E. coli* to develop resistance to NAg through increased flagellin production, a primary flagella protein attached to the cell

wall¹⁴. The protein was indicated to enhance NAg aggregation, and in turn, passivating the nanoparticle toxicity.

On the other hand, bioinformatic analysis indicates that *S. aureus* has no (BLAST, this study), or very low¹¹, prevalence of these characterised genetically-encoded silver-relevant defence determinants. Further, *S. aureus* does not possess genes encoding for flagellin or pyocyanin proteins which have been demonstrated to be critical for phenotypic emergence of silver resistance. Therefore, this study seeks to investigate and understand if a priority pathogen with no natural presence of any of the known silver defence mechanisms has the capacity to develop resistance to silver *via* specific and novel mechanisms.

To address this, *S. aureus* was subcultured in the presence of increasing NAg concentration, in parallel to increasing ionic silver (Ag⁺) concentrations for 50 days. Following the prolonged exposure, the new adapted growth responses of the passaged strains were studied in the presence of NAg or Ag⁺ and compared to those of the wild-type strain to confirm the resistance acquisition. Growth responses and adaptation to antibiotics were also examined in this study, as well as the potential for silver and antibiotic cross-resistance to give insights into how resistance might be occurring via the novel mechanism

3.3. Experimental Methods

3.3.1. Preparation of antibacterial agents

This study utilized silver nanoparticle (NAg, $d_{\text{TEM}} = \sim 2$ nm, 6.6 wt% Ag homogeneously dispersed on 30 nm TiO₂ support^{12,97}) synthesised by Flame Spray Pyrolysis (FSP) at University of New South Wales (UNSW) and silver nitrate (AgNO₃; Ajax Finechem) as the ionic silver (Ag⁺) source. In addition, this study also used ciprofloxacin (Sigma Aldrich), methicillin (Sigma Aldrich), vancomycin (Sigma Aldrich), cefotaxime (Sigma Aldrich) and lysostaphin (Sigma Aldrich). NAg stock was always prepared fresh in Cation-adjusted Mueller-Hinton Broth (Ca-MHB; BD, Australia) prior to each experiment whereas the rest of antibacterial agents were dissolved in autoclaved water and stored in room temperature (AgNO₃), 4°C (antibiotics) or -20°C (lysostaphin). To ensure a homogeneous suspension, NAg suspension was ultra-sonicated (Vibra-cell, Sonics & Materials, Inc.) at 50% output control for 20 s prior to use. Experiments with NAg and Ag⁺ were performed under dark conditions to render the TiO₂ support of the NAg particles photocatalytically inactive and to prevent Ag⁺ reduction to Ag⁰, respectively⁹⁷.

3.3.2. Determination of Minimum Inhibitory Concentration (MIC)

MIC determination was adapted from broth microdilution methods reported by Clinical and Laboratory Standard Institute (CLSI). Briefly, an overnight culture of *S. aureus* ATCC25923 (henceforth known as the WT strain) in Ca-MHB was diluted to $\sim 5 \times 10^5$ CFU/mL and exposed to various dosages of an antibacterial agent in Ca-MHB in a 96-well microplate at 160 μ L total volume. Upon incubation in a static humidified incubator at 37°C for 24 h, optical densities were measured in a microplate reader (Spark 10M, Tecan Trading AG) at 600 nm for Ag-compound and antibiotic and 650 nm for lysostaphin. The experiments were performed in three biological replicates, each with three technical replicates. Antibacterial-only (cell-free) and cell-only (antibacterial agent-free) controls were employed to serve as a reference to obtain the optical density reading corresponding to bacterial growth in respective dosages and biological replicates, respectively. The MIC was defined as the minimum antibacterial concentration that exhibited $\geq 95\%$ growth inhibition activity relative to the cell-only control.

3.3.3. Visual observation of lysostaphin-suspended *S. aureus*

Cell pellet from 1 mL *S. aureus* overnight culture (37°C, 200 RPM) was resuspended in 0.5 mg/mL lysostaphin (Sigma Aldrich) and incubated (static) for 30 min at 37°C, prior to taking images. Bacterial lysis by lysostaphin is shown by a clear transparent suspension whereas a turbid suspension displays no or less bacterial lysis.

3.3.4. Resistance development of *S. aureus* in response to NAg, Ag⁺ and ciprofloxacin exposure

Resistant *S. aureus* to either NAg, Ag⁺, or ciprofloxacin was obtained by serially culturing the bacteria in Ca-MHB containing the respective antibacterial agents. This study was adapted from Ling *et al.*¹¹⁰. On day 1, the overnight culture of the WT strain was inoculated by 1:100 dilution ($\sim 10^7$ CFU/mL) to increasing concentrations of antibacterial agents, e.g. 0.25x, 0.5x, 1x, and 2xMIC in a 24-well plate at 1 mL total volume (**Figure 3.1**). Following 24 h incubation at 37°C and 150 RPM, the optical density were measured in a microplate reader (Spark 10M, Tecan Trading AG) at 600 nm. Each concentration of antibacterial-only (cell-free) and cell-only (antibacterial agent-free) control were served as references to obtain the optical density reading corresponding to the bacterial growth. Cultures from the highest concentration that allow 80% growth relative to the control were sequentially passaged by 1:100 dilutions to higher ranges of antibacterial concentrations e.g. 0.5x, 1x, 2x, and 3xMIC. The study was conducted for 50 days and 30 days for the silver compound and ciprofloxacin,

respectively, with two independent biological replicates and three technical replicates each. The cell-only control (antibacterial-free) was also sequentially passaged for 50 days. NAg was sterilized for microbial work via gamma irradiation (Co-60 irradiation source at Australia's Nuclear Science and Technology Organisation (ANSTO) at a rate 6 Gy/min for 1 h)¹².

3.3.5. Stabilization of resistance genes and new MIC determination for of the resistant strains

The passaging cultures exposed to NAg, Ag⁺, and ciprofloxacin from selected days (see green, blue, and red circle, diamond, and triangle in **Figure 3.4**) were inoculated to the respective antibacterial-incorporated Cation-adjusted Mueller-Hinton Agar (MHA; BD, Australia) plate at the concentration the cultures were sampled from to select for resistant colonies. The colonies were grown by serial overnight culturing (three times, 37°C and 200 RPM) in an antibacterial-free Ca-MHB to stabilize the putative resistance genes (see **Figure 3.2**). The new MIC for the passaged strains in NAg, Ag⁺, antibiotics (ciprofloxacin, vancomycin, cefotaxime and methicillin) and lysostaphin, as well as the visual observation of lysostaphin-treated bacteria were performed as described previously.

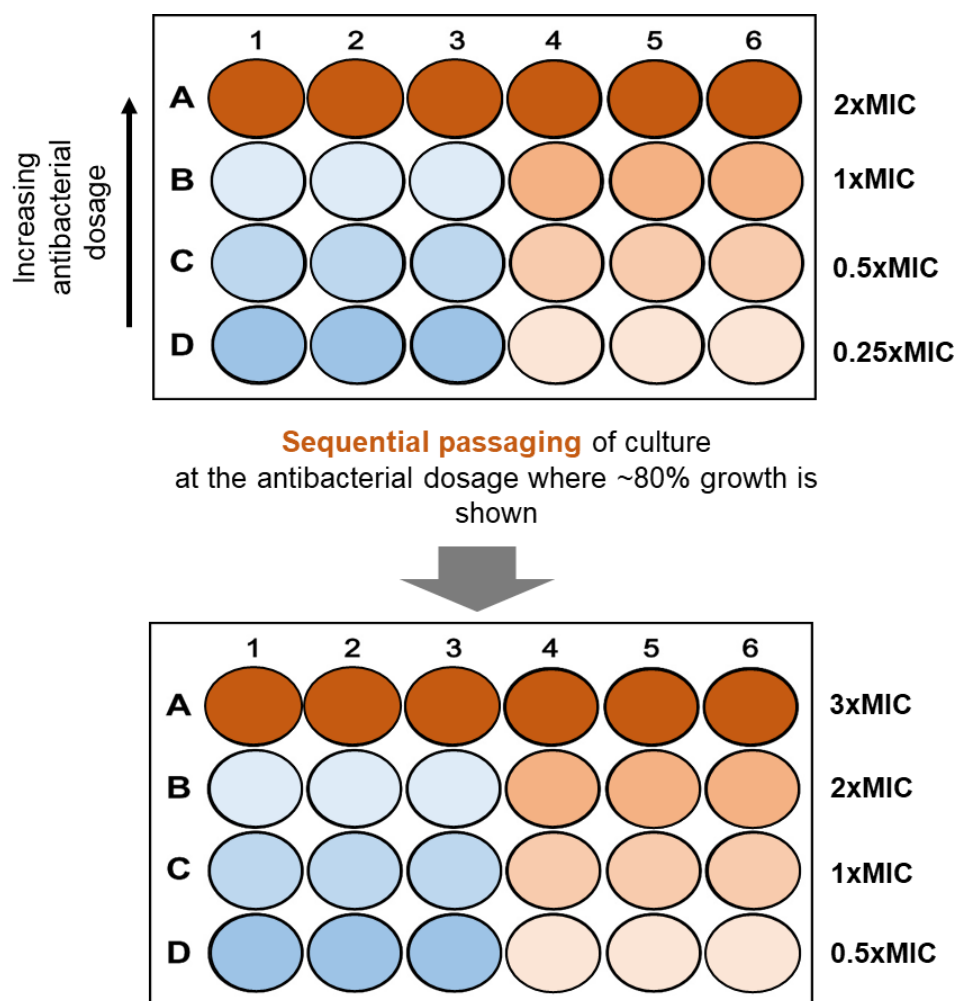


Figure 3.1. Schematic diagram of *S. aureus* resistance development methodology in increasing concentration of antibacterial agents. Shades of blue represents the presence of bacterial growth and shades of orange represents the antibacterial suspensions-only control.

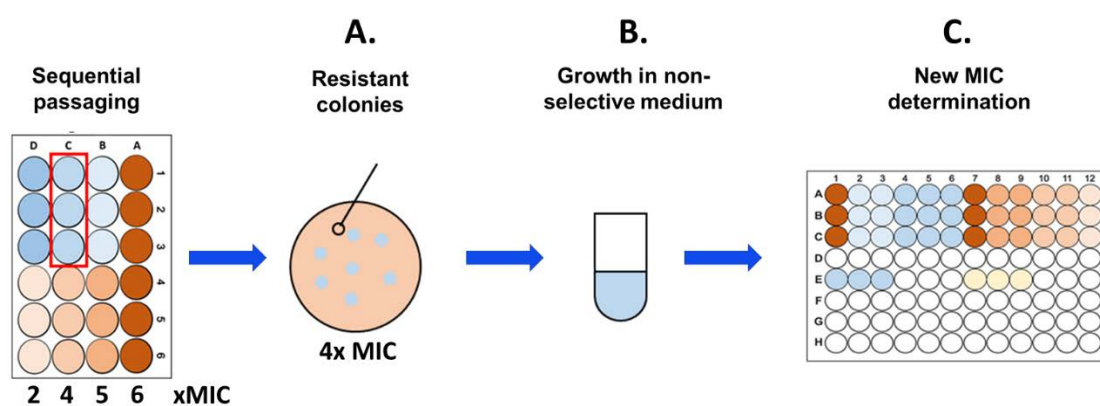


Figure 3.2. Sampling methods prior to the new MIC determination following the prolonged antimicrobial exposures (passaging) of *S. aureus*. Cultures that exhibited ~80% growth (red box) were inoculated on an agar plate (A) containing the respective antimicrobial. Grown colonies were further cultured (B) in antimicrobial-free liquid medium (3 days sub-culturing) prior dose-response growth study for 'new' MIC determination (C) of the antimicrobials. (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

3.4. Results and Discussion

3.4.1. Minimum inhibitory concentration (MIC) of NAg, Ag⁺, and ciprofloxacin on *S. aureus* WT

Minimum inhibitory concentration (MIC) is a standardized parameter to determine the susceptibility of bacteria to specific antimicrobial agents¹¹¹. In this study, MIC is defined as the minimum concentration that inhibits ~95% of bacterial growth relative to the no antimicrobial (cell-only) control following 24 h incubation.

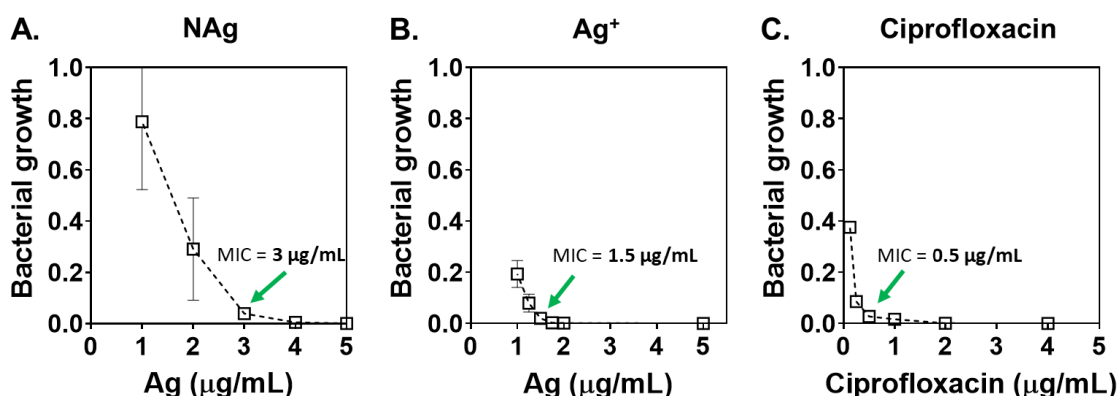


Figure 3.3. Dose-dependent growth response of *S. aureus* WT growth relative to cell-only control in (A) NAg (1 to 5 µg Ag/mL), (B) Ag⁺ (1 to 5 µg Ag/mL), and (C) Ciprofloxacin (0.25 to 4 µg Ag/mL) to determine their respective minimum inhibitory concentration (MIC). The data points represent the mean of nine measurements (three biological replicates, each with three technical replicates), with error bars representing the standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

The extent of bacterial growth relative to the control lessen with increasing NAg dosage (**Figure 3.3A**), in other words, exhibiting a dose-dependent growth effects. At 1 µg Ag/mL exposure, 80% growth was observed relative to the control, then only 5% growth at 3 µg Ag/mL, and ultimately, a complete growth inhibition at >3 µg Ag/mL. The NAg MIC for the WT strain was therefore determined to be at 3 µg Ag/mL. Earlier studies have reported a wide range of NAg MIC, from 1.7 to 100 µg Ag/mL on *S. aureus*, and this variation is likely to result from variations in the particulates' physicochemical properties, including the size²⁵, shape^{28,29} and presence of surface functional groups^{7,25}. The NAg MIC in this study is consistent with the lower range of the published MICs, most likely due to the small particle size.

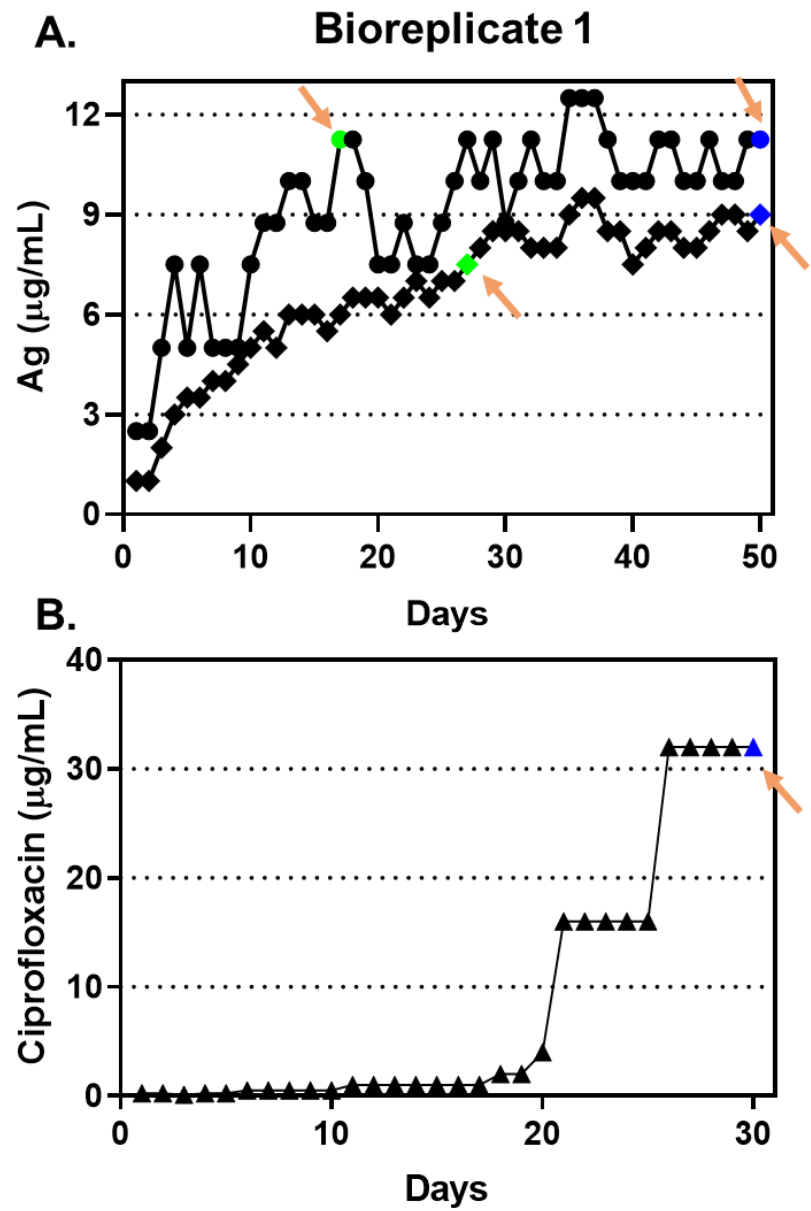
Exposure of *S. aureus* WT to increasing Ag⁺ concentrations also saw a dose-dependent growth inhibition (**Figure 3.3B**). At 1 µg Ag/mL, only ~20% of WT growth relative to the control was detected while doubling the Ag⁺ dosage resulting in complete inhibition of the bacterial growth. The Ag⁺ MIC for the WT strain was determined to be at 1.5 µg Ag/mL, in agreement with a previous study⁴⁹. These results indicate that the

bacterium demonstrates different toxicity responses towards the nanoparticle and ionic forms of silver, which have indeed been identified by many studies including the different MICs^{97,99,112}. This difference is likely to result from their unique origins of antibacterial activity. As described earlier (Chapter 2), for nanoparticles, both the leached soluble silver species and the solid particulates that remained after leaching, contribute to the overall toxicity¹⁸. The kinetic of silver dissolution is affected by the physicochemical characteristics of the nanoparticle, and further interactions of the leached silver with complexing/precipitating moieties in the aqueous environment (e.g. the presence of halides and sulphides) will govern the resultant toxicity of the so-called bioavailability of the silver species¹¹². Exposure of bacteria to ionic silver, on the other hand, is only associated with presence of soluble silver, which will also interact with the aqueous moieties therefore, affecting their bioavailability¹¹².

The growth inhibition study for ciprofloxacin was also carried out in parallel with NAg and Ag⁺ (**Figure 3.3C**). Ciprofloxacin was selected as the model antibiotic for the resistance development study as ciprofloxacin-resistant *S. aureus* have been reported previously^{76,113}, therefore could provide a validation for the methodology. At a concentration of 0.125 µg ciprofloxacin/mL, the WT strain could proliferate to 40% growth relative to the control. Nevertheless, a sharp growth reduction was observed at 0.25 µg ciprofloxacin /mL, then only ~5% growth at 0.5 µg ciprofloxacin/mL. The MIC was determined to be at 0.5 µg ciprofloxacin/mL, also consistent with a previous study¹¹⁴. Considering this low MIC (based on the micromolar concentration comparison), the results herein indicate that ciprofloxacin is more effective on the WT *S. aureus*, when compared to NAg or Ag⁺. This also confirms that the strain used in the present study is a ciprofloxacin-susceptible strain as the MIC is in agreement with the susceptibility classification reported by CLSI (MIC ≤ 1 µg ciprofloxacin /mL)¹¹¹.

3.4.2. Adaptation responses of *S. aureus* to prolonged NAg, Ag⁺ and ciprofloxacin exposure

To investigate the potential for resistance development, *S. aureus* WT was subjected to a continuous 50-days sub-culturing in progressively increasing NAg concentrations. In parallel, the WT strain was also sub-cultured in the presence of Ag⁺ for 50 days and ciprofloxacin for 30 days to study the different defence mechanisms, with and the latter serving as a validation to the resistance acquisition method. Two biological replicates of resistance development (A-B and C-D) in the WT strain upon exposure to NAg, Ag⁺, and ciprofloxacin were performed (**Figure 3.4**). The replicate used different isolates and were performed independently over a separate time period.



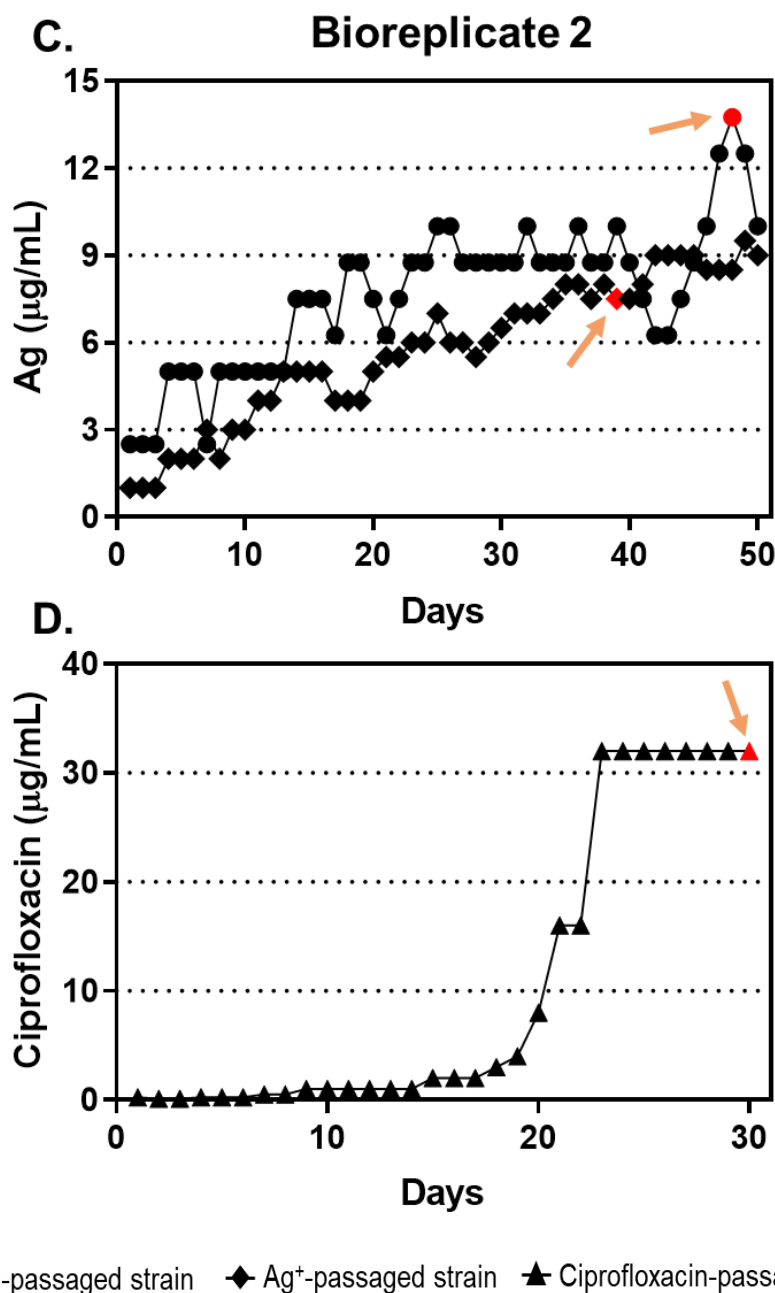


Figure 3.4. Prolonged exposure of *S. aureus* WT to NAg (circle), Ag⁺ (diamond), and ciprofloxacin (triangle) *via* sub-culturing. *S. aureus* WT was exposed for 50 days to NAg and Ag⁺ (A and C) or 30 days to ciprofloxacin (B and D) with progressive increases in antibacterial concentration. Displayed data points are the concentration of the respective antibacterial agents from which the culture showed ~80% growth relative to cell-only control and was passaged to the next day. Experiments were performed with two biological replicates, where one replicate (A-B) was independent to the other (C-D). Colour-filled symbols (green, blue, and red, represented by the orange arrows) represent passaging isolates taken from the respective antibacterial agent for further assessment (new MICs determination and cross-resistance studies, see Section 3.4.3 to 3.4.5). Green, blue and red represent middle of passaging replicate 1 (mid-replicate 1), end of passaging replicate 1 (end-replicate 1), and end of passaging replicate 2 (end-replicate 2), respectively. (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

The 50-days continuous exposure of *S. aureus* WT to NAg saw progressive shifts of NAg dosages at which the bacteria could proliferate at ~80% growth relative to the cell-only control, herein referred to as ‘threshold’. Over time, the NAg threshold increased from 2.5 µg Ag/mL (sub-MIC) on day 1 to ~11 µg Ag/mL on day 17 and was still able to proliferate when exposed to this concentration on day 50 (**Figure 3.4A**, circle). Similarly, progressive shifts of silver threshold were observed in the WT treated with Ag⁺, whereby the threshold increased from 1 µg/mL (sub-MIC) on day 1 to 9 µg Ag/mL on day 50 (**Figure 3.4A**, diamond). Interestingly, following a rather slow start, the WT when exposed to ciprofloxacin displayed an abrupt “up-shift” of the antibiotic threshold after 3 weeks of exposure (**Figure 3.4B**) from 0.3 µg ciprofloxacin/mL (sub-MIC) to 32 µg ciprofloxacin/mL at 30 days of exposure. Comparable results were observed for the second biological replicate (**Figure 3.4C and D**). These results suggest the potential for *S. aureus* to adapt to silver and antibiotic.

Sub-inhibitory concentration in the initial exposure days, as has been established in many studies, is to facilitate the development of resistance traits in bacteria. The sub-inhibitory exposure does not entirely eradicate the bacterial population, allowing the selection for members of the population with an increased tolerance to the antibacterial agent and ultimately, outperform the susceptible population by the development of resistance traits¹¹⁵. While silver is usually incorporated at high concentrations in commercialized products³⁵, the release of silver into the natural environment (e.g. soil and water streams) and human organs and tissues (e.g. gastrointestinal and urinary tracts), can dilute the bioavailable silver concentration, hence creating favourable conditions to select for resistant bacteria³⁵.

The step-wise increase of ciprofloxacin threshold observed in *S. aureus* could be caused by the antibiotic’s single toxicity DNA-targeting mechanism of action (**Figure 3.4B and D**). A fluoroquinolone-class antibiotic, ciprofloxacin blocks DNA replication by inhibiting the activity of DNA gyrase and the type IV topoisomerase enzymes. These enzymes catalyse the unwinding of double stranded DNA (negative supercoiling) for DNA separation¹¹⁶. This step-wise increase has been previously observed in *S. aureus*, both *in vitro* and clinical settings^{76,113}, indicating the emergence of ciprofloxacin-resistant bacteria and therefore, validating the sequential passaging methods used in this study.

On the other hand, the slower increase of the silver threshold is likely to result from the multiple toxicity mechanisms. These include the physical disruptions of *S. aureus* cell surface⁵⁴, damage to DNA and proteins^{68,69}, as well as the stimulation of lethal levels of cellular oxidative stress³¹. These observations in resistance acquisition (**Figure 3.4A**

and C) likely stem from the complex antibacterial mechanisms of silver. Simply said, it was speculated that the bacterial population would take time to adapt to all toxicity targets and therefore, a gradually developed resistance.

3.4.3. Adaptation responses from the ‘resistant’ *S. aureus* to NAg, Ag⁺, and ciprofloxacin

To confirm that the prolonged sub-culturing had altered the bacteria’s susceptibility, new MIC assays were performed on the passaged strains at selected time points and compared to the WT strain. The selected strains are marked by the colour-filled symbols (green, blue, and red circle, diamond and triangle in **Figure 3.4**, see arrow) and were chosen to represent approximately the middle and the end of the passaging timeline from the two independent replicates. Prior to the new MIC determination, the passaged cultures were inoculated into the respective antibacterial-agar plate to select for resistant colonies. To ensure that the resistance traits were stable, indicative of heritable mutations, grown colonies were then passaged in non-selective media (see **Figure 3.2**).

MIC determination is a common assay clinically¹¹⁷ to investigate the level of bacterial phenotypic resistances against antimicrobial agents relative to the susceptible counterpart strain. Resistant strains are distinguished with a higher MIC, corresponding to higher dosages of antimicrobial required to inhibit bacterial growth^{118,119}. The increasing MIC also validates that the ability of the strains to grow in the presence of antimicrobial agent is not due to tolerance or persistence adaptation¹¹⁸. Tolerance describes the ability of the general bacterial population to endure longer antibacterial exposure whilst persistence, also known as ‘heterotolerance’, characterizes a part of bacterial population that has an improved survivability compared to the general population, resulting in an even longer antimicrobial treatment^{118,119}. These are marked by the minimum duration to kill 99% (MDK₉₉) or 99.99% (MDK_{99.99}) of the total population without the change of MIC, respectively^{118,119}.

The growth of the ciprofloxacin-passaged strain in increasing concentrations of ciprofloxacin was first investigated to confirm that *S. aureus* had developed resistance to the antibiotic. The ciprofloxacin-passaged strain in blue and red triangle in **Figure 3.5** were sampled from passaging replicates marked by blue (end-replicate 1) and red (end-replicate 2) triangle in **Figure 3.4B** and D, respectively. Compared to the WT strain, the growth of the passaged strains was significantly different as the strain was now able to proliferate at significantly higher concentrations than the WT strain. The MIC for the passaged strain had increased by >64-fold, from 0.5 µg ciprofloxacin/mL to

>32 µg ciprofloxacin/mL, which indicates a resistant strain¹¹⁸ as also defined by the CLSI guidelines¹¹¹. These results are consistent with previous studies on the development of ciprofloxacin-resistant *S. aureus* via prolonged exposure¹¹³, which further validate the current sequential passaging methodology for the investigation of bacterial adaptive responses to toxic agents.

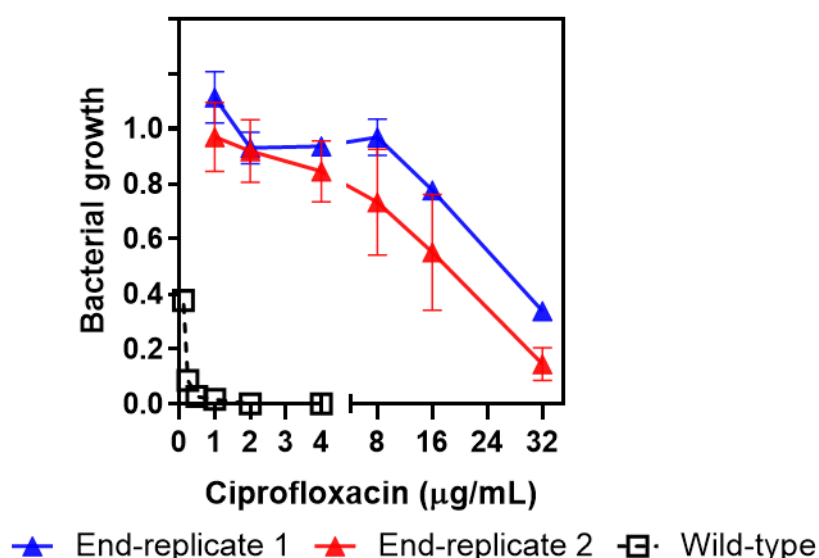


Figure 3.5. Growth responses of ciprofloxacin-passaged (triangle) and WT (square) strain to increasing concentration of ciprofloxacin. The blue and red triangle represent the strains sampled from passaging replicates shown in blue and red triangle in **Figure 3.4B** and **D**, respectively. Each data point represents the mean of nine measurements (three biological replicates and three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

For the silver-passaged strains, first, it is noteworthy to mention that their growth in the absence of silver were comparable to that of the WT strain (see Chapter 6 Section 6.4.1), indicating that any changes in survival in the presence of silver was not due to fitness cost adaptation after previous prolonged growth in the presence of silver.

Exposure of the NAg-passaged strains from different passaging replicates and days to increasing NAg concentrations, all resulted in higher extent of growth relative to the WT strains (**Figure 3.6**). The passaged strains (green, blue and red circle in **Figure 3.6**) were sampled from passaging replicates marked by green (mid-replicate 1), blue (end-replicate 1) and red (end-replicate 2) circle, respectively, in **Figure 3.4A** and **C**. These strains were able to proliferate at >40% growth when exposed to the otherwise toxic 5 µg Ag/mL NAg dosage. Note that as mentioned earlier, the growth of the WT was already inhibited at 3 µg Ag/mL (MIC of NAg for the WT), whereas the NAg-passaged strains were only inhibited at >10 µg Ag/mL. This denotes the increase of MIC by 3-7-fold, and therefore, an emergence of stable resistance to NAg in *S. aureus*.

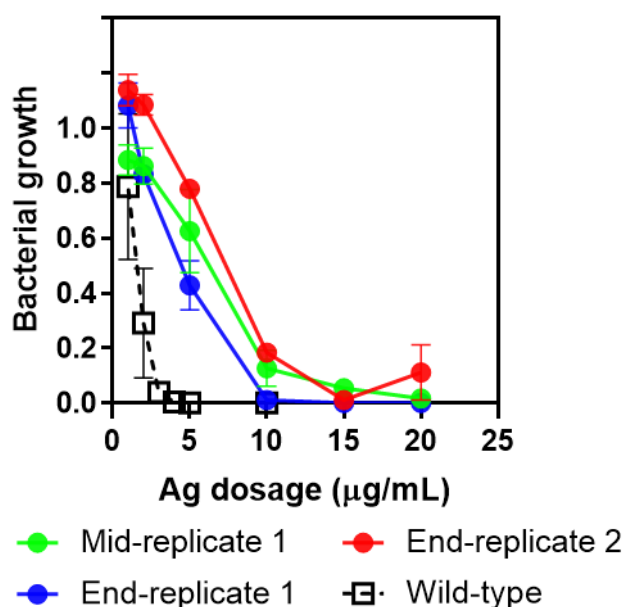


Figure 3.6. Growth responses of NAg-passaged (circle) and WT (square) strain to increasing concentration of NAg. The green, blue, and red circle represent the strains sampled from passaging replicates and days shown in green, blue, and red circle in **Figure 3.4A** and **C**, respectively. Each data point represents the mean of nine measurements (three biological replicates and three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

A higher growth response was also observed upon re-exposure to increasing Ag⁺ concentrations of all Ag⁺-passaged strains from different passaging replicates and days when compared to the WT strain. The passaged strains (green, blue and red diamond in **Figure 3.7**) were sampled from passaging replicates marked by green (mid-replicate 1), blue (end-replicate 1) and red (end-replicate 2) diamond, respectively, in **Figure 3.4A** and **C**. Similarly, all passaged strains showed proliferation yielding >40% biomass at 2 µg Ag/mL of Ag⁺, the concentration that caused complete inhibition in the WT strain (**Figure 3.7**). The new MIC of the Ag⁺-passaged strains have increased to 5 µg Ag/mL from 1.5 µg Ag/mL of the WT, which corresponds to ~3-fold increase, hence, the emergence of stable Ag⁺-resistant *S. aureus*. This finding is in contrast with a previous study that could not obtain Ag⁺-resistant *S. aureus* after 42 days of exposure¹²⁰. The study, however, did not include a non-selective culturing step to stabilise any resistance traits.

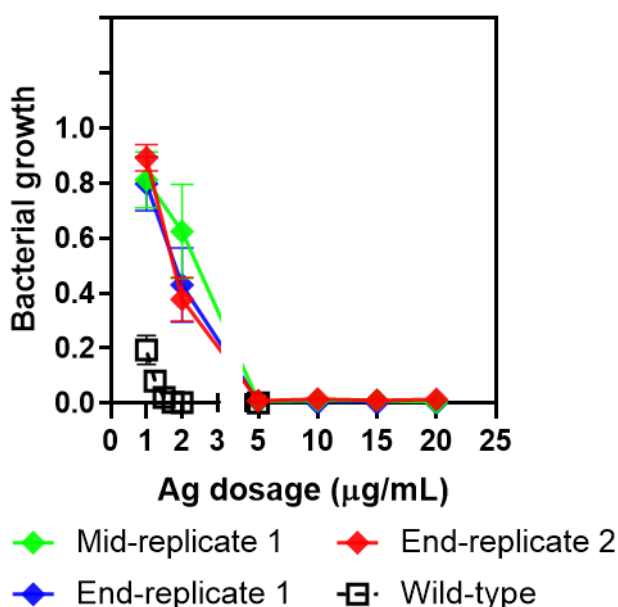


Figure 3.7. Growth responses of Ag⁺-passaged (diamond) and the WT (square) strain to increasing concentration of Ag⁺. The green, blue, and red diamond represent the strains sampled from passaging replicates and days shown in green, blue, and red diamond in **Figure 3.4A** and **C**, respectively. Each data point represents the mean of nine measurements (three biological replicates and three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

The results indicate that *S. aureus* are more readily able to develop resistance to ciprofloxacin than NAg or Ag⁺. This was expected, considering the single toxicity target by the antibiotic, in contrast to the multiple targets by the silver antibacterials.

The findings imply that *S. aureus* developed modest resistance phenotype to NAg, as also consistent with a recently published study reporting 2- to 4-fold NAg MIC increase in 4 out of 20 *S. aureus* strains investigated¹³. The findings also suggest that *S. aureus* are more prone to develop resistance to NAg than Ag⁺. A recent study in different bacteria reported 4- to 16-fold NAg MIC increase with *E. coli* and 32-fold NAg MIC increase with *P. aeruginosa* while no MIC increase for Ag⁺ was observed with either of the Gram-negative bacteria¹⁴.

3.4.4. NAg and Ag⁺ cross-resistance

To investigate the potential for silver cross-resistance, the NAg-resistant *S. aureus* was exposed to Ag⁺ and vice versa, the Ag⁺-resistant strain was exposed to NAg. The NAg-resistant strains (green, blue and red circle in **Figure 3.8A**, sampled from passaging replicates marked by green (mid-replicate 1), blue (end-replicate 1) and red (end-replicate 2) circle, respectively, in **Figure 3.4A** and **C**) showed higher extent of growth at 1-2 µg Ag/mL when compared to the WT strain in the presence of Ag⁺, nevertheless

growth was observed to sharply decline towards 5 µg Ag/mL (**Figure 3.8A**). The NAg-resistant strains were associated with a 3-6-fold MIC Ag⁺ increase indicating that the strains are also resistant to Ag⁺. Interestingly, the growth responses were similar to that of the Ag⁺-resistant strains when exposed to Ag⁺. This may imply that the mechanism underlying the NAg and Ag⁺ toxicity mechanism share similar virtues.

Next, the Ag⁺-resistant strains (see green, blue, and red diamond in **Figure 3.8B** sampled from passing replicates marked by green (mid-replicate 1), blue (end-replicate 1) and red (end-replicate 2) diamond, respectively, in **Figure 3.4A** and C) were exposed to NAg. In general, the growth response of the Ag⁺-resistant strain to handle increasing NAg concentration was not significantly different to that of the WT in the presence of NAg (**Figure 3.8B**). Nevertheless, at 3 µg Ag/mL, only 5% growth of WT was observed while the Ag⁺-resistant strain grew at 30-70% extent of biomass. The NAg MIC changes for the Ag⁺-resistant strains were ~1.5-3-fold higher than that of the WT strain in NAg, indicating a possible cross-resistance phenomenon to NAg. Compared to NAg-resistant strains in Ag⁺, the magnitude of MIC changes in Ag⁺-resistant strains in NAg was slightly less. This could be due to, at least in part, the nanoparticle's more complex toxicity targets in bacteria than Ag⁺, as mentioned earlier.

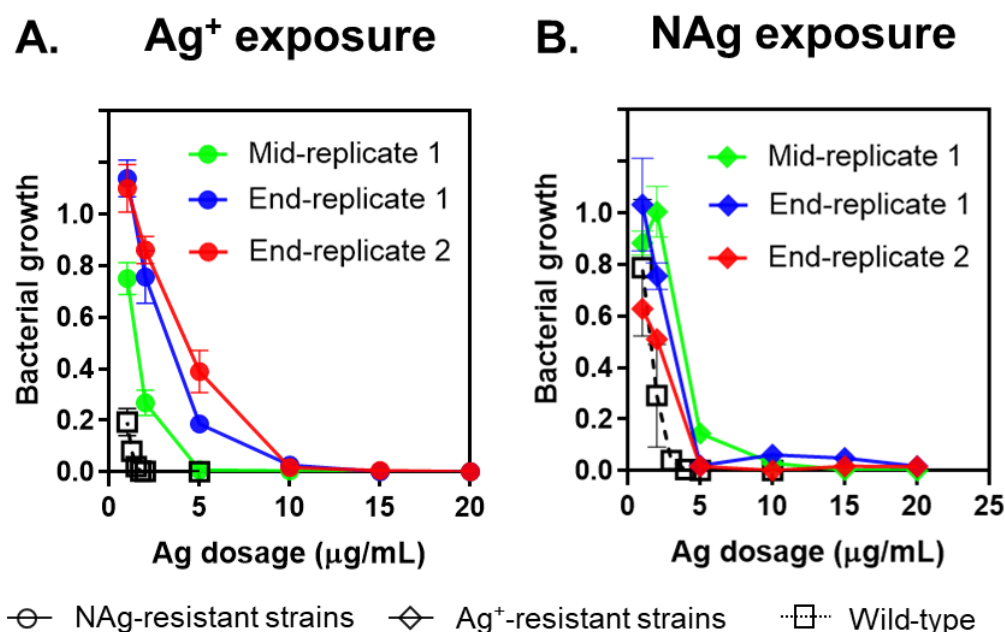


Figure 3.8. Growth responses of NAg-resistant strain (circle) in increasing concentration of Ag⁺ (A) and Ag⁺-resistant strain (diamond) in increasing concentrations of NAg (B) as compared to the WT strain (square). The green, blue, and red circle or diamond represent the strains sampled from passing replicates shown in green, blue, and red circle or diamond in **Figure 3.4A** and C. Each data point represents the mean of nine measurements (three biological replicates with three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

To determine if ciprofloxacin resistance also confers cross-resistance to NAg and Ag⁺, the ciprofloxacin-resistant strains were exposed to NAg or Ag⁺ (**Figure 3.9A** and **B**). These strains displayed similar growth responses as the WT strain (MIC 3 and 1.5 µg/mL for NAg and Ag⁺, respectively), indicating lack of cross-resistance between ciprofloxacin and silver. This is likely as NAg and Ag⁺ (silver) and ciprofloxacin have different antibacterial target. In response to prolonged ciprofloxacin exposure, *S. aureus* have previously been known to develop point mutations in *grlA/grlB* or *gyrA/gyrB*, responsible for altering a subunit in topoisomerase IV and gyrase, respectively¹²¹, and the results herein showed that these mutations do not provide protection to silver toxicity.

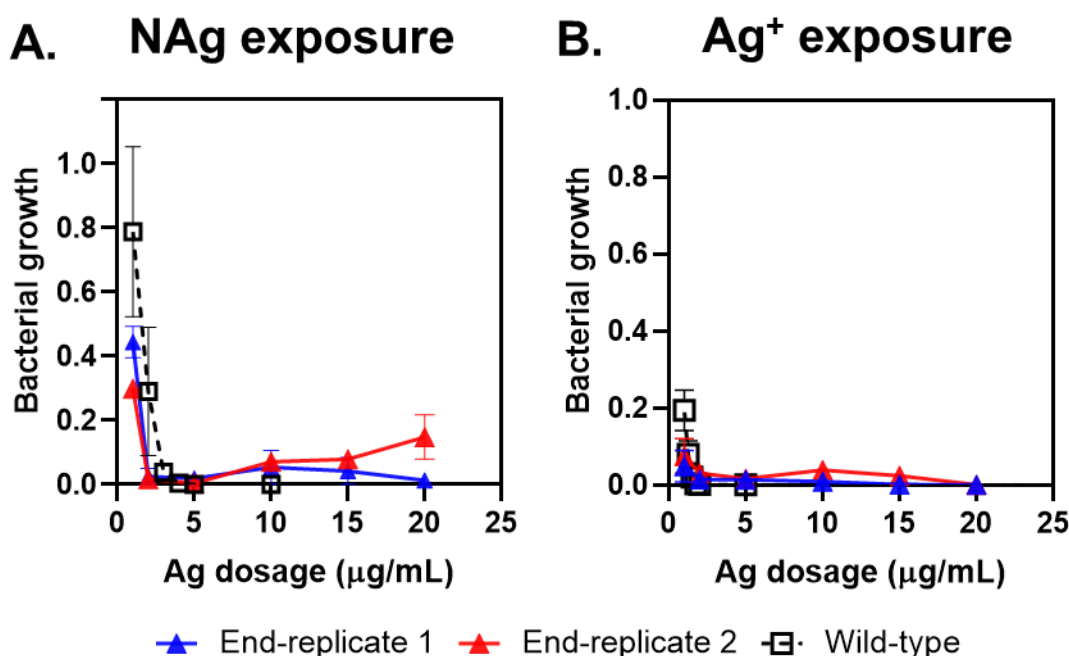


Figure 3.9. Growth responses of ciprofloxacin-resistant (triangle) and WT (square) strain to increasing concentration of NAg (A) and Ag⁺ (B). The blue and red triangle represent the strains sampled from passaging replicates shown in blue and red triangle in **Figure 3.4B** and **D**. Each data point represents the mean of nine measurements (three biological replicates with three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

3.4.5. Responses to other antibiotics

Silver is generally non-degradable in the environment and usually present at a very low concentrations (ng/L)¹²², providing favourable conditions for inadvertent selection of silver-resistance in bacteria¹¹⁵. Studies have shown that heavy metals such as silver, copper, or mercury could select for antibiotic resistance determinants^{123,124}, which is a concerning issue should NAg and/or Ag⁺ be used as a substitute to traditional antibiotics.

To investigate whether silver resistance could confer cross-resistance to other antibiotics, the NAg- and Ag⁺-resistant bacteria were exposed to standard antibiotics - cell-wall synthesis inhibitors with different mechanisms of action as well as ciprofloxacin. The first cell-wall synthesis inhibitor tested was the glycopeptide vancomycin, the “last line of defence” antibiotic to treat infections caused by *S. aureus*¹⁰⁴. The other two inhibitors are β -lactam antibiotics, methicillin and cefotaxime, as this antibiotic class is the most administered antibiotic¹²⁵. Methicillin is a first-generation narrow-spectrum antibiotic, to which some *S. aureus* strain have developed high level of resistance and has caused more than 10,000 mortality cases per year in the United States¹⁰³. Whereas, cefotaxime, a cephalosporin, was also included to compare the growth responses of the silver-resistant strains to a broad spectrum β -lactam antibiotic¹²⁵.

NAg- and Ag⁺-resistant *S. aureus* exposed to vancomycin (**Figure 3.10B**) displayed similar growth response to the WT strain. Comparable MICs were observed for the silver-resistant and the WT strains (MIC = 2-4 μ g vancomycin/mL) and considered as susceptible according to the CLSI¹¹¹. The glycopeptide antibiotic vancomycin works by binding to the two terminal D-alanyl-D-alanine residues (D-Ala(4)-D-Ala(5)) of the *S. aureus* peptidoglycan peptide chains, preventing the cross-linking of the pentaglycine bridge of one glycan strand to the D-Ala(4) residue of another glycan strand¹⁰⁴ (**Figure 3.11**). Studies have detected increased production of ‘free’ D-alanyl-D-alanine residues in vancomycin-resistant *S. aureus*, which is thought to act as decoys to hinder the binding of the antibiotic to its intended D-alanyl-D-alanine target in peptidoglycan^{126,127}, which is most likely non-existent in the defence mechanism of NAg- and Ag⁺-resistant bacteria.

Similarly, the silver-resistant strains also showed comparable growth responses relative to the WT strain when exposed to cefotaxime (**Figure 3.10C**, MIC = ~2 μ g cefotaxime/mL). β -lactam antibiotics work by hindering the cross-linking of the pentaglycine bridge. As a structural analog of the D-alanyl-D-alanine residues, the antibiotic competitively binds to a transpeptidase (also known as the penicillin binding proteins or PBPs) to inhibit the enzyme activity and in turn, the cleaving of the D-Ala(5) residue of the peptidoglycan peptide chain as part of the cross-linking process¹²⁵ (**Figure 3.11**). The susceptibility to cefotaxime implies that the silver-resistant bacteria neither modify their PBPs (to prevent the binding of cefotaxime) nor produce beta-lactamase enzymes (to hydrolyse cefotaxime’s β -lactam ring). *S. aureus* has been known to develop these capabilities as resistance mechanism¹²⁸.

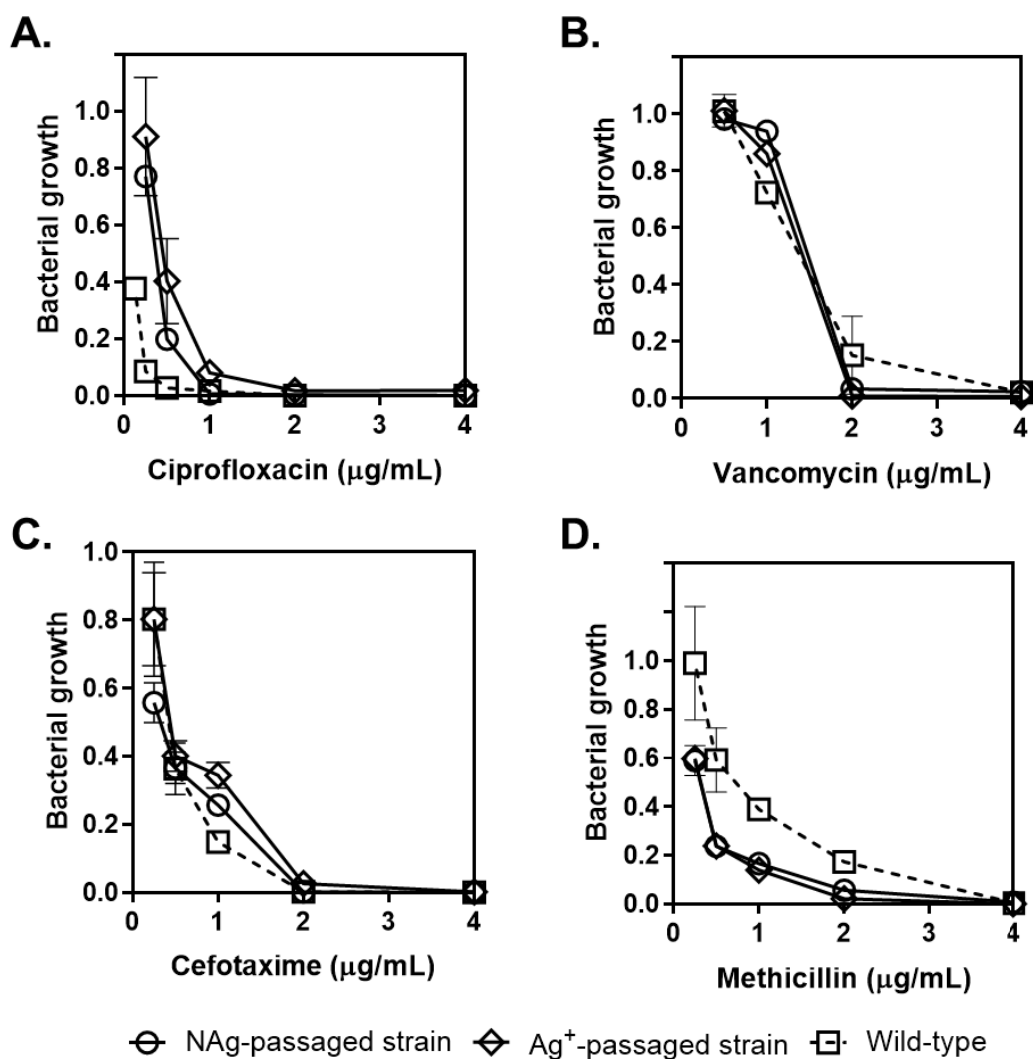


Figure 3.10. Growth responses of NAg-resistant strain (circle), Ag⁺-resistant strain (diamond) and *S. aureus* WT strain (square) to increasing concentration of A) ciprofloxacin, B) vancomycin, C) methicillin, and D) cefotaxime. The resistant strains were isolates sampled from mid-passaging bioreplicate 1 experiment (mid-replicate 1, green circle and diamond in **Figure 3.4A**). Each data point represents the mean of nine measurements (three biological replicates with three technical replicates each). The error bars represent standard error of measurement (SEM). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

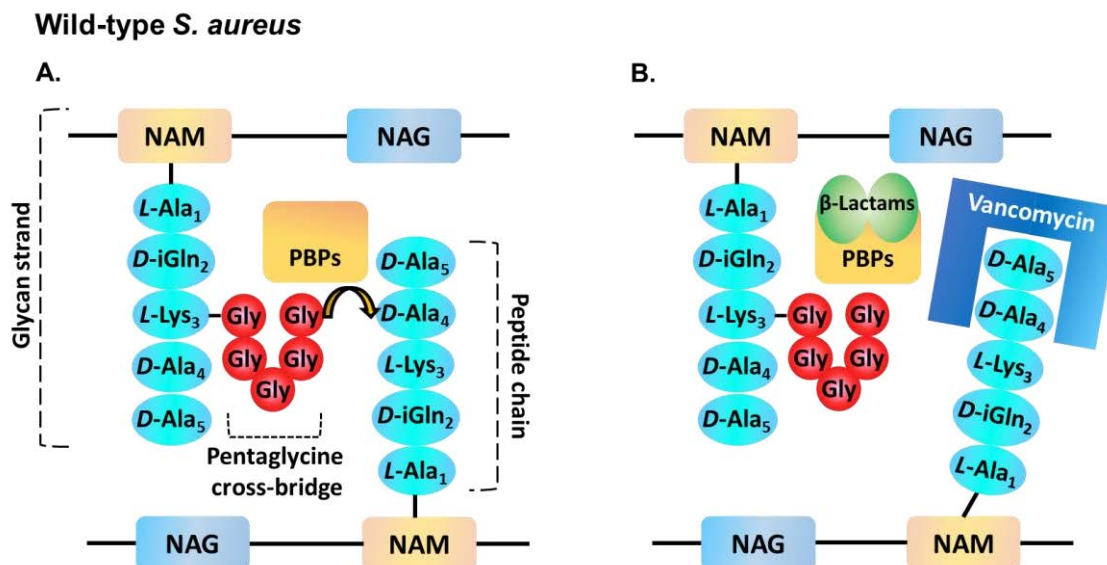


Figure 3.11. The resistance mechanism of β -lactams antibiotics and vancomycin in *S. aureus*. A) Peptidoglycan structure of wild-type *S. aureus*. The structure is made of cross-linked glycan strands; each strand is an alternating co-polymer of two sugar derivatives N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) linked by the covalent β -(1,4)-glycosidic bond. Attached to the NAM moiety is five amino acid (peptide) chain consisting of *L*-alanine(1), *D*-isoglutamine(2), *L*-lysine(3), *D*-alanine(4) and *D*-alanine(5). The glycan strands are cross-linked by a pentaglycine cross-bridge, with the process being catalysed by the transpeptidase PBPs (penicillin binding proteins) to connect the pentaglycine (*L*-lys(3) in one glycan strand to *D*-ala(4) in another strand¹²⁹. B) The antibiotic vancomycin as well as the β -lactams cefotaxime and methicillin target the pentaglycine cross-linking, with vancomycin binding to *D*-ala(4)-*D*-ala(5), while the β -lactams competitively bind to PBPs and inhibit the enzymes^{104,125}. (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Interestingly, different growth response to other β -lactam antibiotic, methicillin, was observed. A moderate increase in susceptibility was seen with both the NAg- and Ag⁺-resistant strains (MIC = ~2 μ g methicillin/mL), relative to the WT strain (MIC = ~4 μ g methicillin/mL; **Figure 3.10D**). Studies regarding ‘hyper-susceptibility’ to methicillin in *S. aureus* have identified the formation of a monoglycine instead of pentaglycine cross-bridge in the bacteria’s peptidoglycan^{130–132}. This defective monoglycine cross-bridge has been known to render the bacterium resistant to the action of lysostaphin, a glycylglycine endopeptidase enzyme and an antistaphylococcal agent. Lysostaphin works by cleaving the pentaglycine cross-bridge of *S. aureus*, leading to cell lysis¹³¹. Indeed, the NAg- and Ag⁺-resistant *S. aureus* displayed higher extent of growth to increasing lysostaphin concentrations when compared to the WT strain (**Figure 3.12A**). Further, visually evident is the turbidity (indicating less cell lysis) of the silver-resistant bacteria suspensions unlike that observed with the WT strain (clear bacterial suspension indicating more cell lysis, **Figure 3.12B**). These findings suggest the presence of a monoglycine cross-bridge in the peptidoglycan structure of the silver-resistant bacteria, which can be examined by comparing cell wall composition of the WT and the resistant strain using reversed-phase high performance liquid

chromatography (HPLC) and amino acid analyser for mucopeptide pattern analysis and glycine content measurement, respectively¹³¹. In some cases, lysostaphin-resistant *S. aureus* have been associated with a decreased cell wall turnover^{130,131}, which has also been seen in cefotaxime-susceptible *S. aureus* when compared to their resistant counterpart¹³³. Note that, as herein observed, the silver-resistant strains were also susceptible to cefotaxime (**Figure 3.10C**). Cell wall turnover is a bacterial physiological process for self-degradation and recycle of their cell wall components¹³³. Taken together, the different growth behaviour of the silver-resistant strains in cefotaxime (susceptible) and lysostaphin (increased toughness) indicates a decreased cell wall turn over for the strains. Lower cell wall turnover has been associated with a stronger cell wall that are less prone to chemical bond cleaving^{130,131}, and therefore, could potentially contribute to the bacteria's resistance to silver, with both the nanoparticle and ionic forms of silver already indicated to target the glycan strand in *S. aureus*⁵⁴ (Chapter 2) To confirm the hypothesis, the rate of cell wall turnover between the WT and the resistant strain could be compared *via* measurement of tritium-labelled N-acetylglucosamine, following methods published^{134,135}. Additionally, investigation on cell wall thickness *via* electron microscopy could be conducted to the WT the resistant strain as bacteria with lower cell wall turnover was shown to have a thicker cell wall¹²⁶.

Up to this stage, the study has shown the capability of *S. aureus* to develop resistant to NAg and Ag⁺, shown by the increasing MIC values when compared to the WT strain (**Table 3.1**). The resistance in silver, however, was not observed to alter the susceptibility of the resistant strain in commercial antibiotics (**Table 3.1**), suggesting a distinct antibacterial mechanism between silver compounds and antibiotics.

Table 3.1. Summary of the minimum inhibitory concentration (MIC) value (in µg/mL) of the wild-type, NAg-resistant and Ag⁺-resistant strain in different antimicrobial agents used in this study.

Strain	MIC (µg/mL)					
	NAg	Ag ⁺	Ciprofloxacin	Vancomycin	Cefotaxime	Methicillin
Wild-type	3	1.5	0.5	2 – 4	~ 2	~ 4
NAg-resistant	10 – 15	~ 5	~ 1	~ 2	~ 2	~ 2
Ag ⁺ -resistant	5 – 10	~ 5	1 – 2	~ 2	~ 2	~ 2

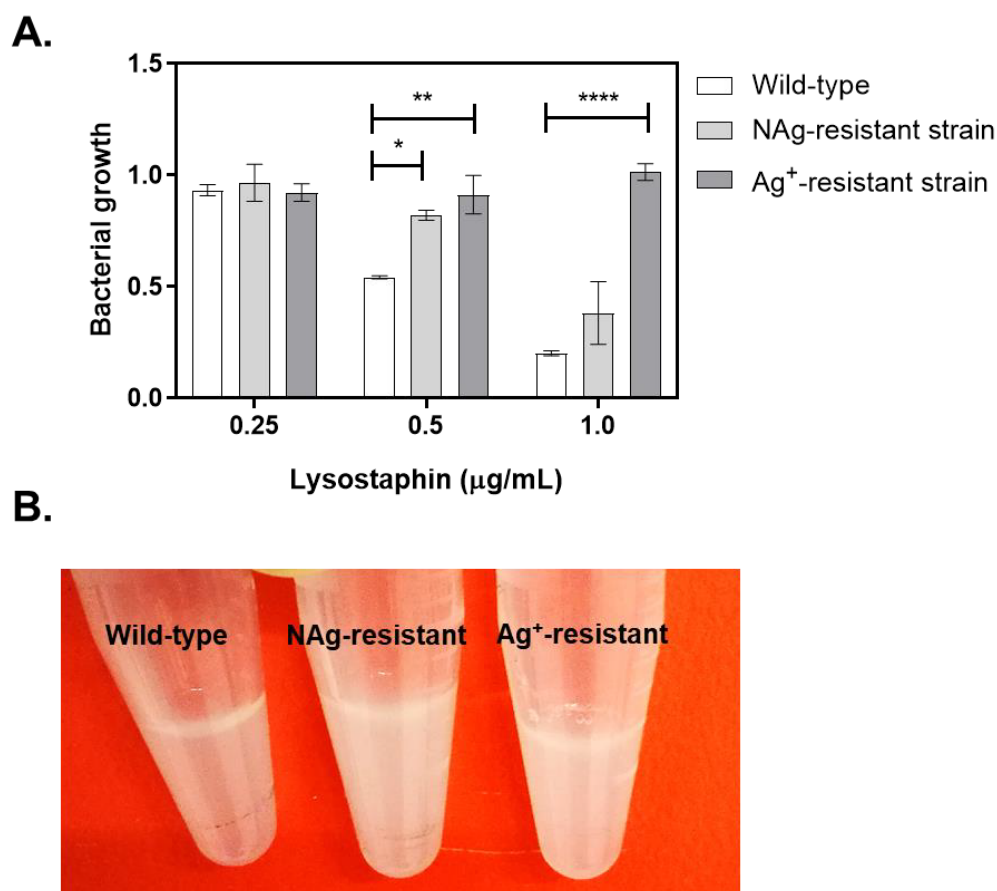


Figure 3.12. Effect of antistaphylococcal agent to silver-resistant *S. aureus*. A) The NAg- and Ag⁺-resistant bacteria were treated with the enzyme lysostaphin (0.25 to 1 µg/mL). Each data point is the mean of nine measurements (three biological replicates with three technical replicates each), with error bars representing the standard error of measurement (SEM). Two-way ANOVA statistical analysis was used to confirm the effects of lysostaphin on the silver-resistant strains relative to the WT with *, **, **** represent $p < 0.05$, 0.01 , and 0.0001 , respectively. B) The visual observations of the lysostaphin-treated bacterial suspensions (0.5 mg/mL, 30 min, 37°C). (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

3.5. Conclusions

To conclude, this study reported the ability of the WHO-listed priority pathogen *S. aureus* to develop stable resistance characteristics to NAg and ionic silver (Ag⁺). With no native presence of any of the known silver-relevant defence mechanisms, *S. aureus* in this study was able to proliferate at silver concentrations that would be otherwise toxic to the wild-type (WT) strain, reducing the efficacy of the silver antibacterials. Both of the NAg and Ag⁺-resistant strains also displayed cross resistance traits between one silver forms to the other, which implies a shared mechanism of silver toxicity on *S. aureus*. The silver resistance, however, did not provide cross-protection to the tested antibiotics (ciprofloxacin, vancomycin, and cefotaxime), most likely due to different antibacterial mechanisms. Interestingly, both silver-resistant strains exhibited higher susceptibility to methicillin and resistance to lysostaphin when compared to the WT

strain. These findings may shed a light in the silver resistance mechanisms in *S. aureus*. To investigate this, a whole genome sequencing approach was carried out to reveal a global snapshot of *S. aureus* genetically-encoded defences to the NAg and Ag⁺. This will be the focus of the next chapter.

Chapter 4

Identification of genetic adaptation in NAg- and Ag⁺-resistant *S. aureus* strains

4.1. Summary

Chapter 3 observed the phenotypic resistance of clinically-relevant pathogenic Gram-positive bacteria, *Staphylococcus aureus*, to silver nanoparticle (NAg) and ionic silver (Ag⁺), indicating the ability of genetically diverse bacteria to become resistant to silver. Both phenotypically and genotypic adaptations for silver resistance have been reported for phylogenetically diverse bacteria. However, it is currently unknown how well conserved these silver resistance mechanisms are between species, or whether *S. aureus* employs completely novel resistance mechanisms. Therefore, whole genome sequencing of the silver adapted strains (Chapter 3) was performed to investigate if the resistance mechanism(s) in *S. aureus* was genetically encoded. In Chapter 4, genomic sequencing reported mutations in genes encoding for the purine operon regulator and L-cystine transporter in the NAg-resistant strain as well as the cysteine metabolism regulator in the Ag⁺-resistant strain. These mutations were hypothesised to affect the cellular process of nucleotide synthesis and to defend against the generated oxidative stress by synthesizing iron-sulphur (Fe-S) clusters or thioredoxin, in response to NAg and Ag⁺ toxicity. Interestingly, some genes in both the NAg- and Ag⁺-resistant strains were mapped to similar functional pathways, hence may explain the cross-resistances phenomenon observed in Chapter 3. Due to time constraints, the investigation to understand the role of the mutations and validate these hypotheses *via* clustered regularly interspaced short palindromic repeats interference (CRISPRi), unfortunately, has not been able to be completed yet. An alternative study using *S. aureus* JE2 (methicillin-resistant strain) mutants were thought to be inconclusive at this stage. Altogether, this study highlights unique and heritable silver mechanisms of resistance by a pathogenic Gram-positive bacterium with no previously described mechanisms for silver resistance. Nevertheless, future studies are crucial to ultimately uncover and elucidate the molecular mechanisms of resistance to NAg and Ag⁺.

4.2. Introduction

One of the first identified silver resistant bacteria was already reported in the 1970s. It was *Salmonella typhimurium*, isolated from patients in a burn unit where silver nitrate (AgNO₃) was used as a topical treatment, that conferred resistance to Ag⁺ as well as antibiotics⁹¹. Therefore, this occurrence of resistant bacteria along with other reports^{136,137}, have raised the importance of investigating the molecular mechanism of silver resistance.

To date, the Ag⁺ resistance in Gram-negative bacteria has been identified to be *via* restricting accumulation of silver intracellularly. The chromosomal-encoded OmpC and OmpF (outer membrane protein) involved in the passive diffusion of non-specific molecule intracellularly, was observed to loss its function and prevent Ag⁺ influx in *E. coli*⁸⁴. Two other mechanisms of resistances have been reported: the chromosomal-encoded Cus protein (Cus-RSCFBA)^{58,107} and plasmid-encoded Sil protein (Sil-CFBAGPRSE)⁹² that orchestrate Ag⁺ efflux *via* ATP-binding cassette (ABC) transporters and chaperone proteins. Studies have reported that the deletion of the genes encoding for these proteins resulted in loss of resistance to silver^{85,88,92}.

A study by Graves *et al.* in NAg-resistant *E. coli* identified three chromosomal mutations in *cusS*, *purL*, and *rpoB*, encoding for an Ag⁺ sensor protein in the cus system, an enzyme phosphoribosylformylglycinamide subunit L in the purine biosynthesis process, and RNA polymerase synthesis protein, respectively¹⁶. In contrast, a recent study did not identify genomic changes in NAg-resistant *E. coli* instead, the study observed an increase production of flagellin, a protein used for the formation of bacterial flagella, that facilitated NAg aggregation and rendered the particle ineffective¹⁴.

While progressive evidences of NAg- and Ag⁺-resistant mechanism have been reported in pathogenic Gram-negative bacteria, there is still lack of study and understanding in the molecular mechanisms of silver resistance in pathogenic Gram-positive bacteria. This is of particular interest for *Staphylococcus aureus* as the bacteria was identified bioinformatically to have no (BLAST, this study), or very low¹¹, capacity of the known silver resistance determinants. Additionally, this study could highlight if the mechanism of resistance was conserved or specifically-related to a species. Following up from the discovery of stable NAg and Ag⁺ resistance in *S. aureus* in Chapter 3, the study utilized a whole genome comparison study between the resistant strains and the wild-type (WT) strain to identify genomic changes and further, to uncover and elucidate the resistance mechanism(s) of NAg and Ag⁺.

4.3. Materials and Methods

4.3.1. Whole genome comparison study

4.3.1.1. NAg- and Ag⁺-resistant *S. aureus* strain for whole genome comparison study

Silver nanoparticle (NAg) and ionic silver (Ag⁺) resistant strains were obtained from the resistant strain cultured and studied in Chapter 3. Briefly, upon prolonged exposure to either NAg or Ag⁺, the cultures from selected days (see green, blue, and red circle, and diamond in **Figure 3.4A** and **C**) were passaged in a selective media with the respective antibacterial agent followed by a non-selective media to stabilize the resistance genes (see the methods in **Figure 3.2**) prior the new minimum inhibitory concentration (MIC) determination. The strains demonstrated higher MIC than that of the susceptible counterpart (see **Figure 3.6** and **Figure 3.7**), indicating the phenotypic-resistance characteristic to the respective antibacterial agent.

S. aureus ATC25923 (henceforth will be mentioned as wild-type (WT) strain) and no-treatment bacterial control strains from the respective days of the passaging were also included in this study. The latter was serially passaged in parallel with the resistant strains without the presence of antimicrobial agents (cell-only). Unless otherwise stated, overnight inoculation of the NAg-resistant strain, Ag⁺-resistant strain, the WT strain, and the control strain was performed in Cation-adjusted Mueller Hinton Broth (Ca-MHB, BD, Australia) at 37°C, 200 RPM.

4.3.1.2. Whole genome DNA extraction

Genomic DNA from overnight culture (37°C, 200 RPM, silver-free) of NAg- and Ag⁺-resistant *S. aureus* were extracted. Briefly, the resuspended cell pellets from the overnight culture were lysed by incubating the cells with lysostaphin (0.5 mg/mL, 37°C, 30 min) (Sigma Aldrich) followed by proteinase K (1.9 mg/mL, 55°C, 30 min) (Sigma Aldrich) and purified using PureLink™ Genomic DNA Mini Kit (Invitrogen) following the manufacturer's instructions. The DNA concentrations and purity were analysed *via* Qubit and Nanodrop (Thermo Fisher) following the manufacturer's instructions.

4.3.1.3. Whole genome sequencing (WGS)

DNA samples were sequenced on an Illumina MiSeq platform at the Ramaciotti Centre for Genomics, Sydney with 250 bp paired-end sequence (coverage ~170x). Sequences were assembled and analysed using CLC Genomics Workbench 8.0 (Qiagen) at Singapore Centre for Environmental Life Science Engineering (SCELSE), Nanyang University of Technology (NTU), Singapore. The complete *S. aureus* ATCC25923

reference genome (accession number: CP009361) from NCBI database was used for mapping and annotation¹³⁸. The threshold variant frequency was set as 35% and synonymous mutations with coding regions were filtered out. To select for a mutant-specific variant, the variants occur in resistant passaged samples were compared to that of the WT strains and cell-only (silver-free) culture.

4.3.1.4. Validation via Sanger sequencing

To confirm that the genetic mutations occur were conserved in different colonies from the same culture, Sanger sequencing was performed at the Ramaciotti Centre for Genomics, Sydney. Polymerase chain reaction (PCR) was used to produce amplicons of specific genes using primers (**Table 4.1**, Integrated DNA Technologies), Phusion High-Fidelity DNA polymerase and GC buffer (New England Biolabs, Inc.) with the following conditions: 98°C for 30 s, 30 amplification cycles (98°C for 5 s, annealing temperature for 30 s (see **Table 4.1**), 72°C for 30 s), and finished by 72°C for 5 min. The amplicons were extracted from an agarose gel (1% agarose in TBE buffer with GelRed (Biotium), submerged in 1xTBE buffer), and cleaned up using Monarch® Gel Extraction Kit (New England Biolabs, Inc.) following the manufacturer's instructions.

Table 4.1. List of primers used to validate the identified mutations in the NAg- and Ag⁺-resistant strains

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Annealing Temp (°C)
<i>purR</i>	CGCAAGTGGTGGTGTACG	CACCATTGATAGAGCCACCA	50
<i>tcyA</i>	GCAGGGCGTTTTGATGTAAT	AGAAACATCTTGACCAAACCA	50
<i>cymR</i>	GTTTTTGCGCAGGTGGTT	AAAAAGAGGGGCAAGGATGT	50
<i>uppS</i>	TTCCAAGTAAAATTGGTCAAGA	TCGTCAAAGTCAGGCCATAA	50
<i>rsbU</i>	CAATGCTTAAACAGATATTCCACA	TCAGTCACACCATCCGTTAAA	50
<i>tcyP</i>	GCAACGCCGAATGAACTAAT	GCATTCACATCACGCTCAAG	50
<i>trxB</i>	GTATTCCAGGCGGTCAAATG	TGAGTCACCACCACCGATAA	52
<i>apT</i>	TGCTGCTTCAATCGTACCAC	TCGTGAGCAATTTAGGAGGAA	50
<i>mprF</i>	TTCATTCCGGCTAAAGATGTG	TGATAATCGAATAACCACGCAAT	50
<i>csbA</i>	GTCTGCTTCCACCGCTACTC	TTCGTCCACCACATCGTAAA	50
<i>cds</i>	CCCTGCACCTACAACCAATA	TCATTGGCGAAGTTGTTGAA	52

4.3.2. Gene inactivation via CRISPR interference (CRISPRi)

Clustered Regularly Interspaced Short Palindromic Repeat interference (CRISPRi) was conducted in several steps (**Figure 4.1**), starting from the single guide RNA design to the phage transduction to the target *S. aureus* strain. Each of this step is presented in details in the following subsections.

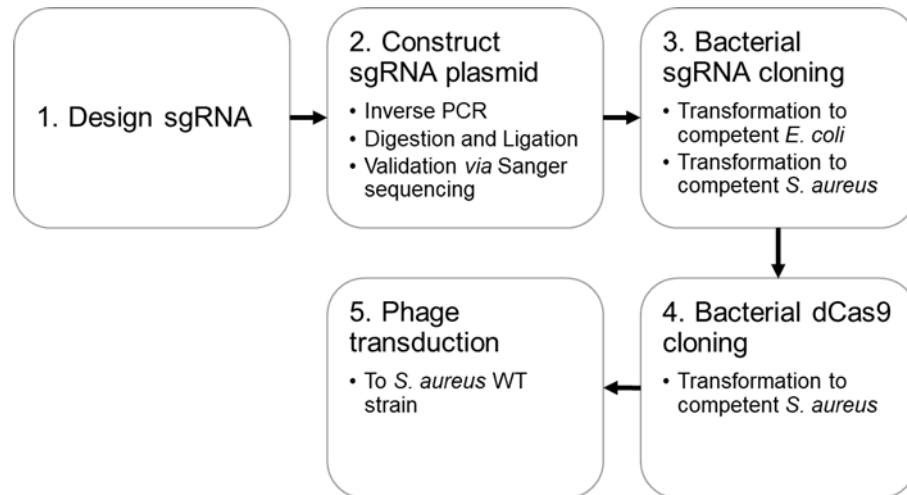


Figure 4.1. Process flow diagram for gene inactivation via CRISPRi

4.3.2.1. Single guide RNA (sgRNA) plasmid construction

The sgRNA plasmid construction was adapted from Larson *et al.*¹³⁹. First, a sgRNA sequence that block the transcription elongation was designed by manually identifying the protospacer adjacent motive (PAM) sequences (5'-NGG-3') from the template strand of the target gene region. Subsequently, 20-25 nt upstream of the PAM sequence were added to the 3' end; this sequence is known as sgRNA base-pairing region. The reverse complementary sequence of the base-pairing region was annealed to a dCas9 handle sequence (5'-GTTTAAGAGCTATGCTGGAAACAGC-3') to form the sgRNA oligonucleotide. A BLAST search and a secondary structure prediction using RNAfold from the ViennaRNA package¹⁴⁰ were performed to identify the specificity and the structure of the sgRNA oligonucleotide.

To construct a sgRNA linear plasmid, inverse PCR was performed using *pbp1* sgRNA, obtained from Morten Kjos¹⁴¹ as template plasmid (**Figure 4.2A**, base-pairing region matches 20 bp of *pbp1* gene sequence), sgRNA oligonucleotide and universal primer as forward and reverse primer, respectively (**Table 4.2**, Integrated DNA Technologies), Phusion High-Fidelity DNA polymerase and GC buffer (New England Biolabs, Inc.) with following conditions: 98°C for 60 s, 25 amplification cycles (98°C for 20 s, annealing temperature for 30 s (**Table 4.2**), 72°C for 3 min), and finished by 72°C for 10 min. Following confirmation of the PCR product *via* gel electrophoresis with ~6 Kbp expected size (1% agarose in TBE buffer with GelRed (Biotium), submerged in 1xTBE buffer), the mixture of sgRNA linear plasmid and template plasmid was purified using Monarch® PCR and DNA cleanup kit (New England Biolabs, Inc.) following the manufacturer's instruction (see **Figure 4.3**). Mixture without DNA polymerase enzyme was included as a control.

To degrade the template plasmid, the mixture were added with DpnI enzyme, which only recognises methylated DNA (New England Biolabs, Inc.) and incubated at 37°C for 2 h, followed by 85°C for 20 min to inactivate the enzyme. This mixture was subsequently added with T4 DNA ligase enzyme (New England Biolabs, Inc.) and was set up at 16°C overnight to circularize the sgRNA linear plasmid. Heat inactivation was again performed afterwards at 60°C for 10 min to inactivate the ligation enzyme. Mixture of sgRNA without ligase enzyme was included as a control. Upon transformation, the presence of the newly constructed base-pairing region was confirmed *via* Sanger sequencing performed at the Australian Genome Research Facility (AGRF) using a primer 5'-AAATCTCGAAAATAATAGAGGGA-3' (Integrated DNA Technologies).

Table 4.2. List of primers used to construct sgRNA plasmid. Base-pairing sequences for each gene target were highlighted in bold.

Gene	Forward Primer (5'→ 3')	Reverse primer (5'→ 3')	Annealing temperature (°C)
<i>purR</i>	ATGAAACTTAGATAAACTGTTGCCGT TTAAGAGCTATGCTGGAAACAGC	TATAGTTATTATACAG GGGACAGTGC	60
<i>tcyA</i>	TTATATTTCTTTCTCTATCGTTAAGA GCTATGCTGGAAACAGC		
<i>cymR</i>	ATTCAATACTTTCAACAAATGTAATGTT TAAGAGCTATGCTGGAAACAGC		

4.3.2.2. Preparation of *E. coli* DH5α competent cells

Overnight culture of a single colony *E. coli* DH5α (37°C, 200 RPM) were inoculated into 400 mL Luria-Bertani Broth (LB, 1 L contains 10 g NaCl, 5 g yeast extract, and 10 g tryptone) to reach a starting OD₆₀₀ of 0.05. Following 2-3 h incubation (37°C, 200 RPM) to reach mid exponential stage (OD₆₀₀ = 0.5-0.7), the cultures were cooled down on an ice-slurry (ice and water) for 15 min and spun down at 4°C, 4600 RPM for 10 min. The cell pellets were then washed with 30 mL ice cold sterile water 3 times and spun down (4°C, 4600 RPM, 10 min). Cells were pooled together and glycerol was added to reach a final concentration of 10%. Cells were then immediately used for transformation (Section 4.3.2.4).

4.3.2.3. Preparation of *S. aureus* RN4220 competent cells

A single colony *S. aureus* RN4220 was inoculated in 400 mL Brain Heart Infusion media (BHI, Oxoid) at 37°C, 200 RPM for 10 – 12 h. The overnight culture was diluted to a fresh 400 mL BHI to OD₆₀₀ = 0.1 and incubated for 60 – 90 min at 37°C, 200 RPM. The bacterial cultures were centrifuged (4600 RPM for 10 min at room temperature). The cell pellets were then washed with 25 mL autoclaved water for 3 times, followed by

10 mL 10% glycerol stock for 2 times prior to cell pellets collection (4600 RPM for 10 min at room temperature). All cells were pooled together and 10 % glycerol was added and immediately used for transformation (Section 4.3.2.4).

4.3.2.4. Plasmid transformation via electroporation

S. aureus RN4220 is a good intermediate cloning host as it can easily be transformed with DNA, including plasmids, from *E. coli*. However, this strain contains a number of genetic mutations to allow transformation techniques to be possible, and therefore is not a suitable strain to use to understand silver restriction mechanisms. Therefore, plasmid DNA was transferred from *S. aureus* RN4220 to ATCC25923 via phage transduction.

Competent cells (50 µL) were inoculated with 5 – 15 µL of the sgRNA plasmid mixture (**Figure 4.2A**, base pairing region matches the target genes' sequence: *purR*, *tcyA*, or *cymR*) or dCas9 plasmid (**Figure 4.2B**) and transferred to BioRad Gene Pulse electroporation cuvette. Competent cells without plasmid addition served as a negative control. The mixture was electroporated with 2 kV, 25 µF and 200 Ω or 2.5 kV, 25 µF and 100 Ω and immediately added with ice-cold LB or room-temperature BHI prior incubation at 37°C, 200 RPM for 1 h and 3 h for *E. coli* DH5α and *S. aureus* RN4220, respectively. The cell culture was spread to an LB or BHI plate supplemented with appropriate antibiotic (**Table 4.3**) and incubated at 37°C for 24 – 72 h to select for potential transformants.

Table 4.3. The list of antibiotic used for plasmid selection.

Plasmid	Antibiotic
sgRNA plasmid	Ampicillin 100 µg/mL (<i>E. coli</i>) or chloramphenicol 10 µg/mL (<i>S. aureus</i>)
dCas9 plasmid	Erythromycin 5 µg/mL and lincomycin 25 µg/mL (<i>S. aureus</i>)

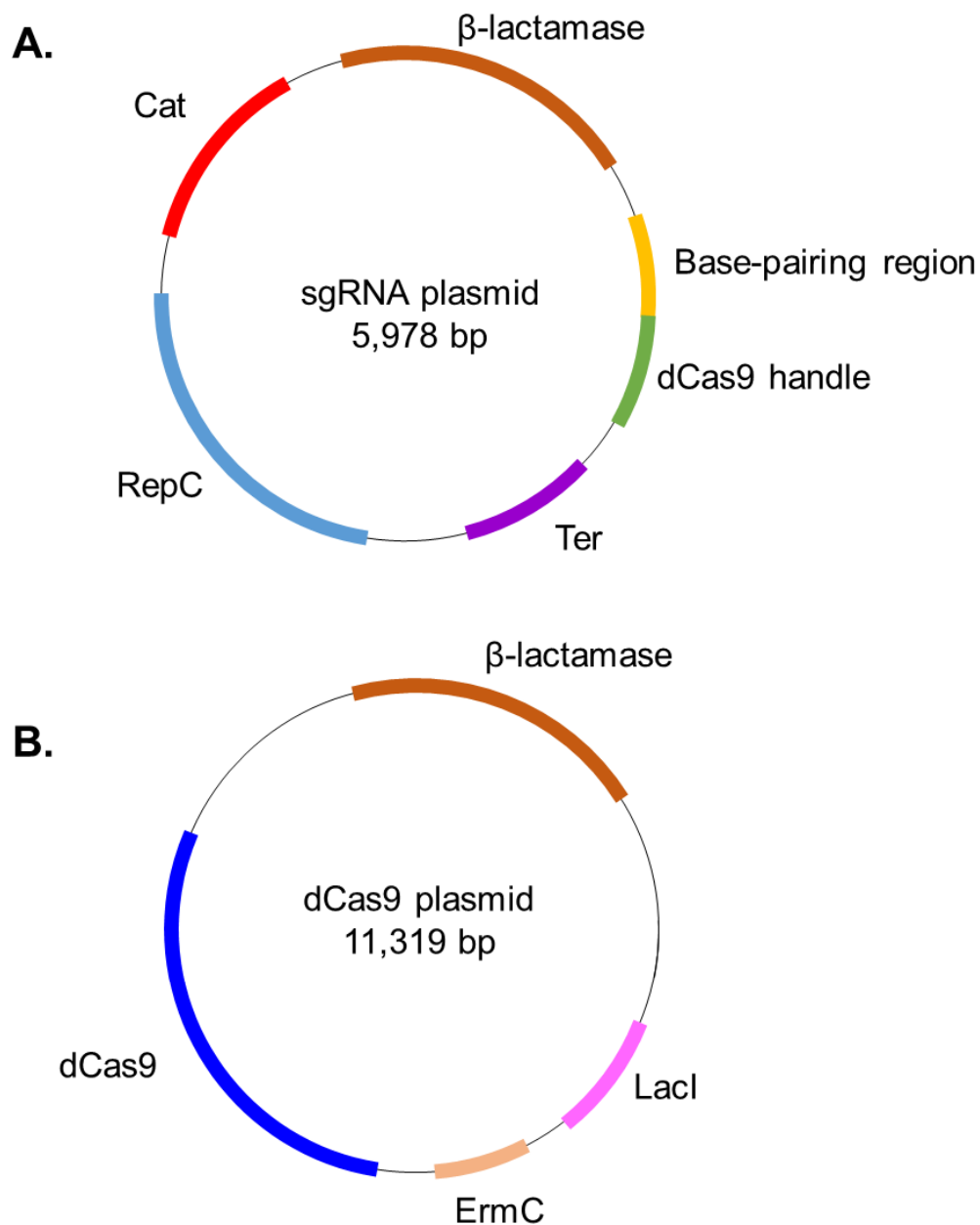


Figure 4.2. Map of A) sgRNA plasmid and B) dCas9 plasmid for CRISPRi. The dCas9 plasmid was obtained from Morten Kjos¹⁴¹. RepC: replication initiation, Cat: chloramphenicol resistance, Ter: DNA replication terminus, ErmC: erythromycin resistance, LacI: lactose operon repressor.

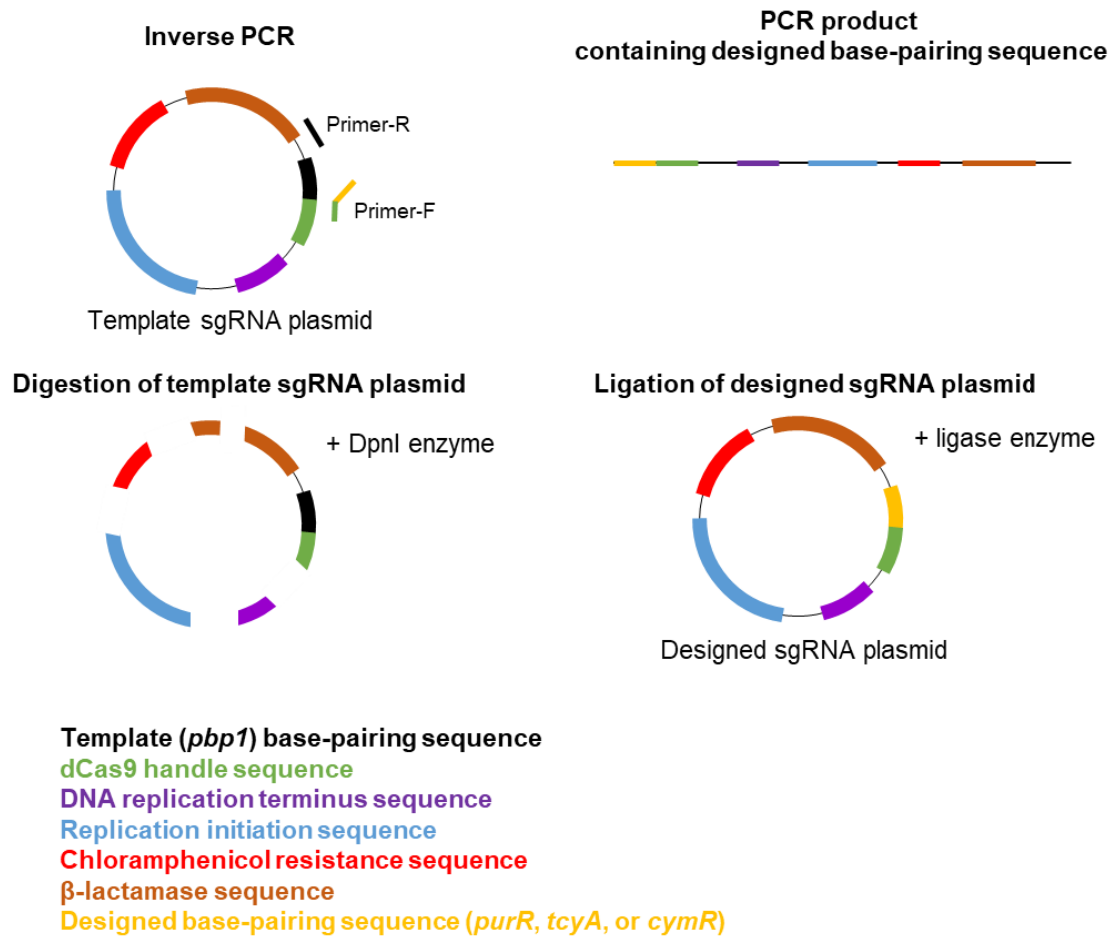


Figure 4.3. Schematic diagram of sgRNA plasmid construction. The sequences in the plasmid are colour-coded and explained in the bottom left corner. Primer-R: reverse primer, Primer-F: forward primer.

4.3.2.5. Phage transduction to *S. aureus* ATCC25923

To prepare the phage lysate, 150 µL of the donor overnight strain culture (*S. aureus* RN4220 with sgRNA or dCas9 plasmid) was added to a mixture of 5 mL BHI, 5 mL filtered phage (Φ) buffer (1 mM MgSO₄, 4 mM CaCl₂, 50 mM Tris-HCl pH 7.4, 0.6% w/v NaCl, and 0.1% w/v Gelatin), and 100 µL of Φ11. This mixture was statically incubated at room temperature until suspension was clear (~4 h) and was spiked again with another 150 µL of the donor strain culture to increase phage titer, prior to overnight incubation at room temperature. The following day, cell debris was removed with 0.2 µm filter and lysate was stored at 4°C prior to use.

Phage infection of the recipient strain (*S. aureus* ATCC25923) will result in the transduction of the desired plasmid from phage into *S. aureus*. Firstly, cell pellets of *S. aureus* were prepared from the 50 mL overnight culture in LK medium (10 g/L tryptone, 5 g/L yeast extract, and 7 g/L KCl) at 37°C, 200 RPM. Following the resuspension of the cell pellet in LK, 10 µL of 1 M CaCl₂ and 500 µL of Φ11 lysate containing the desired plasmid was added. The mixture without lysate served as the negative control.

The mixture was statically incubated at 37°C for 25 min followed by 37°C at 200 RPM for 15 min.

To eliminate remaining $\Phi 11$, the mixture was added with 0.02 M Na-citrate. Upon collecting the cell pellets and resuspension with 0.02 M Na-citrate, the suspension was kept on ice for 45 min. Cells were allowed to recover on LK bottom agar (2% agar) incorporated with 0.05% Na-citrate for 2 h at 37°C, and positive colonies were selected by overlaying the agar with LK top agar (0.7% agar) incorporated with 0.05% Na-citrate and appropriate antibiotic at 3 times concentration (**Table 4.3**). Plates were incubated at 37°C for 24-48 h to select for potential colony incorporated with the plasmid. This procedure was repeated sequentially with both the sgRNA plasmid and dCas9 plasmid were to create strains that contained both plasmids.

4.3.3. Determination of minimum inhibitory concentration (MIC) of *S. aureus* JE2

4.3.3.1. Growth of *S. aureus* JE2 and JE2 transposon mutant

The following strains (**Table 4.4**) were provided by the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) for distribution by BEI Resources, NIAID, and NIH from the United States of America (USA). All the strains have MLST profile (ST 8), SCCmec (subtype IV), agr group, and spa motif (MBQBLO), and usually carry Panton-Valentine leucocidin (PVL) toxin genes and arginine catabolic mobile element (ACME). The transposon mutant library was created by the Centre for Staphylococcal Research (CSR) at the University of Nebraska Medical Centre, USA.

Table 4.4. List of *S. aureus* JE2 (USA300 JE2) strains used in this study

Strain	Remarks
<i>Staphylococcus aureus</i> JE2, NR-46543	Parent strain for transposon mutant
<i>Staphylococcus aureus</i> JE2, Transposon Mutant NE1237 (SAUSA300_0473), NR-47780	Transposon inserted to <i>purR</i> gene region
<i>Staphylococcus aureus</i> JE2, Transposon Mutant NE1592 (SAUSA300_2359), NR-48134	Transposon inserted to <i>tcyA</i> gene region
<i>Staphylococcus aureus</i> JE2, Transposon Mutant NE1293 (SAUSA300_1583), NR-47836	Transposon inserted to <i>cymR</i> gene region

To grow *S. aureus* JE2, a single colony was inoculated to a 5 mL Ca-MHB and incubated at 37°C, 200 RPM whereas to grow the transposon mutant strains, the growth media was added with 5 µg/mL erythromycin to maintain the transposon insertion.

4.3.3.2. Preparation of silver antibacterial agent and MIC determination

The MIC determination of *S. aureus* JE2 wild-type and selected transposon mutants to NAg and Ag⁺ was conducted by Dr. Amy Bottomley. The preparation of silver antibacterial agent and the determination of the MIC were performed as explained previously (see Section 3.3.2).

4.4. Results and Discussion

4.4.1. Genetic adaptation of NAg- and Ag⁺-resistant *S. aureus* strain

To investigate if the resistance to NAg and Ag⁺ shown by the passaged *S. aureus* was conferred by genetic changes, whole genome sequencing and comparison was performed. The strains selected (see green, blue, and red circle, and diamond in **Figure 3.4A** and **C**), cultured from two passaging bioreplicates and from different passaging days, were previously confirmed to have higher minimum inhibitory concentration (MIC) than that of the wild-type (WT) strain (see **Figure 3.6** and **Figure 3.7**), indicating resistance characteristic¹¹⁸.

The whole genomic sequencing was performed, not only on the resistant strain and wild-type (WT) parent (unevolved), but also to the control strain. The WT strain is the parental strain that serves as the initial inoculum for the resistance development study and was shown to be susceptible to silver (see **Figure 3.3**). The control strain is the silver-free (cell-only) culture passaged in parallel with the silver-exposed culture. The inclusion of these two strains in the genomic studies is to identify and exclude any random 'background' mutations that may already initially present in the first inoculum (the parent WT strain) or due to repeated sub-culturing (the control strain)¹⁴², respectively. Indeed, the genomic sequencing displayed that the WT strain harboured a single nucleotide variant (SNV) at a gene encoding for a hyperosmolarity resistance protein (Ebh). Some of the control strains were also identified to harbour an SNV at a gene encoding a histidine kinase protein. This finding may suggest that *S. aureus* potentially attempted to adapt to its growth environment.

The non-synonymous mutations (mutations that give rise to changes in amino acid sequence in the encoded protein) were identified upon comparing the SNV identified in the resistant strains, the WT strain and the control strain from the respective passaging days. These mutations were further validated by PCR amplification and sequencing the gene of interest (Sanger sequencing) in multiple bacterial isolates to avoid false-positive results.

Table 4.5. Number of non-synonymous mutations identified in NAg- and Ag⁺-resistant strain. The mutations were confirmed to present in multiple isolates upon validation *via* Sanger sequencing.

Strains	Non-synonymous mutations
NAg-resistant strain	4
Ag ⁺ -resistant strain	7

The study detected 4 and 7 non-synonymous mutations in NAg and Ag⁺-resistant strains, respectively (**Table 4.5**). These mutations were identified in multiple isolates, however, not all of them were shown in the bioreplicates of the passaging experiments (see **Table 4.6** and **Table S4.1** for mutations in NAg-resistant strain, **Table 4.7** and **Table S4.2** for mutations in Ag⁺-resistant strain). Although no multiple mutations found in one gene or genes conserved between the NAg- and Ag⁺-resistant strain, the study identified that some pathways were common, e.g. purine nucleotide synthesis, cysteine metabolism, and cell envelope metabolism; these pathways previously reported as being associated with silver antibacterial mechanisms. The mutations in NAg- and Ag⁺-resistant strains herein described in more detail are the mutations identified in both independent bioreplicates of the passaging experiments (**Table 4.6** and **Table 4.7**).

NAg-resistant bacteria harboured a single nucleotide mutation in *purR* gene (locus tag KQ76_02295) that encodes a putative purine synthesis repressor protein PurR. The TCT-to-TAT mutation (**Table 4.6**) results in amino acid change (serine at position 169 to tyrosine, Ser169Tyr) within the phosphoribosyl pyrophosphate (PRPP) putative binding motif of PurR¹⁴³. The PRPP binding motif is a candidate for direct interaction of PurR with the purine operon to downregulate the expression of enzymes that synthesise inosine monophosphate (IMP), a precursor for the DNA purine nucleotides adenosine monophosphate (AMP) and guanosine monophosphate (GMP) (**Figure 4.4A**)^{143,144}. Whilst both serine and tyrosine are polar amino acids, this mutation results in the replacement of an uncharged R group (serine) to an aromatic ring R group (tyrosine), which may affect steric bulk or conformation within the PRPP binding pocket. The observed mutation was thought to lower the binding affinity, resulting in increased purine nucleotide synthesis. Purine synthesis is a major cellular process, involved in synthesizing substrates for DNA replication and repair as well as in the synthesis of amino acids and energy production^{145,146}. Indeed, research inquiries have identified DNA and proteins as major NAg targets in *S. aureus*^{8,68,69}. Thus, the mutation in *purR* could alleviate nanoparticle toxicity through higher purine production levels. In *E. coli*, resistance to NAg has been linked to an SNV in *purL* that encodes the PurL enzyme subunit¹⁶, which is also important in the early stages of purine synthesis (the enzyme catalyses the ATP-dependent conversion of phosphoribosyl formylglycinamide

ribonucleotide (FGAR) to formylglycinamidine ribonucleotide (FGAM)). Thus, it would be reasonable to assume that the purine metabolism may be a conserved pathway playing a role in silver resistance mechanism between different species.

A second mutation, a single nucleotide deletion, was found in *tcyA* gene (locus tag KQ76_12595) of the NAg-resistant *S. aureus* (**Table 4.6**). The observed ATC-to-TC mutation in the *tcyA* shifted the reading frame of the amino acid coding and was projected to result in early termination of the protein synthesis (originally 21 amino acids after Ile237 to only 5 amino acids after Ser237 in the resistant mutants), thus generating a truncated, most likely non-functional protein. This gene encodes a putative L-cystine binding protein (TcyA) that imports extracellular cystine into bacteria for reduction to the amino acid cysteine. Cysteine forms the active site of the thioredoxin that combats oxidative stress in Gram-positive bacteria, including those that are stimulated by NAg exposure^{31,65,74}. Cysteine is also one of the precursors for Fe-S cluster synthesis¹⁴⁷. Upregulation of cysteine synthesis genes have been seen in *E. coli* when exposed to NAg⁷², possibly to also compensate for the nanoparticle-mediated damage to iron-sulphur (Fe-S) clusters that are present in many proteins^{75,147,148}. However, intracellular cysteine levels must be tightly regulated. Recent studies have reported that excess levels of cysteine can trigger the generation of oxygen radicals in bacteria^{78,149}. Cysteine reduces ferric ion (Fe³⁺) to ferrous ion (Fe²⁺), the latter then reacts with cellular peroxide (H₂O₂) *via* a Fenton-type reaction to release highly reactive hydroxyl radicals (•OH). Additionally, excess cysteine can lead to elevated cellular H₂S levels, in turn promoting the formation of the damaging peroxide radicals⁷⁸. Therefore, the loss of TcyA function could help maintain cysteine levels in such a way that protect the cells from high levels of oxidative stress (**Figure 4.4B**). Taken together, it seems that the influence to cysteine pathway to silver resistance mechanism is not specific to *S. aureus* only, although the way it responds by protein function alteration may be unique to *S. aureus*.

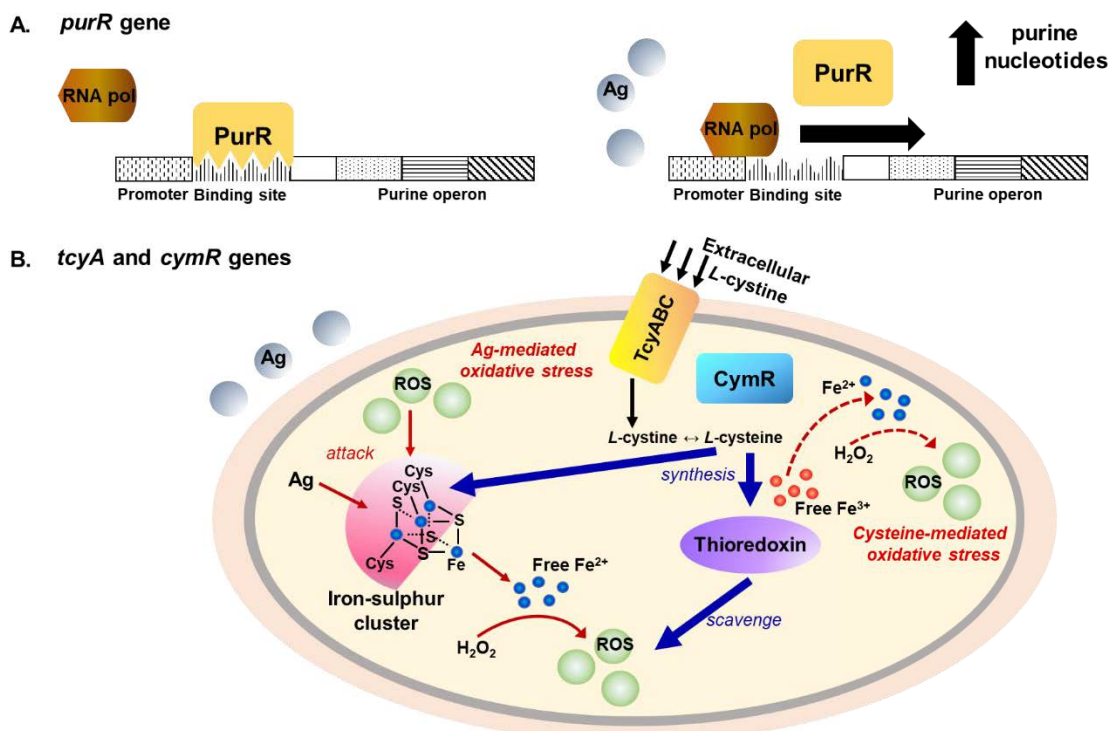


Figure 4.4. The observed mutations and hypothesised effect in the genes encode for putative purine operon repressor PurR in NAg-resistant strains (A), L-cystine binding protein TcyA and cysteine metabolism regulator CymR (B) identified in NAg and Ag⁺-resistant strains isolates. The mutations in *purR* was hypothesised to increase the synthesis of purine nucleotides as a defence mechanism to cope with NAg damage on DNA and proteins. The mutation would reduce the affinity of PurR with its corresponding DNA binding site, allowing increased transcription of the purine operon transcription by RNA polymerase. The mutation in the genes associated with cysteine metabolism (*tcyA* and *cymR*) were hypothesised to minimize cysteine-mediated redox imbalance that may be linked to the mechanism of silver toxicity. Silver has been indicated to damage iron-sulphur clusters in proteins either through direct or indirect attack (via ROS), releasing the Fenton-active Fe²⁺ ions that subsequently react with cellular H₂O₂ to generate hydroxyl radicals (*OH). In *S. aureus*, this silver-mediated oxidative stress naturally triggers a defence process that leads to, among other mechanisms, an increased pool of cellular cysteine most likely for the synthesis of the thioredoxin, as well as, to re-supply the damaged iron-sulphur clusters. Mutations in *tcyA* that imports the extracellular cystine for conversion to cysteine inside the cell, and on the regulator *cymR*, were thought to reduce the influx of extracellular cystine, lowering the cellular cysteine pools. Cysteine has been indicated to involve in a Fenton-type reaction by first reducing the Fe³⁺ ions to Fe²⁺ to then cause a cellular oxidative stress. The iron-sulphur cluster (Fe-S) illustration was adapted from ⁷⁵. (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

For Ag⁺-resistant mutants, a single nucleotide variation was detected in *cymR* that is also involved in cysteine metabolism. The GGT-to-GAT mutation (Table 4.7) resulted in amino acid change (Gly63Asp) in the putative operon repressor protein CymR. Substitution of the non-polar glycine to a negatively-charged aspartate likely results in misfolding of the protein, affecting functionality. The protein regulates cysteine synthesis in *S. aureus*, including the expression of *tcyA*¹⁵¹. It has been previously shown that exposure to toxic concentrations of Ag⁺ increased cysteine pools in bacteria⁷². Therefore, as with NAg, it was hypothesised that the mutation could dampen the cysteine-mediated cellular redox imbalance (Figure 4.4B).

From the whole genomic studies, it is clear that there are unique mutations that potentially contribute to the individual resistance mechanisms to either the nanoparticle or ionic silver. For example, the nucleotide synthesis *purR* mutation was only detected in the NAg-resistant strain but not in the Ag⁺-resistant strain. This most likely explains the observed differences in the extent of resistance development to the different forms of silver. There are also mutations in genes that encode functionally related proteins in the resistant bacteria, such as the cysteine metabolism-related *tcyA* and *cymR* mutations that was thought to contribute in mediating oxidative stress, found in NAg and Ag⁺-resistant mutant, respectively. These 'common' mutations could be a factor in the observed phenomenon of nanoparticle/Ag⁺ cross-resistance that was observed previously (Chapter 3).

The mutations identified in the resistant strains demonstrated that *S. aureus* is capable to develop a unique and stable hereditary resistance characteristic, indicating an acquired resistance mechanism¹⁵², to NAg and Ag⁺. Unlike a previous study that identified an epigenetic-type (no gene mutation) of resistance characteristic in NAg-resistant *E. coli*¹⁴, this genetic changes is likely the underlying reason for *S. aureus* resistance to NAg and Ag⁺ as the strains might change the protein functions or bacterial physiology to accommodate the silver toxicity.

The findings here could demonstrate the unique characteristic of silver resistance in *S. aureus* as no known silver-resistant mechanisms (*omp*, *cus*, and *sil* system) in *S. aureus* ATCC25923 could be identified (BLAST, this study). Although an indication of a low prevalence of *sil* genes in clinically- and environmentally-isolated methicillin-resistant *S. aureus* (MRSA) has been reported previously by Loh *et al.*, they did not specify the genes' location, be it on plasmid or chromosome¹¹. Considering the source of the isolates, it is reasonable to speculate that the isolates could undergo a horizontal gene transfer (HGT) process and acquire foreign DNA containing *sil* genes from the environment. Note that the mutations found here were obtained in a tightly-controlled exposure environment starting with a pure bacterial culture, therefore, preventing an HGT process. The mutations were also identified in *S. aureus* chromosome, instead of plasmid, based on the mapping reference. Altogether, it might be interesting and more environmentally relevant to conduct a mixed-culture studies to investigate the impact of mixed-culture population to the possibility of resistance generation or acquisition.

At this stage, it was shown not only that *S. aureus* was capable of developing resistance to NAg and Ag⁺, but also was likely to resist the otherwise toxic silver antibacterial agent by acquiring genomic mutations. The mutations identified in NAg-resistant strain operated in nucleotide and cysteine metabolism pathway. The latter

was also identified for Ag⁺-resistant strain. The mutations were thought to mediate NAg toxicity by increasing purine nucleotides and managing oxidative stress that may originate from the silver exposure and abundant cysteine uptake. It is essential to validate these hypotheses as to understand and confirm the role of the mutations. This will be the focus of the next section.

Identification of genetic adaptation in NAg- and Ag⁺-resistant *S. aureus* strains

Table 4.6. Mutations in NAg-resistant *S. aureus* genome

Nucleotide change							Amino acid change	Pathways
Gene locus	^a Position in genome	Mutation type	Original	Variation	f (%)	Gene annotation (gene name)		
KQ76_02295	483,828	SNV	C	A	100	Purine operon repressor (<i>purR</i>)	Ser169Tyr	Nucleotide metabolism
KG76_12595	2,446,154	Deletion	A	-	99.6	L-cystine binding protein (<i>tcyA</i>)	Ile237Ser	Cysteine metabolism

^aIn *S. aureus* ATCC25923

SNV (single nucleotide variants) denotes substitution-type change of a single nucleotide at a specific position in the genome, f = frequency of the nucleotide changes occurring in a bacterial isolate (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Table 4.7. Mutations in Ag⁺-resistant *S. aureus* genome (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Nucleotide change							Amino acid change	Pathways
Gene locus	^a Position in genome	Mutation type	Original	Variation	f (%)	Gene annotation (gene name)		
KQ76_08480	1,689,735	SNV	G	A	100	Cysteine metabolism regulator (<i>cymR</i>)	Gly63Asp	Cysteine metabolism

^aIn *S. aureus* ATCC25923

SNV (single nucleotide variants) denotes substitution-type change of a single nucleotide at a specific position in the genome, f = frequency of the nucleotide changes occurring in a bacterial isolate (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

4.4.2. The role of the mutations to NAg and Ag⁺ resistance

To investigate the role of the mutations identified in NAg- and Ag⁺-resistant strain, this study sought to manipulate the genes of interest (*purR*, *tcyA*, and *cymR*) in the WT strain by depletion or deletion of the gene and subsequently, determine the bacteria's susceptibility level to NAg and Ag⁺ via minimum inhibitory concentration (MIC) assessment. One of the most commonly used gene manipulation techniques is the homologous recombination, however, this has been known to be a particularly challenging technique for *S. aureus* due to its low cloning success rate, rendering the technique laborious and time costly¹⁵³. Therefore, studies have attempted to develop different methods to overcome the aforementioned drawbacks, including the use of the Clustered Regularly Interspaced Short Palindromic Repeat (CRISPR) technique¹⁵⁴. CRISPR is a naturally-occurring protection mechanism identified in prokaryotes to recognize and cleave foreign DNA, using CRISPR-associated nucleases (Cas) protein¹⁵⁵. This defence system has been adapted into a molecular microbiology tool through the use of artificially made single guide RNA (sgRNA) that contains the complementary sequences of the target genes. Briefly, upon the introduction of the sgRNA and Cas protein intracellularly, a sgRNA-Cas complex is formed. This complex is then 'guided' to the target genes due to the presence of complimentary sequences in the sgRNA, upon which the Cas protein will perform nuclease activity on the target gene and prevent its transcription (see **Figure 4.5**). The now widely-used Cas protein is the Cas9 protein, originating from *Streptococcus pyogenes*¹⁵⁴.

For the NAg- and Ag⁺-resistant strain, this study intended to perform gene inactivation to the target genes because of the single variant mutations identified in each of the gene were hypothesised to result in a loss of function in the translated proteins. The use of CRISPR is made possible the current case by employing a catalytically-inactive Cas9 protein, known as 'dead' Cas9 (dCas9), of which cleaving ability was disabled through mutations in both of its RuvC and HNH domain¹⁵⁵. Instead of cleaving the target genes as what the original Cas9 protein would do, dCas9 'interferes' with the transcription process by binding to the target gene and physically blocking RNA polymerase, hence the system is called CRISPRi (CRISPR interference) (see **Figure 4.5**).

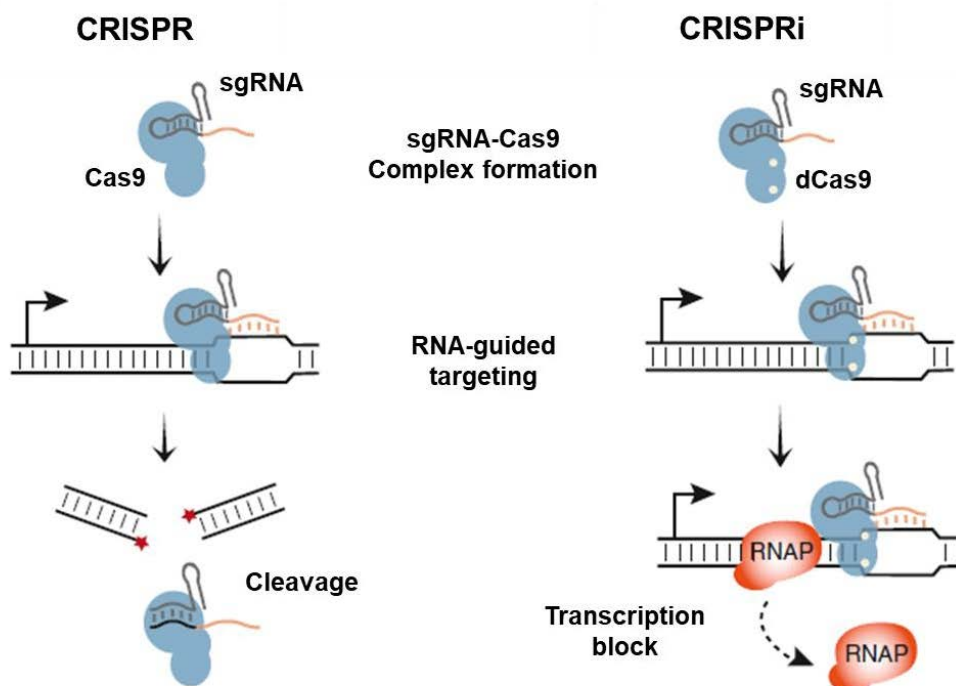


Figure 4.5. Schematic diagram of CRISPR and CRISPRi. RNAP: RNA polymerase. (Adapted from ¹⁵⁴ with permission from Elsevier).

To perform CRISPRi, firstly, sgRNA primer was designed for each gene of interests to construct the sgRNA plasmid (see **Figure 4.2A**). Upon the confirmation that the plasmid contains the designed sequence, corresponding to the reverse complement of part of the target gene sequences, competent *E. coli* DH5 α cells were transformed with the plasmid and the transformants was selected by its antibiotic-resistant marker. *E. coli* DH5 α is a common competent *E. coli* strain that has been widely used for initial transformation and storage of plasmid prior to the later transformation of sgRNA plasmid to the target bacteria. This is made possible as a result of *recA1* and *endA1* mutation in *E. coli* that reduces plasmid recombination and endonuclease activity, respectively, hence a more efficient and stable bacterial host system for plasmid transfer¹⁵⁶.

The next step is to transfer the sgRNA plasmid to introduce DNA from *E. coli* into lab strain *S. aureus* RN4220. This strain is commonly used as an intermediate system for *S. aureus* genetic manipulation process because of its *sau1hsdR* mutation, blocking the bacterium's restriction-modification function¹⁵⁷, thus, allowing easy access for foreign DNA uptake by the bacterium. The sgRNA plasmids (each for the *purR*, *tcyA*, and *cymR* genes) and the plasmid dCas9 were transformed individually to competent *S. aureus* RN4220 cells. Upon transformation, the transformants were selected *via* its antibiotic resistant marker.

The plasmids transfer between the *S. aureus* strains (RN4220 to the model ATCC25923 WT strain for the silver studies) is facilitated by phage transduction, the most common horizontal gene transfer (HGT) process in *S. aureus*^{158,159}. This technique utilizes the commonly occurring phenomena of bacteriophages (viruses that infect bacteria) to (accidentally) take up DNA of the donor *S. aureus* strain (in this case the RN4220 transformed with each sgRNA plasmid) during infection, and subsequently, transferring the bacterial DNA to the recipient strain (in this case the *S. aureus* WT strain)¹⁵⁸. Successful transduction process results in the recipient bacteria containing the designated plasmid, of which will be selected using its antibiotic resistance marker.

This study aimed to introduce both sgRNA and dCas9 plasmid to the WT strain. This was done in a sequential manner whereby the sgRNA plasmid was initially introduced into WT, resulting in positive colonies on the selective agar. However, attempts to introduce the dCas9 plasmid into this strain has not been successfully performed, even after several attempts using gold-standard protocol. It was established prior to the experiment that the donor and recipient strain were susceptible to the phage used (Φ 11), demonstrated by a clear lysis area in an agar plate inoculated by bacterial growth (data not shown). It was also established that the CRISPRi system worked under our laboratory conditions, as construction of knockdown strains using an *ftsZ* or *pbp1* sgRNA plasmid in *S. aureus* 8325-4 showed the expected phenotype (Dr. Amy Bottomley, personal communication). The underlying reason for this unsuccessful transduction is currently unclear, nevertheless, it was thought that the differences in structure of the *S. aureus* ATCC25923 strain's wall teichoic acid (WTA) might play a role in the efficiency of plasmid transfer, as has been reported previously¹⁶⁰. Due to time constraints, further optimization on the experimental conditions has not been completed yet. These include technical optimization of the phage transduction step e.g. adding more bacteriophage volume or longer exposure time to the donor strain, taking a step back to investigate if accurate transformation was performed, changing the phage to Φ 29¹⁶¹, as well as considering electroporation^{153,162}.

To pursue the investigation on the mutations' role in a timely manner, this study decided to utilize the publically available *S. aureus* JE2 mutant library, developed by the Centre for Staphylococcal Research (CSR) at the University of Nebraska Medical Centre and manufactured by BEI Resources. This strain, also known as USA300 JE2, is a methicillin-resistant strain that causes nosocomial MRSA infection¹⁶³. The construction of the mutant library was carried out by the insertion of transposons that disrupts non-essential genes, causing the transposon mutant to lose the proteins'

function. Therefore, it was thought that using JE2 mutant would be a suitable alternative study at this stage to understand the role of the mutations in NAg- and Ag⁺-resistant strains.

S. aureus parents (JE2) and three mutants (JE2 *purR*, *tcyA*, and *cymR*) were subjected to MIC determination studies performed by Dr. Amy Bottomley. Similarly as explained in Chapter 3, the MIC was defined as the minimum concentration that inhibits 95% of bacterial growth when compared to the untreated control. The MIC values of the mutants were compared to that of the parents.

In contrast to that of the *S. aureus* WT (ATCC25923) and the silver-resistant strains, *S. aureus* JE2 parents and mutants were observed to be eradicated completely by NAg and Ag⁺ (**Figure 4.6**), using the same concentration range as tested for *S. aureus* WT (0.5 – 5 µg Ag/mL). The results were thought to be due to the use of two different strains with different hereditary characteristics. As mentioned previously, *S. aureus* JE2 is a methicillin-resistant strain (MRSA) whereas the *S. aureus* WT was demonstrated in this study (Chapter 3) and others^{164,165} as a methicillin-susceptible strain (MSSA). Therefore, it is likely that the resistance characteristics or mechanisms of an MRSA may contribute in granting higher susceptibility in silver than that of the MSSA. Further investigation will need to use a lower NAg and Ag⁺ concentration range as to determine the exact susceptibility level of *S. aureus* JE2 parents and mutant strains.

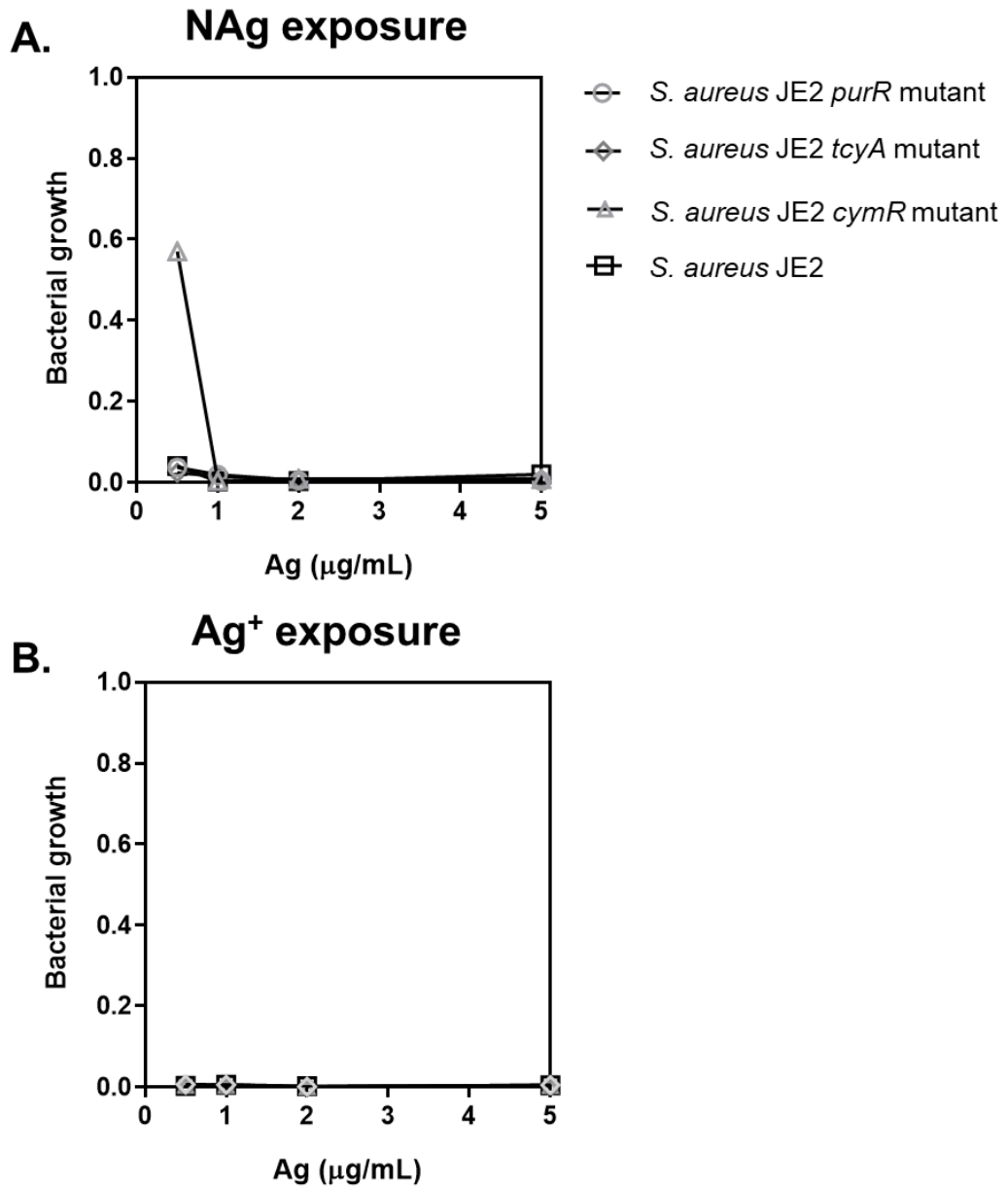


Figure 4.6. Growth responses of *S. aureus* JE2 (square) and JE2 mutants (*purR* mutant: grey circle, *tcyA* mutant: grey diamond, and *cymR* mutant: grey triangle) in increasing concentration of NAg- (A) or Ag⁺ (B) when compared to the cell-only control. Each data point represents the mean of three measurements (one biological replicate with three technical replicates).

One distinct growth response was observed in *S. aureus* JE2 *cymR* mutant (**Figure 4.6A**). When exposed to 0.5 µg/mL of NAg, the mutant displayed ~60% growth relative to the untreated control, indicating a slightly lower susceptibility when compared to the *S. aureus* JE2. Interestingly, the mutation was only associated with Ag⁺-resistant *S. aureus* (**Table 4.7**), however, it impacted the growth response in NAg. This result suggests that there is a difference in susceptibility when certain genes are knocked out, at least in *S. aureus* JE2, and indicates that certain genes may contribute to the cross-resistance phenomenon observed between NAg and Ag⁺ in *S. aureus* WT (see Chapter 3). Again, comparing the growth responses when *S. aureus* JE2 parents and mutants

exposed to NAg and Ag⁺ at a lower concentration range should be considered for future work.

4.5. Conclusion

In conclusion, the study identified unique genomic adaptations in the NAg- and Ag⁺-resistant *S. aureus* despite lacking any of the previously determined silver resistance determinants. The identified mutations, which were thought to govern the resistance traits, are involved in nucleotide and cysteine metabolism pathways and were hypothesized to play a role in synthesizing more nucleotides for cellular processes and mediating oxidative stress in response to silver toxicity. Different mutations between NAg- and Ag⁺-resistant strains may explain the different extent of resistance, nevertheless, mutual functionally-related mutated genes between the two strains may signify the cross-resistance phenomenon observed in Chapter 3. To validate the hypotheses regarding the role of the mutations, the study has attempted to perform CRISPRi, although this has not completed yet due to time constraints. An alternative study using *S. aureus* JE2 strain showed the inactivated target genes have no difference in susceptibility to NAg or Ag⁺ at the concentration tested, however, it is currently inconclusive. The study acknowledges that it is essential to confirm the hypotheses, therefore, future study *e.g.* further optimization of CRISPRi experimental methods and specifying the susceptibility level of *S. aureus* JE2 strain at a lower NAg and Ag⁺ concentration range, was suggested.

4.6. Supplementary Information

Table S4.1. Additional mutations in NAg-resistant *S. aureus* genome

Nucleotide change						Gene annotation (gene name)	Amino acid change	Pathways
Gene locus	^a Position in genome	Mutation type	Original	Variation	f (%)			
KQ76_06080	1,232,347	SNV	G	T	100	UDP pyrophosphate synthase (<i>uppS</i>)	Asp195Tyr	Cell envelope metabolism
Hypothesised mechanism: The UppS protein functions as a catalyst for undecaprenyl diphosphate (UPP) biosynthesis, a precursor for lipid II, an important component in the second-half of peptidoglycan synthesis process ¹⁶⁶ . The mutation may decrease the UPP levels and lead to less lipid II synthesis, consequently elevating the intermediates from the first-half of the peptidoglycan synthesis process ¹⁶⁷ . A study has shown that reduced UppS levels was observed to increase susceptibility to β -lactam antibiotics (competitively bind with lipid II), as also observed in this study ¹⁶⁸								
KQ76_10725	2,102,445	SNV	G	A	100	Phosphoserine phosphatase (<i>rsbU</i>)	Asp134Asn	Virulence factor
Hypothesised mechanism: The RsbU protein activated σ^B , a protein that influences many genes to cope with environmental (e.g. peroxides, UV) and antibacterial stress ¹⁶⁹ . The mutation may alter levels of σ^B to allow cells to survive in the presence of NAg.								

^aIn *S. aureus* ATCC25923

SNV (single nucleotide variants) denotes substitution-type change of a single nucleotide at a specific position in the genome, f = frequency of the nucleotide changes occurring in a bacterial isolate (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Table S4.2. Additional mutations in Ag⁺-resistant *S. aureus* genome (Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Nucleotide change						Gene annotation (gene name)	Amino acid change	Pathways
Gene locus	^a Position in genome	Mutation type	Original	Variation	f (%)			
KQ76_01810	386,992	SNV	G	T	100	L-cystine uptake protein (<i>tcyP</i>)	Met357Ile	Cysteine metabolism
Hypothesised mechanism: Together with the TcyABC protein, the TcyP protein facilitates L-cystine uptake to cytoplasm for the synthesis of L-cystine ¹⁷⁰ , an essential compound to fight off silver-generated ROS ^{65,72,82} . Due to the potential for cysteine-mediated oxidative stress, the mutation could be to reduce the increase in cellular cysteine pools already reported during Ag ⁺ exposure ⁷⁸ .								

KQ76_03830	788,889	SNV	A	G	100	Thioredoxin reductase (<i>trxB</i>)	Tyr13Cys	Oxidative stress
Hypothesised mechanism: The enzyme thioredoxin reductase catalyses the reduction of thioredoxin to its (reduced) active form to scavenge ROS ¹⁷¹ , generated by silver exposure ⁷⁹ . The mutation is thought to provide additional cysteine (a change of Tyr to Cys) in the thioredoxin reductase active site ⁶⁵ , possibly to cope with the Ag ⁺ binding to the 'original' cysteine in the active site.								
KQ76_00825	1,700,650	SNV	G	C	100	Adenine phosphoribosyltransferase (<i>apt</i>)	Met1Ile	Nucleotide metabolism
Hypothesised mechanism: The Apt protein catalyses the formation of adenine (purine nucleotide) from adenosine monophosphate (AMP) ¹⁷² . The mutation results in the absence of methionine (Met) in initiation codon that could lead to a very low degree of the Apt protein synthesis ¹⁷³ , which in turn may alter the energy pools available to support growth in the presence of NAg ¹⁷⁴ .								
KQ76_06710	1,354,358	SNV	T	G	100	Phosphatidylglycerol lysyltransferase (<i>mprF</i>)	Cys380Trp	Cell envelope metabolism
Hypothesised mechanism: The enzyme catalyses the transfer of a lysyl group from L-lysyl-tRNA(Lys) to phosphatidylglycerol to generate the positively-charged lysylphosphatidylglycerol in <i>S. aureus</i> cell membrane ¹⁷⁵ . The mutation could lead to an increased net positive charge of the cell envelope ^{176,177} , thereby repelling the Ag ⁺ ion ⁶⁸ . A mutation to this gene was found to associate with a reduced resistance to the β -lactam oxacillin and methicillin ¹⁷⁸ .								
KQ76_10800	2,116,579	SNV	T	C	100	RNA helicase (<i>cshA</i>)	Ser451Pro	RNA metabolism
Hypothesised mechanism: The protein CshA is an ATP-dependent RNA helicase, an enzyme that is involved in RNA metabolism, including in RNA splicing and translation initiation ¹⁷⁹ . The mutation is thought to reduce energy usage for RNA metabolism ¹⁷⁹ .								
KQ76_04420	903,745	SNV	C	T	100	CoA-disulphide reductase (<i>KQ76_04420</i>)	Gln141*	Cell homeostasis
Hypothesised mechanism: The enzyme catalyses the formation of CoA-disulfide from CoA (coenzyme A) with the expense of the cofactor NAD ⁺ or NADP ⁺ . The process is key in the regulation of thiol (sulphur) metabolism to maintain cellular redox balance ¹⁸⁰ .								

^aIn *S. aureus* ATCC25923

SNV (single nucleotide variants) denotes substitution-type change of a single nucleotide at a specific position in the genome, f = frequency of the nucleotide changes occurring in a bacterial isolate,

* = stop codon.

(Reproduced from ¹⁵⁰ with permission from the Royal Society of Chemistry)

Chapter 5

Toxicity Responses of *S. aureus* to NAg or Ag⁺

5.1. Summary

Silver nanoparticle (NAg) is one of the most effective antibacterial agent due to its properties and its numerous antibacterial mechanisms. Despite many single targeted experiments conducted, it is still a challenge to fully understand NAg antibacterial mechanism, particularly in the individual role of solid particulate and ionic silver (Ag⁺). This study in Chapter 5, utilizing the unbiased and untargeted approach of whole transcriptomic analysis, has seen the upregulated expression of genes responsible for siderophore biosynthesis, ROS scavenger compound, teichoic acid D-alanylation, and metal transporters upon exposure of *Staphylococcus aureus* to NAg. From this observation, it was hypothesised that to cope with oxidative stress, *S. aureus* may increase iron uptake and ROS scavenger genes. It was also thought that the bacterium may alter the surface charge by incorporating D-alanine in teichoic acid to reduce NAg-cell interaction as well as increase the silver efflux *via* metal transporters. Further study is essential to confirm these hypotheses. Interestingly, some genes were observed to be expressed by both NAg- and Ag⁺-exposed *S. aureus*, indicating the role of Ag⁺ in NAg toxicity. Unlike Ag⁺-exposed *S. aureus*, NAg-exposed *S. aureus* also expressed a different set of genes from different pathways. It may suggest that NAg toxicity does not solely originate from Ag⁺, but could also be from the solid particle. Altogether, the findings here support the silver antibacterial mechanisms already reported and the individual role of Ag⁺ ions and solid particle contributing to NAg toxicity.

5.2. Introduction

NAg has been proven by many to display a great antibacterial activity and efficacy. One of the reasons is the multifaceted antibacterial mechanisms *e.g.* cell-particle interaction that ruptures cell surface, DNA and protein structure and function disruption, and generation of reactive oxygen species (ROS)^{31,54,56}. Unlike the primary single target offered by many antibiotics, NAg capability to provide various antibacterial mechanisms offer a promising antibacterial agent due to its multi-target mechanisms.

Nevertheless, research efforts have found it challenging to fully understand the NAg antibacterial mechanisms. Many studies have conducted a number of bottom-up and

single targeted experiments, e.g. cell surface investigation using transmission electron microscopy (TEM), probe to detect the presence of ROS, or a proteomic study on a specified gene target^{56,58}. Despite that, it has been acknowledged that the NAg antibacterial mechanism is currently still a work in progress¹⁸¹.

Further, one of the most discussed issues in the area of NAg antibacterial mechanism is the role of soluble ionic silver (Ag⁺) and the solid particle. A study by Xiu *et al.*, for example, reported that the solid particle did not play a role in NAg toxicity to *E. coli* and only Ag⁺ ions contribute to the toxicity. This was demonstrated by the ability of bacteria to grow in an anaerobic setting, which under these condition prevents particle oxidation and Ag⁺ release²⁰ (see Chapter 2). In contrast, Faiz *et al.* showed that, in order to reach similar growth inhibition of *B. subtilis*, at least a 2-fold higher Ag⁺ concentration was required than that of NAg⁹⁹, suggesting the particle does contribute to cell toxicity. Taken together, with the lack of top-down approach to understand NAg antibacterial mechanisms, particularly in the role of the solid particle versus ionic silver in NAg toxicity, this study aimed to investigate and understand the contribution of each individual component of NAg in an unbiased way.

To address that, this study used a whole transcriptomic approach *via* ribonucleic acid sequencing (RNA-seq) as it provides unbiased and untargeted global gene expression profiling¹⁸², requiring no prior knowledge on target genes. Therefore, this technique may reveal not only the previously-known antibacterial targets, but also novel mechanisms. A transcriptomic study by Singh *et al.* in the biofilm of NAg-susceptible *S. aureus* exposed to non-inhibiting NAg concentration showed that NAg affects genes associated with biofilm formation, metabolism, virulence, and transporter¹⁸³. A similar study in planktonic Gram-negative bacteria, *E. coli* and *P. aeruginosa*, has also been reported^{72,184}. However, to the author's knowledge, the study in planktonic pathogenic Gram-positive bacteria using this approach has never been conducted before. Therefore, this study could be a valuable insight to address the aforementioned challenges and issues, particularly in a pathogenic Gram-positive bacterium.

Here, *Staphylococcus aureus* was exposed to a sub-inhibitory NAg or Ag⁺ concentration prior to RNA extraction and sequencing. Upon quality control of the RNA-seq raw data, differentially expressed genes (DEGs) were identified and mapped to functional pathways. The study then performed biological interpretation of the pathways, that was used to deduce the toxicity response, and in turn, the NAg antibacterial mechanism.

5.3. Experimental Methods

5.3.1. Preparation of antibacterial agents

Silver nanoparticle (NAg, $d_{\text{TEM}} = \sim 2$ nm, 6.6 wt% Ag homogenously dispersed on 30 nm TiO₂ support^{12,97}) was synthesised by Flame Spray Pyrolysis (FSP) at University of New South Wales (UNSW) and silver nitrate (AgNO₃; Ajax Finechem) was used as ionic silver (Ag⁺) source. NAg stock was always prepared fresh in Cation-adjusted Mueller-Hinton Broth (Ca-MHB; BD, Australia) prior to each experiment whereas AgNO₃ stock were dissolved in autoclaved water and stored at room temperature. To ensure a homogeneous suspension, NAg suspension was ultra-sonicated (Vibra-cell, Sonics & Materials, Inc.) at 50% output control for 20 s prior to use. Experiments with NAg and Ag⁺ were performed under dark conditions to render the TiO₂ support of the NAg particles photocatalytically inactive and to prevent Ag⁺ reduction to Ag⁰, respectively⁹⁷.

5.3.2. Determination of NAg and Ag⁺ concentration for the transcriptomic study

The determination of NAg and Ag⁺ concentration used to treat the *S. aureus* ATCC25923 (henceforth known as the wild-type (WT) strain) was performed *via* growth curve comparison. Briefly, an overnight culture was inoculated in a 25-mL Ca-MHB with starting OD₆₀₀ = ~ 0.05 . Upon reaching mid-exponential stage (2 h, 37°C, 200 RPM, OD₆₀₀ = ~ 0.4), NAg or Ag⁺ was added accordingly to reach the designated final concentration. To construct the growth curve, an aliquot was taken from each treatment hourly and the OD₆₀₀ was measured with a spectrophotometer. Growth curve of the untreated WT strain was performed in parallel to serve as a comparison. The experiments were performed at least in two biological replicates.

5.3.3. Sample preparation for RNA extraction

A single colony of the WT strain was inoculated and grown overnight in a 5 mL Ca-MHB at 37°C and 200 rpm. The overnight culture was sub-cultured to a 25 mL MHB with starting OD₆₀₀ = ~ 0.05 for 2 h at 37°C 200 rpm to reach mid-exponential phase (OD₆₀₀ = ~ 0.4). The culture was treated with NAg or Ag⁺ for 30 min at half of their respective minimum inhibitory concentrations (MICs) (**Table 5.1**). Untreated WT strain was also conducted in parallel to serve as a control. These experiments were performed in three biological replicates.

Table 5.1. Treatment conditions used for transcriptomic study in the WT strain

	Treatment at 0.5xMIC
Silver nanoparticle (NAg)	1.5 µg Ag/mL
Ionic silver (Ag⁺)	0.75 µg Ag/mL

5.3.4. RNA extraction

Cell pellets from each treatment were washed with PBS and resuspended in 1 mL Trizol (Invitrogen) prior transferring to a 2-mL Lysing Matrix B tubes, containing 0.1-mm-diameter silica spheres (FastPrep MP Biomedicals). To obtain intracellular compounds, cell suspensions were mechanically lysed using high speed homogenizer (FastPrep MP Biomedicals) performed three times (6.5 m/s for 45 s and 2-3 min on ice in between). Supernatants were collected upon centrifugation (10,000 g at 4°C for 5 min) and mixed with 200 µL chloroform to remove protein and DNA. Crude RNA was collected from the upper aqueous phase following a centrifugation (12,000 g at 4°C for 15 min). RNA extraction was performed using miRNeasy Mini Kit (Qiagen) following manufacturer's instructions. The isolated RNA was further purified with Turbo DNA-free kit (ThermoFisher) for genomic DNA removal and *via* magnetic beads treatment using Agencourt RNAClean XP kit (Beckman Coulter), both following the manufacturer's protocol.

5.3.5. RNA quality control

The total RNA quantity and purity was measured using NanoDrop spectrophotometer (Thermo Fisher) and Qubit 2.0 Fluorometer (Thermo Fisher) using Qubit RNA and DNA HS Assay Kit (Thermo Fisher). The integrity of the RNA was determined using a 2200 TapeStation system (Agilent Technologies) with RNA Analysis ScreenTape (Agilent Technologies). These quality controls were performed following the manufacturers' protocol.

5.3.6. RNA sequencing (RNA-seq) and read mapping

The RNA library preparation and sequencing (RNA-seq) were performed at Singapore Centre of Environmental and Life Science Engineering (SCELSE), Nanyang University of Technology (NTU), Singapore. The preparation follows the TruSeq Stranded mRNA protocol (Illumina) without oligo-dT mRNA purification. Sequencing was conducted using the HiSeq2500 sequencer (Illumina) at a 100 bp paired end read length.

The quality of Illumina pair-ended reads (approximately 19 to 34 million PE) was first assessed using FastQC¹⁸⁵. Prior mapping of the non-ribosomal RNA (non-rRNA) to the

reference genome using HISAT2 version 2.1.0¹⁸⁶, ribosomal RNA (rRNA) reads were discarded using SortMeRNA version 2.1¹⁸⁷ and the non-rRNA reads were trimmed using Trimmomatic version 0.38¹⁸⁸ to remove adapters, low quality reads, and reads under 36 bp. The reference genome used is published on NCBI (accession number: CP009361.1 and CP009362.1 for chromosome and plasmid, respectively)¹³⁸. The quality assessment and read mapping were performed by Dr. Xianghui Liu and Dr. Xinyi Zhu at SCELSE.

5.3.7. RNA-seq data analysis

Count reads were extracted from the SAM files generated by HISAT2 software using R package Rsubread version 1.34.7¹⁸⁹. Differential expression (DE) analysis was performed upon extraction using the DESeq2 pipeline¹⁹⁰. The cut-off threshold to define the differentially expressed genes between the designated pairwise comparisons was log2 fold-change value > 2 and the associated adjusted P-value < 0.05. This was performed following normalization of the counts obtained to the total library size and dispersion estimation. This analysis was performed by Dr. Xianghui Liu and Dr. Xinyi Zhu at SCELSE.

Significantly differentially expressed genes (DEGs) were further annotated and mapped to functional pathways of *S. aureus* ATCC25923 using BioCyc Omics Dashboard¹⁹¹. Due to limitations of ATCC25923 database, the annotations and mapping of some genes were performed using the combination of BioCyc database¹⁹², NCBI database¹⁹³, and manual interpretation based on published literatures on *S. aureus* (most are different strains with some studies on *B. subtilis*, also a Gram-positive bacterium).

5.4. Results and Discussion

5.4.1. Determination of NAg and Ag⁺ concentration to treat the WT strain

Growth curve comparison between the treated (silver exposure) and untreated (no silver) WT strain was performed as a preliminary study to determine the suitable NAg and Ag⁺ working concentration (**Figure 5.1**) for the transcriptomic study. The silver exposure concentrations are to trigger the toxicity responses, while at the same time, not altering bacterial growth. This is to ensure unbiased results for the analysis of silver toxicity and to exclude any overlapping responses that may originate from changes in growth behaviour.

When exposed to NAg at sub-inhibitory concentration (1.5 $\mu\text{g Ag/mL}$; 0.5xMIC), the WT strain has a comparable growth rate (0.52 1/h) as that of the untreated one (0.54 1/h) (**Figure 5.1A**). Similar observation was found upon treating the WT strain with Ag⁺ (**Figure 5.1B**), whereby the presence of sub-inhibitory Ag⁺ concentration (0.75 $\mu\text{g Ag/mL}$; 0.5xMIC) did not affect the WT growth following 30 min exposure (**Figure 5.1B** inset), although a slightly lower growth rate (0.40 1/h) was observed when compared to those of the untreated one. Exposure of the WT strain to NAg and Ag⁺ at their respective inhibitory concentration (3 $\mu\text{g Ag/mL}$ and 1.5 $\mu\text{g Ag/mL}$; 1xMIC) saw the bacteria ceased to proliferate after 1 h of exposure, unable to reach the exponential stage. Therefore, for the transcriptomic study here, the working NAg and Ag⁺ concentration to treat the WT strain was determined to be at half of the respective MIC.

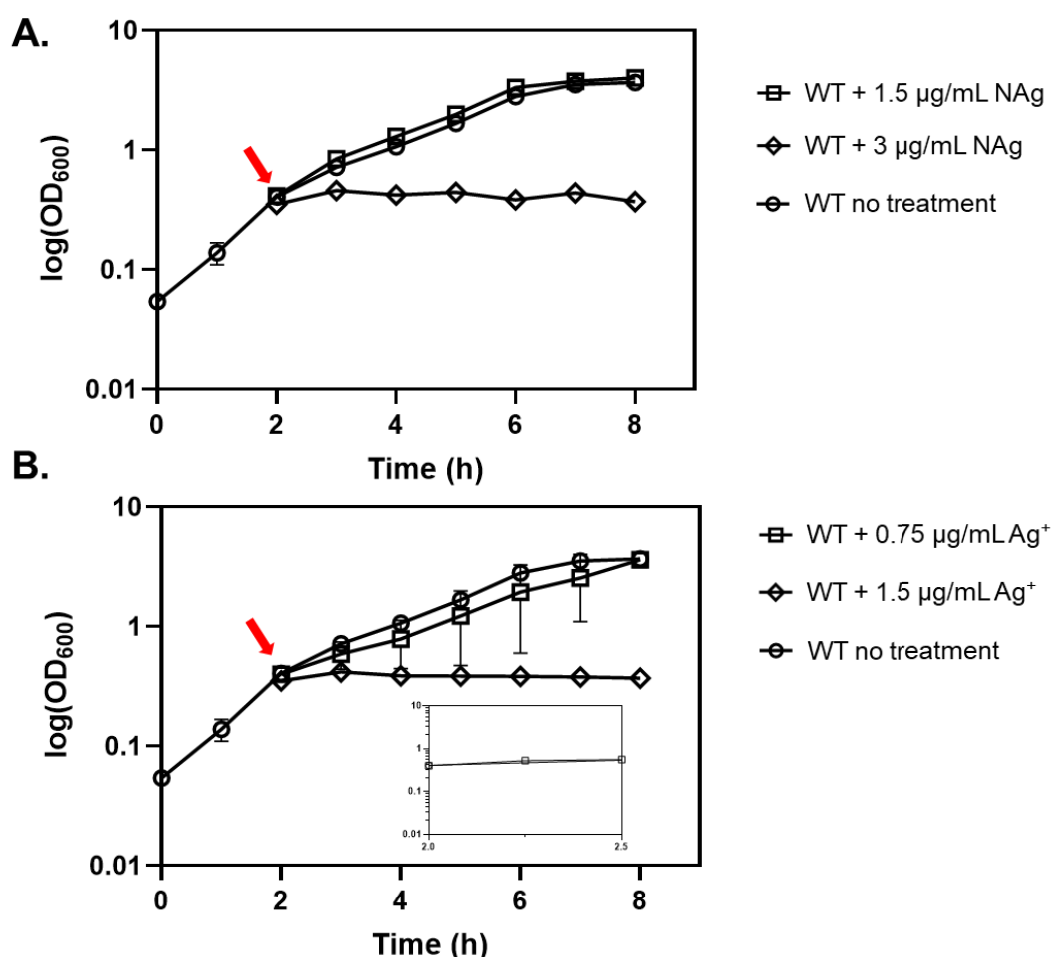


Figure 5.1. Growth curve of the WT strain treated with (A) NAg and (B) Ag⁺ at different concentrations (0.5xMIC of NAg = 1.5 $\mu\text{g Ag/mL}$, 0.5xMIC of Ag⁺ = 0.75 $\mu\text{g Ag/mL}$, square; 1xMIC of NAg = 3 $\mu\text{g Ag/mL}$, 1xMIC of Ag⁺ = 1.5 $\mu\text{g Ag/mL}$, diamond) compared to the untreated (circle) as measured by the optical density at 600 nm. The red arrow denotes the time when NAg or Ag⁺ was added to the culture. RNA was extracted 30 min after the addition of silver. Each data point represents the mean of at least two biological replicates, with error bars representing the standard error of measurement (SEM). The inset shows an expansion of range between 2.0 – 2.5 h to display comparable growth between 0.5xMIC and untreated WT strain (symbols are overlapped).

5.4.2. Variation analysis between samples

Variation analysis was conducted *via* principal component analysis (PCA), a technique that simplify large data sets, *e.g.* gene expressions, into 2 dimensional views with a purpose of examining the extent of variation between samples and classifying the data at a glance¹⁹⁴. In this study, the analysis, performed by Dr. Xianghui Liu and Dr. Xinyi Zhu at SCELSE, displayed the variations of the RNA-seq data between the treatment groups as well as between biological replicates in each group (**Figure 5.2**). The different colour represent the treatment groups (no treatment, NAg or Ag⁺ treatment in orange, blue, and green, respectively) whereas the circle represents their respective biological replicates.

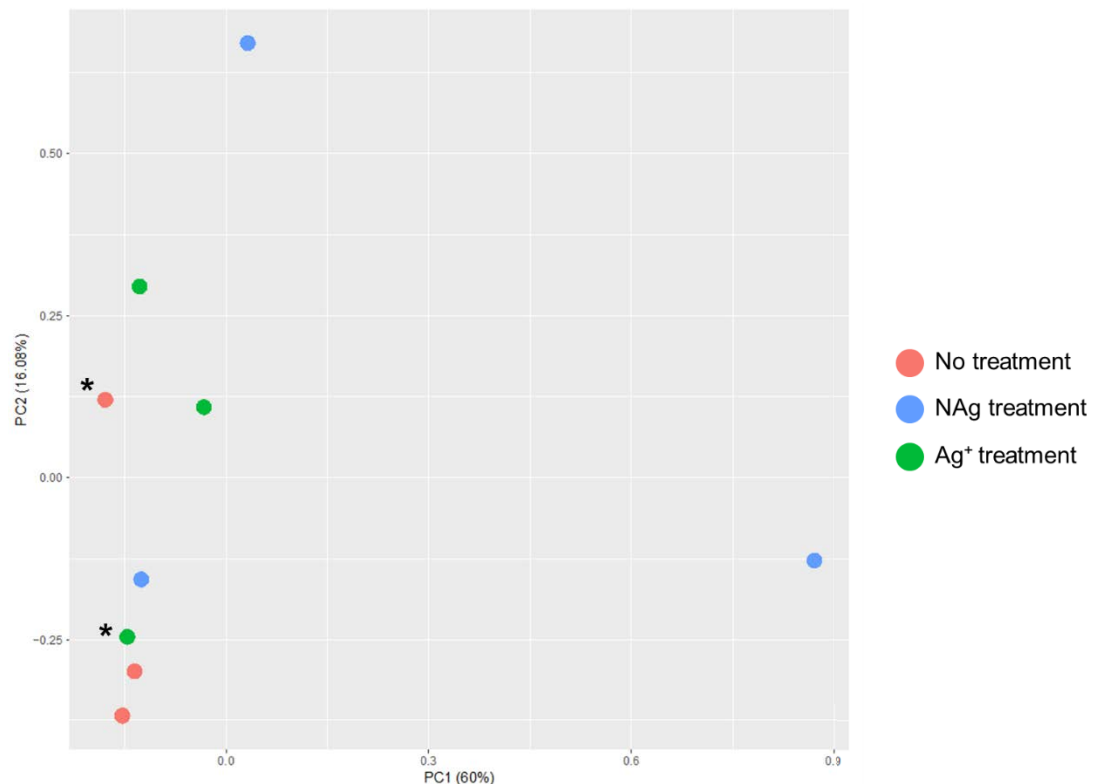


Figure 5.2. Principal component analysis (PCA) of different treatment groups (no treatment, NAg treatment and Ag⁺ treatment) to *S. aureus* WT strain and its biological replicates.

The positions of each biological replicate data for the no treatment group are relatively close to each other and similarly, those of the Ag⁺ treatment group. Even though there is one of the replicates from the no treatment and Ag⁺ treatment group that is distant from the rest (see * in **Figure 5.2**), it did not show high variation in the x-axis (60%). This indicates minimum variability among biological replicates in the no treatment and Ag⁺ treatment group. For the NAg treatment group, the PCA analysis saw a potential high variation data from a biological replicate in the x-axis (60%) that might cause an issue in RNA-seq analysis. After careful consideration, it was decided to still include the data in the transcriptomic study with a caution that follow up studies are essential.

5.4.3. Comparison of differentially expressed genes in the WT strain treated with NAg and Ag⁺

To investigate the toxicity response of *S. aureus* to NAg and Ag⁺, *S. aureus* WT strain was treated with 0.5xMIC of NAg and Ag⁺ for 30 min and the gene expression was measured and compared to the control (untreated *S. aureus* WT, WT NT). The total number of the differentially expressed genes (DEGs) number in each treatment followed cut off values of log2 fold change > 2.0 and p-adjusted value < 0.05 (**Table 5.2**).

Table 5.2. Number of upregulated and downregulated genes in the WT strain treated with NAg and Ag⁺ compared to the no-treatment.

	Upregulated	Downregulated	Total
NAg-treated <i>S. aureus</i> (NAg-WT)	43	1	44
Ag ⁺ -treated <i>S. aureus</i> (Ag-WT)	11	0	11

RNA-seq analysis showed 44 and 11 genes being differentially expressed for the NAg-treated (henceforth will be mentioned as NAg-WT) and Ag⁺-treated (henceforth will be mentioned as Ag-WT) *S. aureus* when compared to the control (no treatment), respectively (**Table 5.2**). Earlier studies have reported a higher variability of the DEGs in other bacteria in their planktonic form (*E. coli*, *P. aeruginosa*, and *B. cereus*), as indicated by their large number of DEGs (> 200 genes) at various silver exposure durations (10 – 120 min)^{72,184,195}. This difference might due to the species-specific response to NAg and Ag⁺ and/or the length of exposure.

Of these 44 transcripts from NAg-WT, 43 transcripts were upregulated and 1 was downregulated (**Table 5.2**) whereas all transcripts were upregulated for Ag-WT. The difference in the number of gene expressions between exposure to NAg and Ag⁺ could be attributed to the unique toxicological responses to the different forms of silver. A previous transcriptomic study in *E. coli* exposed to NAg also observed higher number of DEGs to that of Ag⁺⁷². The results may suggest additional toxicity effects of NAg relative to Ag⁺, as discussed next in more detail.

The biological interpretation of NAg and Ag⁺ toxicity to the WT strain was analysed through mapping and annotation of the genes' locus tag onto functional pathways of *S. aureus* ATCC25923 in BioCyc Omics Dashboard and NCBI database (**Table S5.1** and **Table S5.2**, respectively). Due to the limited pathway database for *S. aureus* ATCC25923 in BioCyc, any genes that do not map to any functional pathway were interpreted manually using published literatures. The discussion in this chapter

focusses on **the interpretation of the pathways**, rather than a single gene, because the changes in a number of genes within a pathway are thought to provide greater insights into the mechanisms of NAg and Ag⁺ toxicity.

However, as this is an initial study to understand a global toxicity response, it is important to later validate the interpretation of the RNA-seq data: to measure the level of expression *via* quantitative polymerase chain reaction (qPCR) and followed by a pathway-specific study. Due to time constraint, these follow up work have not been performed in this study. Nevertheless, suggested future works tailored for the targeted pathway are included herein following a biological interpretation of each pathway.

The transcriptomic data revealed an interesting finding on NAg and Ag⁺ toxicity. It was observed that there were 9 DEGs shared between these two silver exposures (**Figure 5.3**), of which represent the majority (82%, 9 out of 11) of the DEGs of Ag-WT. These genes encode protein for siderophore synthesis (*sbnA*, *sbnC*, *sbnD*, *sbnE*, and *sbnH*), copper transport (*copA* and *copZ*), and teichoic acid biosynthesis (*dltA* and *dltX*). Interestingly, the other 2 DEGs for the Ag-WT (*dltB* and *dltD*) are in the same teichoic acid biosynthesis pathway, whereas for the NAg-WT, there were 35 more DEGs that mapped to a different functional pathways e.g. the oxidative stress pathway (see **Table S5.1**, **Table S5.2**, and **Figure S5.1**).

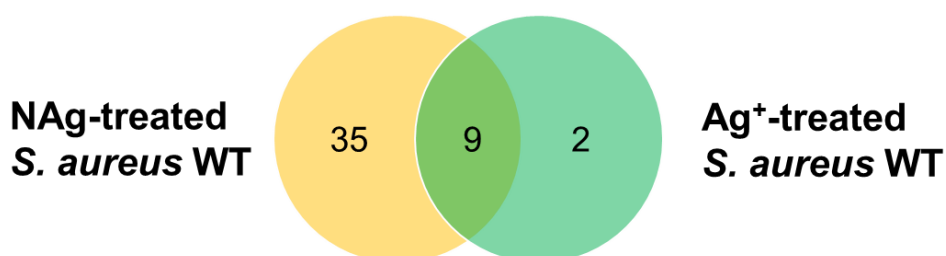


Figure 5.3. The number of differentially expressed genes on the *S. aureus* WT strain treated with NAg and Ag⁺ compared to the untreated WT strain.

The findings here imply that *S. aureus* responses to NAg involve a number of unique pathways, whereas *S. aureus* response to Ag⁺ is conserved within those of NAg. The similar responses might be due to the fact that intracellular presence of Ag⁺ ions has been known to contribute to NAg toxicity^{5,63}. At the same time, the unique responses suggest that NAg source of toxicity does not only originate from the leached Ag⁺ ions, but also from the solid particulate that remain after leaching. Faiz *et al.* reported that at equivalent silver dosages of 1 to 4 µg Ag/mL, exposure of *B. subtilis* to NAg saw higher extent of antimicrobial activity with 20 - 99% growth inhibition effects, compared to 20 - 70% of the Ag⁺ relative to the no silver cell-only control⁹⁹. It suggests the toxicity contribution from the solid silver particulates for the former system⁹⁹. Whilst cell death was also observed in Ag⁺-treated *B. subtilis*, the study detected a considerably low

cellular ROS levels, approximately 6 times lower than that of the NAg, indicating that oxidative stress is likely not the major toxicity mechanisms for Ag⁺⁹⁹. This is consistent with the lack of oxidative stress pathway DEGs identified for the Ag-WT (**Table S5.2**).

Considering the high similarity of Ag-WT DEGs to those of NAg in *S. aureus*, the following section is focused on the detailed explanation of all affected pathways due to NAg exposure, to provide a better understanding of antibacterial mechanisms of NAg (and at the same time, Ag⁺) in *S. aureus*.

5.4.4. Toxicity responses to NAg

Mapping and annotations of NAg-WT DEGs has seen changes in 15 functional pathways from 44 genes (**Figure 5.4A**) upon exposure to 0.5x NAg MIC (1.5 µg Ag/mL), relative to WT NT. The largest proportion, with 17% distribution of the total DEGs, was observed from iron uptake and metabolism pathways, while the largest log₂ fold change was observed in genes associated with the transporter pathway, comprising 15% distribution of the total DEGs. Herein, the potential roles of these DEGs as bacterial responses to NAg toxicity are discussed.

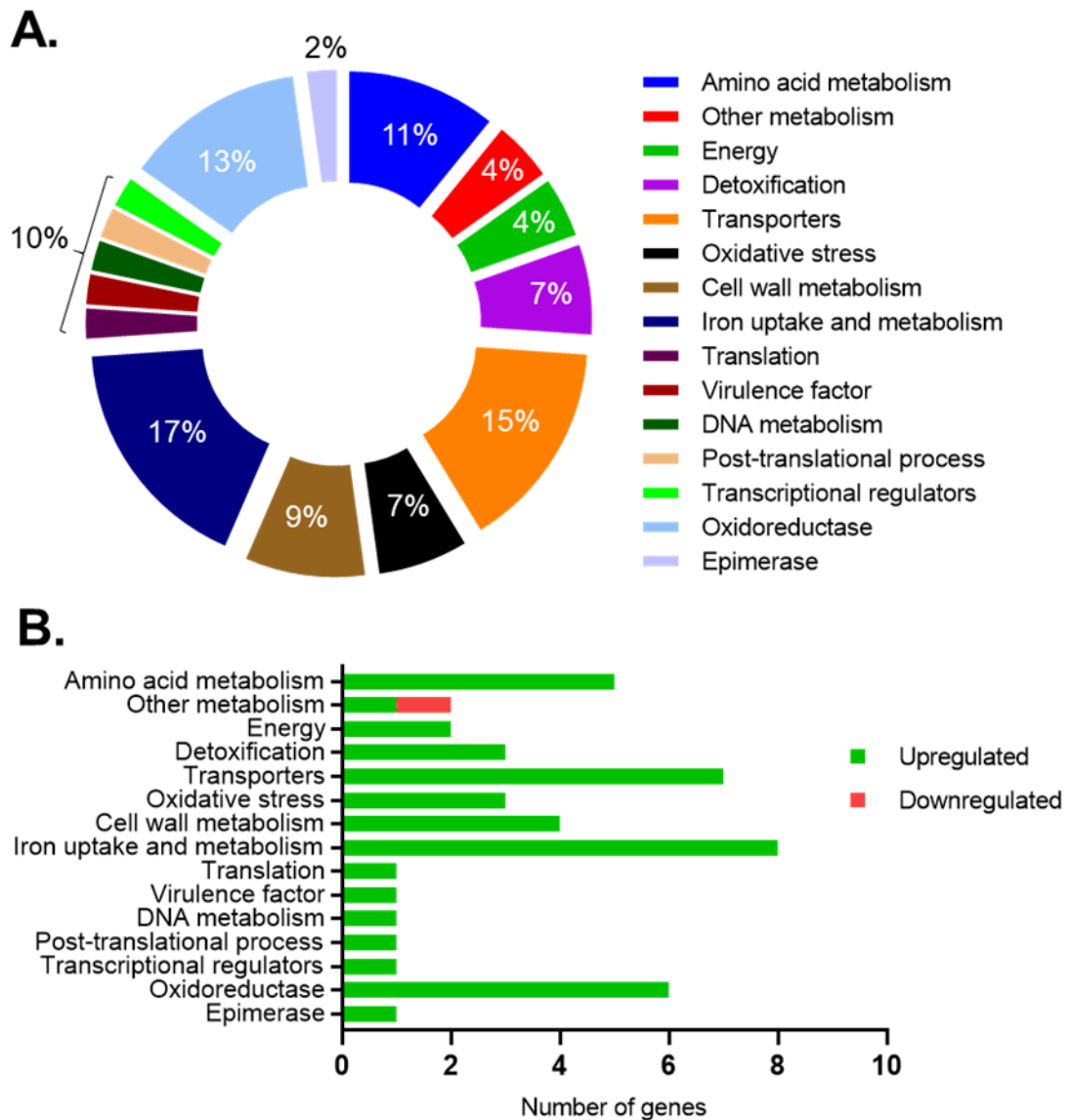


Figure 5.4. The distribution of differentially expressed genes (A) and the number of upregulated (green) and downregulated (red) genes (B) classified based on the functional pathway in NAg-WT when compared to the no-treatment. The functional pathways were classified from BioCyc Omics Dashboard, NCBI database, and manual interpretation.

For the **iron uptake and metabolism pathways**, RNA-seq analysis detected a 2.2 – 3.3 log₂ fold change of *sbnABCDEFGH* in the NAg-WT relative to WT NT, that, interestingly, constitutes 8 out of 9 genes of the *sbn* gene cluster (*sbnABCDEFGHI*), a cluster that encodes the staphyloferrin B biosynthesis (see **Table S5.1** and **Figure 5.4B**). Staphyloferrin B is a siderophore, a low-molecular-weight molecule with high affinity to scavenge ferric ion (Fe³⁺) from various sources in the environment and is produced particularly by pathogenic staphylococcal bacteria¹⁹⁶. Regulated by the *fur* regulon, the biosynthesis of staphyloferrin B is enabled by the sole expression of *sbn* gene cluster which produces the necessary substrates *de novo* (**Figure 5.5**), e.g. citrate that is produced by the SbnG protein from the reaction of oxaloacetate and

acetyl-CoA. The other two substrates, L-2,3-diaminopropionic acid (L-Dap) and α -ketoglutarate (α -KG), can be produced by two subsequent reactions catalysed by SbnA and SbnB. Firstly, SbnA catalyses the O-phospho-L-serine (OPS) and L-glutamate reaction to generate N-(1-amino-1-carboxy-2-ethyl)-glutamic acid (ACEGA) and secondly, SbnB catalyses ACEGA hydrolysis in the presence of NAD⁺. OPS itself is reported to be synthesised by SbnI *via* serine phosphorylation. To generate staphyloferrin B, citrate and L-Dap are firstly condensed by SbnE to generate the intermediate citryl-L-2,3-diaminopropionic acid. Following a decarboxylation of citryl-L-2,3-diaminopropionic acid by SbnH, the citryl-diaminoethane generated was further condensed with the second L-Dap molecule by SbnF and subsequently, with α -KG by SbnC, thus forming staphyloferrin B. Finally, staphyloferrin B is exported out by SbnD^{196–198}.

Upon exposure to NAg, the herein detected upregulation of the *sbm* cluster in *S. aureus* suggests an increased scavenging of the non-bioavailable ferric ion by staphyloferrin B to form the soluble Fe³⁺-siderophore complex that could be utilized for cellular processes. NAg has been known to target the iron-sulphur (Fe-S) clusters that are present in many structural proteins and enzymes^{199,200} and the upregulated expression of the *sbm* cluster was thought to compensate for the NAg-mediated loss of bioavailable iron due to attack on the clusters.

In more detail, research inquiries have indicated both direct and indirect targeting of the Fe-S clusters by NAg⁷⁵. Direct targeting is *via* the activity of the leached Ag⁺ ions^{79,80}. A soft Lewis acid, Ag⁺ has high affinity to the thiol (-SH) donor group in the amino acid cysteine side chain, and in turn, can displace the iron in the Fe-S clusters, releasing the bioavailable ferrous ion (Fe²⁺) into the cytoplasm. This Ag⁺ direct targeting on the Fe-S clusters is consistent with the 2.2 – 3.0 log₂ fold upregulation of 4 out of 9 genes (***sbmACDE***, **Table S5.2**) in the *sbm* cluster detected for the Ag-WT.

Indirect targeting, on the other hand, is *via* the activity of reactive oxygen radical. NAg exposure studies in both Gram-positive and Gram-negative bacteria have reported inhibition of the inner membrane-bound respiratory chain enzymes, causing premature leakage of electrons, and in turn, reducing molecular oxygen (O₂) to O₂^{•-} radical^{8,9,75,80,201}. This generation of O₂^{•-} can disrupt the Fe-S clusters, again, releasing the Fe²⁺ ion into the cytoplasm.

Ferrous ion is a Fenton-active moiety, undergoing reduction in the presence of cellular hydrogen peroxide (H₂O₂) to transform into the insoluble Fe³⁺, and in the process also generating the very reactive hydroxyl (OH[•])²⁰². This later generation of oxygen radical

can further attack the Fe-S clusters, and therefore, forming an 'amplification loop' for the release of Fe²⁺ from the clusters. Note that both O₂^{•-} and OH[•] are one-electron oxidants that can oxidize cysteine's thiol group to thiol radicals (RS[•]) in the clusters²⁰³.

These Fe-S clusters are abundant, for example, in dehydratases, an enzyme family that has key roles in biosynthetic and catabolic pathways^{204,205}. This hypothesis is supported by Faiz *et al.* that reported an elevated generation of oxygen radicals in *B. subtilis* upon NAg exposure⁹⁹, of which major radical species later identified to be both the O₂^{•-} and OH[•] radicals⁹⁸. In regards to the Ag⁺ exposure under comparable conditions, lower levels of oxygen radicals were detected in *B. subtilis*, than those of the NAg exposure⁹⁹. This was thought to result, at least in part, from the absence of the leaching phenomenon for the Ag⁺ exposure (in the form of the soluble silver salt AgNO₃).

Using electron paramagnetic resonance (EPR) spectroscopy, Gunawan *et al.* detected generation of oxygen radicals by NAg during its leaching process, in the absence of cells, causing damages on the cell envelope components including the major phospholipid that composed the inner membrane⁹⁸. This inner membrane disruption is consistent with the earlier mentioned inhibition of respiratory enzyme that leads to the O₂^{•-} generation due to electron leakage²⁰⁶. To simply put, the Ag⁺ exposure, due to lack of the extracellularly generated oxygen radicals, was possibly associated with less attack on the membrane-bound respiratory enzyme, in turn, lower O₂^{•-} generation and less attack on the Fe-S clusters. This can lead to less release of the Fe²⁺ ion into the cytoplasm, which is again, consistent with the less number of *sbn* cluster genes being upregulated for Ag-WT.

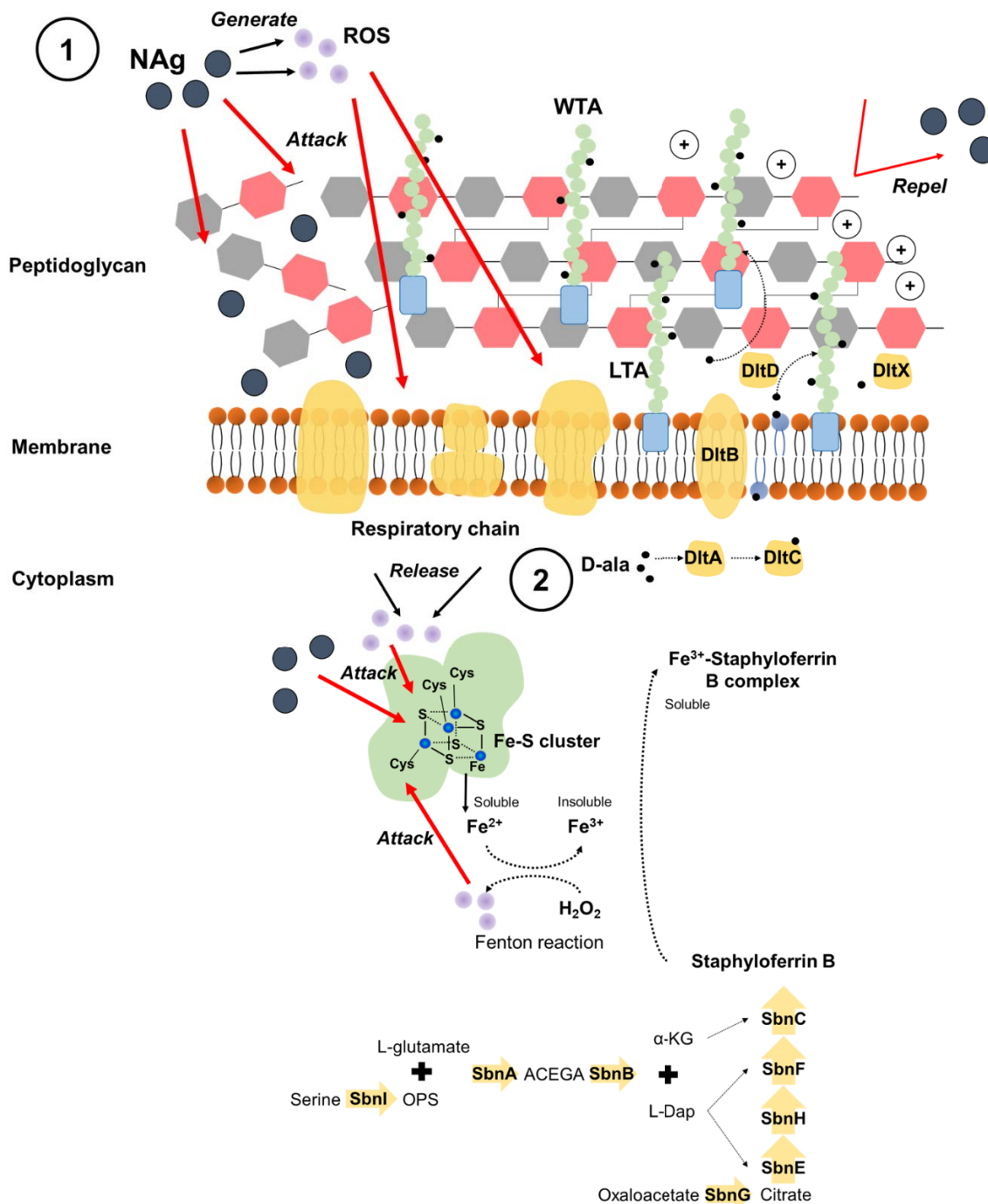


Figure 5.5. A proposed model for *S. aureus* toxicity response to NAg via iron uptake and D-alanylation of teichoic acid. 1) NAg and ROS-generated NAg could attack cell envelope and respiratory chain, generating additional ROS and damaging cell membrane. Once internalized, NAg and ROS may also attack Fe-S cluster, releasing Fe²⁺. Through Fenton reaction, the cell may simultaneously reduce H₂O₂ to ROS and oxidize Fe²⁺ to insoluble Fe³⁺, thereby creating a lack of intracellular iron. The cell, therefore, may synthesise Staphyloferrin B, encoded by the *sbn* cluster, to form soluble complex with Fe³⁺ thus the cell could utilize iron for cellular processes. 2) The incorporation of D-alanine to WTA and LTA is facilitated by the *dlt* cluster. As a result, higher (less negative) net surface charge may be formed in the cell surface to electrostatically hinder particle-cell interaction. Some figures were adapted from ^{75,196,197,207–209}. ROS: reactive oxygen species, WTA: wall teichoic acid, LTA: lipoteichoic acid, Fe-S: Iron-sulphur cluster, OPS: O-phospho-L-serine, ACEGA: N-(1-amino-1-carboxy-2-ethyl)-glutamic acid, α-KG: α-ketoglutarate, L-Dap: L-2,3-diaminopropionic acid.

A previous study in *E. coli* has reported the upregulation of *fur*-regulated genes following 10 min of NAg and Ag⁺ exposure⁷². Fur is a repressor for many iron uptake and metabolism genes e.g. *fep* and *ent* cluster in *E. coli* or *sbm*, *sir*, and *sfa* cluster in *S. aureus*^{72,196}. As a repressor, Fur blocks the transcription of these gene clusters when high level of cytosolic Fe²⁺ concentration is present. In agreement with earlier studies, the upregulation of Fur family transcriptional regulator (KQ76_08115) by 2.0 log2 fold observed for the NAg-WT (**Table S5.1**) here may support the low cytosolic Fe²⁺ concentration upon NAg exposure to *S. aureus*.

To confirm this hypothesis, the level of iron in the NAg-exposed WT strain could be measured *via* Ferrozine assay, and subsequently, the value can be compared to that of the WT NT. Additionally, supplementing the cell and NAg suspension with iron would be interesting to investigate if that would attenuate NAg toxicity to *S. aureus*.

The transcript levels of the **oxidative stress functional pathway** was upregulated in NAg-WT by ~2.1 log2 fold relative to WT NT; these include genes that encode the ROS scavenger: alkyl hydroperoxide reductase system (*ahpF*, *ahpC*) and a thioredoxin reductase (*trxB*) (**Table S5.1**). The genes *ahpF* and *ahpC*, encodes AhpF and AhpC protein, respectively, that work together to reduce H₂O₂ to H₂O and O₂²¹⁰. AhpC contains two redox-active cysteines (contains thiol group, -SH) that reduces peroxide and, in turn, are oxidized to a disulfide bond. AhpF, with NADH as electron donor, then reduces this disulfide bond to re-activate AhpC²¹¹. In aerobic environment, the inevitable presence of cellular H₂O₂ in bacteria can generate ROS *via* a number of mechanisms, such as the oxidation of cysteine residues in enzymes and proteins creating intracellular disulfide imbalance²¹² and undergoing the Fenton reaction with Fe²⁺ ions^{210,213}. Thioredoxin reductase, together with thioredoxin (the latter encoded by *trxA*), function as ROS scavengers in Gram-positive bacteria that in this case, together with AhpF and AhpC, most likely serve to protect *S. aureus* from the NAg-induced oxidative stress^{214,215}. As with the AhpF and AhpC system, thioredoxin reductase maintains the active form of thioredoxin by reducing its disulphide bond to thiol group (-SH)⁶⁵. The upregulation of these enzymes is also consistent with the increased expression of the iron uptake and metabolism *sbm* gene cluster. As earlier described, the latter is thought to compensate for the NAg-induced release of the physiologically essential Fe²⁺ ions from Fe-S clusters, to subsequently generate OH• radical *via* Fenton reaction.

Altogether, it was thought that the presence of NAg rises the intracellular ROS level that consequently leading to an iron-limited condition and trigger the expression of iron uptake and ROS scavenger genes as responses to protect the cell from oxidative

stress. These findings are consistent with a study by Chang *et al.* that observed an upregulated expression of iron uptake genes and oxidative stress genes following H₂O₂-induced oxidative stress in *S. aureus*²¹⁶.

To confirm the NAg-induced generation of cellular ROS, future work could utilize cell permeable oxidative reporter dye H₂DCFDA (2',7'-dichlorodihydrofluorescein diacetate)⁹⁹ to identify the presence of ROS or electron paramagnetic resonance (EPR) spectroscopy with cyclic hydroxylamine (CMH) spin probe to identify a specific type of ROS⁹⁸ in *S. aureus* in the presence and absence of NAg. Another way to investigate the presence of oxidative stress is by the addition of antioxidant compound such as superoxide dismutase (SOD) enzyme to the cell suspension, then the level of growth eradication following NAg treatment and addition of SOD could be compared to the control (absence of NAg).

This study also observed upregulation of genes involved in the **cell wall metabolism**. The RNA-seq data analysis revealed 2.0 – 2.5 log₂ fold change in ***dltXAC*** genes, a part of *dlt* cluster (*dltXABCD*), responsible for incorporating D-alanine (D-ala) into wall teichoic acid (WTA) and lipoteichoic acid (LTA) in bacterial peptidoglycan (see **Table S5.1**). The findings here, to the best of the author's knowledge, have not been identified in any NAg-exposed transcriptomic study.

In more detail, the incorporation process firstly starts with the activation of D-ala *via* adenylation process by the cytoplasmic DltA protein and transported by DltC to the cell membrane (see **Figure 5.5**). DtlB, a membrane protein, has been proposed to aid the transfer of D-ala to a cell wall intermediate, the lipid-linked undecaprenyl phosphate (C₅₅-P). Upon flipping this intermediate compound to face the periplasm, DltD and DltX have been suggested to facilitate the incorporation of D-ala to WTA and LTA^{208,209}.

Studies have previously reported that D-ala has important functions in Gram-positive bacteria, one of the examples is for maintaining bacterial surface charge^{217,218}. Studies have reported an increased sensitivity of *S. aureus dlt* mutant (lack of D-ala incorporation to the teichoic acids) to cationic antimicrobial peptides (CAMP)^{218,219}, suggesting the cell surface charge-altering role of D-ala, in this case, as a response to the positively-charged antibacterial. It has been shown by Peschel *et al.* that the *S. aureus dlt* mutant bound to a lower amount of negatively-charged protein than the wild-type, indicating that the incorporation of D-ala in WTA and LTA increases the global net negative charge of the cell surface²¹⁹. Similar to this, in the presence of NAg, the observed upregulation of the *dlt* system was thought to electrostatically hinder the particle-cell interaction in the cell surface (same charge electrostatic repulsion). Indeed,

Badawy *et al.* observed a surface charge-dependent antibacterial activity of NAg, that is, nanoparticles with net surface charge of the same sign as that of the cell surface were associated with less cell death, indicating a passivated toxicity³⁰. *S. aureus* herein was seen to also express a number of genes in this pathway upon exposure to Ag⁺ (specifically, upregulation of ***dltXABD*** at 2.0 – 2.6 log2 fold-change, see **Table S5.2**). Indeed, electron microscopy studies have reported the ‘affinity’ of Ag⁺ ions towards the bacterial cell surface, being associated with the evident cell wall and membrane damages, leading to cell death^{68,220}. The upregulation of this *dlt* genes was therefore thought to work in a similar manner as that of the positively-charged Ag⁺.

Studies have shown that NAg can disrupt bacterial cell wall by cleaving the glycosidic bond in the peptidoglycan that leads to the formation of the so-called ‘cell pits’, or holes, on the cell surface^{9,54}. Using the attenuated total reflectance – Fourier-transform infrared (ATR-FTIR) spectroscopy, a recent study by Gunawan *et al.* also observed the complete breakdown of LTA, including its D-ala moiety from *B. subtilis*, following NAg exposure⁹⁸. The upregulation of the *dlt* system was also thought as an effort to repair the cell wall structure.

As a follow up study, the structural changes of *S. aureus* cell envelope following NAg exposure could be investigated by utilizing the earlier mentioned technique (ATR-FTIR spectroscopy). At the same time, the study could apply the same approach to *S. aureus* in the absence of NAg, therefore, investigating the changes before and after exposure. Additionally, examining the treated and untreated *S. aureus* under an electron microscopy could be one of the validation studies to perform, as indicated by a previous study that the presence of D-ala would affect cell wall integrity²²¹.

Lastly, the RNA-seq analysis also saw the elevated expression of **copper transporters** by 3.0 – 5.0 log2 fold in the ***copA***, ***copB***, and ***copZ*** gene (**Table S5.1**) relative to the WT NT. CopA and CopB proteins are a chromosomal- and plasmid-encoded P-type ATPase copper-binding efflux transporters, respectively, while CopZ is a metallochaperone, a protein that facilitate intracellular copper transport (all are inner membrane proteins)^{222,223}. Apart from copper, the expression of these genes could also be induced by the presence of iron and silver^{222,224,225}. This findings herein suggest that *S. aureus* is capable of activating the copper transporters to cope with NAg toxicity, possibly to transport silver out of the cell. The upregulation of *copA* and *copZ* were also observed for Ag-WT by 3.5 – 3.7 log2 fold relative to WT NT (**Table S5.2**). These results are consistent with a recent study that observed the upregulation of copper transport genes (*cusRS-cusABCF*, *cueO*, and *copA*) in both NAg- and Ag⁺-exposed *E. coli*⁷². The Cus system, a set of transmembrane proteins, indeed, has been reported to

facilitate Ag⁺ efflux that confer Ag⁺ resistance in *E. coli*^{88,89}. Interestingly, this study also observed the upregulation of **cadmium transporter** gene and its regulator (**cadA** and **cadC**, respectively) by 5.0 – 5.3 log2 fold in NAg-WT (**Table S5.1**), but not in Ag-WT. These cadmium transporter genes have been known to confer resistance to cadmium, a heavy metal pollutant²²⁶, however, to the best of the author's knowledge, no studies have so far reported the association of these proteins to silver transport. Regardless, the findings here suggest the capability of *S. aureus* to utilize transporter system for other metals to transport silver out of the cell system. Although not entirely clear, the phenomenon is thought to involve the tendency of metal ions to form complexes with amino acids in proteins which, in this case, most likely involving the high affinity of the Ag⁺ ion as soft Lewis acid for the NH_x donor groups of histidine (NH⁺), arginine (-NH₂⁺) and lysine (-NH₃⁺) as well as for the thiol (-SH) in cysteine and the thioether sulphur in methionine^{89,225}.

It is important to note that the transporter genes were associated with the highest fold changes in expression (relative to the WT-NT) (**Table S5.1**). This should warrant future investigations on their exact mechanisms in the bacterium's toxicological responses to NAg, which, for example, by creating $\Delta copABZ$ and/or $\Delta cadA$ mutants and subsequently examine potential changes in susceptibility to NAg in comparison to the WT strain. Earlier studies investigating NAg as an antimicrobial agent have reported its multi-targeted antimicrobial mechanisms, e.g. the damage to cell surface and generation of ROS, both potentially originate from the leached soluble silver and the insoluble solid particulate. Aligned to those studies, this study detected the changes in the gene expression profile of *S. aureus* WT upon exposure to NAg that suggesting responses to the NAg toxicity *via* the increased expression of D-alanylation, metal transporter, iron acquisition, and ROS scavenger genes. First, D-alanylation to WTA and LTA in the cell surface was thought to reduce the NAg-cell interaction through electrostatic repulsion. The cell may also seek to expel the leached soluble silver out by activating the metal transporters. Lastly, the findings herein indicate the consequences of oxidative stress due to the generation of ROS potentially drive *S. aureus* to acquire bioavailable iron and scavenge ROS.

5.5. Conclusion

In conclusion, the study in this chapter has reported the changes in gene expression in *S. aureus* WT strain upon exposure to NAg and Ag⁺ which are likely to have important roles in the bacterium toxicological responses to silver. Detected at sub-inhibitory silver concentration (0.5xMIC) relative to the no-antimicrobial control, this study found a number of similar DEGs for the NAg and Ag⁺ exposures. These DEGs include those

that involve in iron uptake and metabolism, cell wall metabolism and metal transport. Interestingly, this study also found a substantial number of DEGs only for the NAg system, while being absent in the Ag⁺ system. These unique DEGs mapped to different cellular functions, including the antioxidant mechanisms. The findings herein indicate the role of Ag⁺ ions, that is, those that leached from the particles, in NAg toxicity. The results also suggest additional toxicity effects and these most likely attributed to the solid particles that remain after leaching, or in other words, a multiple toxicity origins of NAg.

From the biological interpretation of the DEGs for the NAg and Ag⁺ exposures, this study implied that *S. aureus* displayed the necessity to expel silver out of the cell system and to dampen the positively-charged NAg and Ag⁺ to interact with the cell surface. It was also thought that the cell may be required to acquire bioavailable iron due to the direct and indirect interaction of silver-generated ROS, leached soluble silver, and silver solid particulate with the Fe-S clusters that facilitates iron-lacking condition. While these hypotheses were deduced from either the NAg or Ag⁺ exposure, this study also detected that oxidative stress-associated responses when *S. aureus* was exposed to NAg, indicating the cells' immediate effort to scavenge ROS. Altogether, these hypothesis support the known NAg antibacterial mechanisms.

However, it is important to note that as an initial screening for potential genes responding to the silver toxicity, it is required to conduct complementary studies to validate the hypotheses. Future study could potentially start with validating the expression level of target genes *via* qPCR or investigating the protein expression *via* targeted proteomic study. Subsequently, the measurement of intracellular iron concentration *via* Ferrozine assay and NAg-generated ROS, the identification of teichoic acid structural changes *via* Fourier transform infra-red spectroscopy (FTIR), and genes knock-out were some suggested follow-up studies for specifically elucidate the role of the affected pathways in the toxicity responses.

5.6. Supplementary Information

Table S5.1. Functional groups of differentially expressed genes in the *S. aureus* WT exposed to NA_g in comparison to the no-treatment. Highlighted tables are the pathways discussed in the main text.

Locus tag	Log2 fold change	Annotation	Gene
Amino acid metabolism			
KQ76_02075	2.35	Cysteine synthase	<i>cysM</i>
KQ76_02080	2.79	Cysteine gamma-synthase	<i>metB</i>
KQ76_11515	2.41	Acetolactate synthase	<i>alsS / ilvB</i>
KQ76_00355	2.50	diaminopimelate decarboxylase	<i>lysA / sbnH</i>
KQ76_11515	2.41	Acetolactate synthase	<i>alsS / ilvB</i>
Other metabolism			
KQ76_01125	2.57	Choloylglycine hydrolase	<i>yxel</i>
KQ76_12525	-2.24	cobalamin biosynthesis protein	<i>cbiX</i>
Energy			
Fermentation of Pyruvate			
KQ76_11510	2.65	Alpha-acetolactate decarboxylases	<i>alsD</i>
KQ76_11515	2.41	Acetolactate synthase	<i>alsS</i>
Detoxification			
Arsenate detoxification			
KQ76_14270	3.00	Arsenical efflux pump membrane protein	<i>arsB</i>
KQ76_14275	2.89	Arsenate reductase	<i>arsC</i>
KQ76_09220	2.10	Arsenical efflux pump membrane protein	<i>arsB</i>
Transporters			
Cadmium transport			
KQ76_14235	5.08	Cadmium-transporting ATPase	<i>cadA</i>
KQ76_14240	5.32	Transcriptional regulator	<i>cadC/arsR</i>
Copper transport			
KQ76_14375	3.04	Copper-transporting ATPase B	<i>copB</i>
KQ76_13380	4.68	Copper chaperone CopZ	<i>copZ</i>
KQ76_13375	5.07	Copper-translocating P-type ATPase	<i>copA</i>
Sulfonate transporter			
KQ76_00600	2.90	Sulfonate ABC transporter ATP-binding protein	<i>tauB</i>
KQ76_00610	2.73	Sulfonate ABC transporter permease	<i>tauC</i>
Other			
KQ76_01120	2.35	Uncharacterized MFS-type transporter	<i>proP</i>
Oxidative stress			
KQ76_01795	2.04	Alkyl hydroperoxide reductase protein F	<i>ahpF</i>
KQ76_01800	2.05	Alkyl hydroperoxide reductase protein C	<i>ahpC</i>
KQ76_04050	2.08	thiol reductase	<i>trxB</i>
Cell envelope metabolism			
Cell structure (major wall teichoic acid and lipoteichoic acid)			
KQ76_04215	2.49	Teichoic acid D-Ala incorporation-associated protein	<i>dltX</i>
KQ76_04220	2.08	D-alanine-poly(phosphoribitol) ligase subunit 1 protein	<i>dltA</i>
KQ76_04230	2.13	D-alanine--poly(phosphoribitol) ligase subunit 2 protein	<i>dltC</i>

Cell wall organization			
KQ76_01130	2.31	Glycyl-glycine endopeptidase	<i>lytM</i>
Iron uptake and metabolism			
Siderophore biosynthesis			
KQ76_00320	3.25	Siderophore biosynthesis protein	<i>sbnA</i>
KQ76_00325	2.50	2,3-Diaminopropionate biosynthesis protein	<i>sbnB</i>
KQ76_00330	3.22	Siderophore biosynthesis protein	<i>sbnC</i>
KQ76_00335	3.07	Siderophore biosynthesis protein	<i>sbnD</i>
KQ76_00340	2.67	Siderophore biosynthesis protein	<i>sbnE</i>
KQ76_00345	2.30	Siderophore biosynthesis protein	<i>sbnF</i>
KQ76_00350	2.17	Siderophore biosynthesis protein	<i>sbnG</i>
KQ76_00355	2.50	Diaminopimelate decarboxylase	<i>lysA / sbnH</i>
Translation			
KQ76_06575	2.61	30S ribosomal protein S14	<i>rpsN</i>
Virulence factor			
KQ76_12395	2.11	Phage infection protein	<i>yhgE</i>
DNA metabolism			
KQ76_02535	2.02	Excinuclease ABC subunit B	<i>uvrB</i>
Post-translational process			
Protein modification			
KQ76_01400	2.02	Glycine cleavage system protein H	<i>gcvH</i>
Transcriptional regulator			
KQ76_08115	2.02	Fur family transcriptional regulator	<i>fur</i>
Oxidoreductase			
KQ76_01395	2.12	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
KQ76_01805	2.13	NADPH-dependent oxidoreductase	<i>nfnB</i>
KQ76_01885	2.03	NAD(P)-dependent oxidoreductase	<i>ybjT</i>
KQ76_04285	3.05	Pyridine nucleotide-disulfide oxidoreductase	
KQ76_04340	2.16	NADH-dependent flavin oxidoreductase	<i>fadH</i>
KQ76_08465	2.22	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
Epimerase			
KQ76_12780	2.08	epimerase	<i>wcaG</i>

Table S5.2. Functional groups of differentially expressed genes in the *S. aureus* WT exposed to Ag⁺ in comparison to the no-treatment. Highlighted tables are the pathways discussed in the main text.

Locus tag	Log2 fold change	Annotation	Gene
Amino acid metabolism			
KQ76_00355	2.30	Diaminopimelate decarboxylase	<i>lysA / sbnH</i>
Cell envelope metabolism			
Teichoic acid biosynthesis			
KQ76_04220	2.37	D-alanine-(poly)phosphoribitol ligase	<i>dltA</i>
KQ76_04225	2.08	D-alanyl-lipoteichoic acid biosynthesis protein	<i>dltB</i>
KQ76_04235	2.08	D-alanyl-lipoteichoic acid biosynthesis protein	<i>dltD</i>
KQ76_04215	2.56	Teichoic acid D-Ala incorporation-associated protein	<i>dltX</i>
Iron uptake and biosynthesis			

Siderophore biosynthesis			
KQ76_00320	3.04	Siderophore biosynthesis protein	<i>sbnA</i>
KQ76_00330	2.82	Siderophore biosynthesis protein	<i>sbnC</i>
KQ76_00335	2.51	Siderophore biosynthesis protein	<i>sbnD</i>
KQ76_00340	2.15	Siderophore biosynthesis protein	<i>sbnE</i>
KQ76_00355	2.30	Diaminopimelate decarboxylase	<i>lysA / sbnH</i>
Transporters			
KQ76_13375	3.56	Copper-translocating P-type ATPase	<i>copA</i>
KQ76_13380	3.77	Copper chaperone	<i>copZ</i>

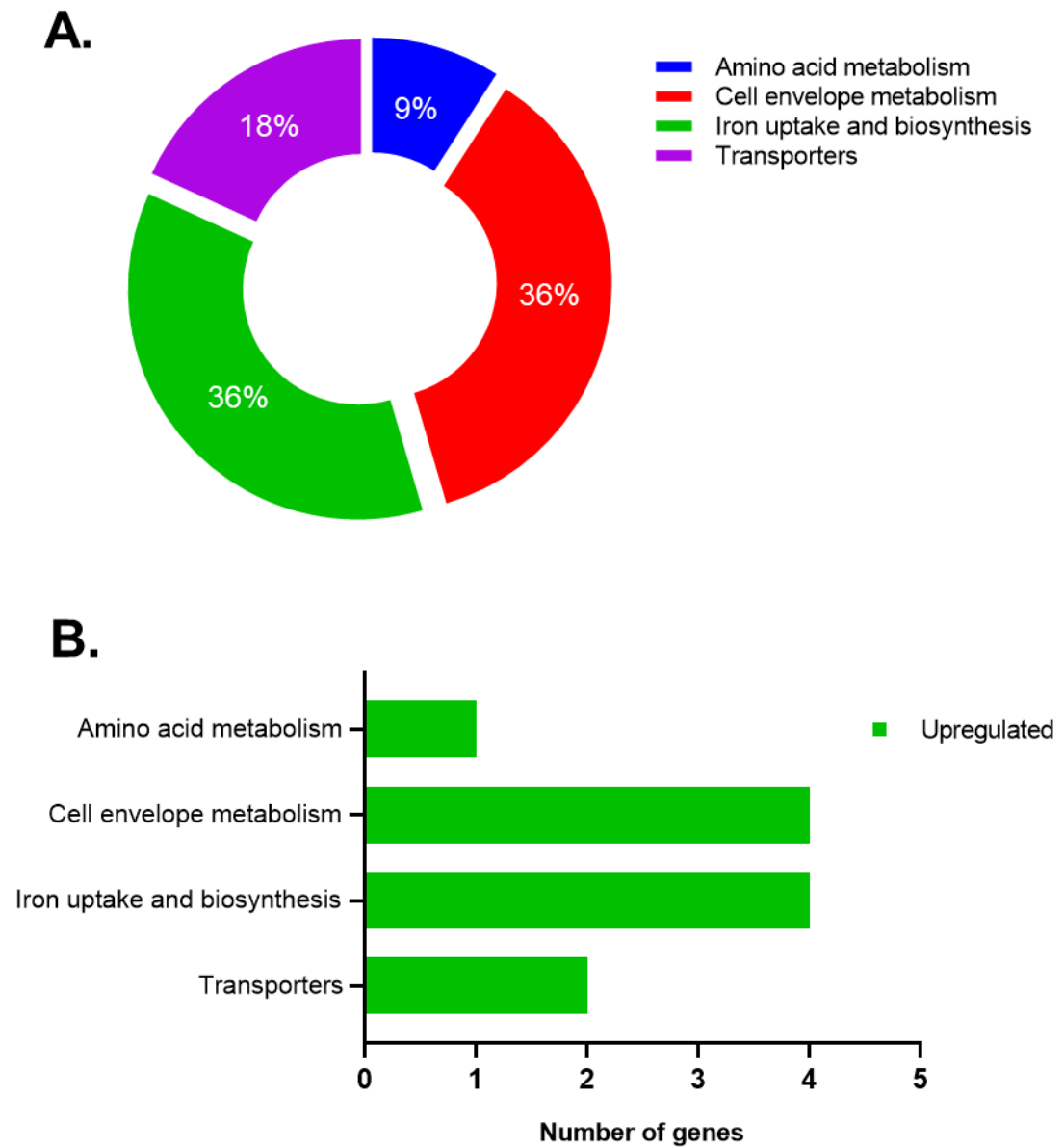


Figure S5.1. The distribution of differentially expressed genes (A) and the number of upregulated (green) genes (B) classified based on the functional pathway in Ag-WT when compared to the no-treatment. The functional pathways were classified from BioCyc Omics Dashboard, NCBI database, and manual interpretation.

Chapter 6

Defence Mechanisms of Resistant *S. aureus* to NAg or Ag⁺

6.1. Summary

Staphylococcus aureus has been reported to develop resistance to silver nanoparticle (NAg) (Chapter 3). It is currently unclear how *S. aureus* could develop resistance to NAg and if these defence mechanisms are species-specific, particularly due to no innate presence of previously-identified molecular mechanisms in *S. aureus*, e.g. *cus*, *sil*, and *omp* system. In addition to the genomic adaptations that potentially contribute to the resistance traits (Chapter 4), this study in Chapter 6 conducted a whole transcriptomic study to investigate physiological adaptations upon a prior prolonged exposure to silver, as well as resistance responses upon re-exposure to silver. In the absence of NAg, the NAg-resistant (NAg-r) strain increased the expression of purine, pyrimidine, and ribosomal protein synthesis genes, as well as repressed the expression of capsular polysaccharide synthesis genes relative to the untreated wild-type (WT) strain. The findings imply that physiologically, the NAg-r strain attempts to recover its fitness and redistribute precursors and energy that allow for growth in response to genetic mutations that accumulate after prior NAg exposure. On the other hand, upon re-exposure to silver, the NAg-r strain upregulated a large set of ROS (reactive oxygen species) scavenger, iron-sulphur (Fe-S) assembly, cysteine metabolism and DNA repair genes, potentially to handle the lethal impact of NAg-generated ROS to the cell. These responses are unique responses identified only when NAg is present, suggesting the importance of defence to oxidative stress as one of the resistance mechanisms. Further study is essential to validate these hypotheses. A large number of differentially expressed genes (DEGs) were commonly shared between the NAg-r and the Ag⁺-resistant (Ag-r) strain, implying the role of soluble silver ion in the NAg antibacterial mechanisms. Nevertheless, unique physiological adaptation and resistance responses identified in either strain are likely to originate from the genomic adaptations or the additional toxicity of silver solid particulate. Lastly, the findings herein suggest that the mechanisms of defence in the NAg-r *S. aureus* are likely to be conserved across different species.

6.2. Introduction

The discovery of silver nanoparticle (NAg) resistance in recent years has been seen as an emerging issue because it may jeopardize NAg efficacy against a wide range of bacteria, including multi-drug resistant bacteria. To prevent or monitor the emergence and spread of resistance, as well as to administer appropriate treatment, it is urgent and necessary to investigate the mechanisms of resistance^{227,228}. Therefore, upon the discovery of NAg-resistant *Staphylococcus aureus* (see Chapter 3), this study aimed for a deeper understanding of NAg resistance mechanism in this bacterium.

Previous studies have reported certain phenotypic resistance mechanism that the bacteria employ to handle NAg toxicity. One of such examples is the elevated level of flagellin, a protein forming bacterial flagella, which was seen to aggregate the NAg particle and render it ineffective to *E. coli*¹⁴. Other has suggested the presence of pyocyanin, an electron carrier, reduces Ag⁺ to Ag⁰, and thus reduces the toxic bioavailable silver in NAg against *P. aeruginosa*¹⁵. The role of pyocyanin was also suggested to mediate Ag⁺ resistance in *P. aeruginosa*¹⁰⁹, in addition to the presence of Cus, Sil, and Omp proteins in *E. coli* that facilitate Ag⁺ efflux^{84,88}.

Up to this stage, evidences of resistance mechanism to both NAg and Ag⁺ were mostly reported in Gram-negative bacteria, however, the resistance mechanism in Gram-positive pathogenic bacteria, *S. aureus*, remains unknown. The molecular basis of silver resistance is of particular interest in *S. aureus* since none of the reported resistance traits important for silver resistance in Gram-negative bacteria molecularly exist in the *S. aureus* genome.

Upon the discovery of silver resistant *S. aureus*, this study has performed and identified genomic mutations that was hypothesised to govern the defence against NAg (see Chapter 4). Whilst these genomic mutations provide valuable insights into the potential role of individual genes in resistance to silver, it does not provide a snapshot of the dynamic physiological responses after silver exposure. Therefore, to address this, transcriptomics was performed with both the NAg- and Ag⁺-resistant strain. To the best of the author's knowledge, the transcriptional profiles of NAg- or Ag⁺-resistant *S. aureus* has not been reported yet.

Whole cell transcriptomics was initially conducted with the untreated NAg- and Ag⁺-resistant *S. aureus* strain as to understand the physiological adaptations that have occurred after a previous prolonged silver exposure that lead to their resistance characteristics. Next, both resistant *S. aureus* strains were exposed to low (sub MIC of the wild-type strain) and high (above MIC of the wild-type strain) NAg and Ag⁺

concentration, to examine the resistance responses in the presence of silver. To ensure reproducibility, each bacteria-silver exposure system have three biological replicates and conducted following a comparable growth rates between NAg-resistant, Ag⁺-resistant, and wild-type strain in the presence or absence of NAg or Ag⁺. The latter particularly ensures that any detected changes in the cellular responses would represent a silver-induced effect, not due to differences in growth characteristics. Following RNA-seq analysis, the study continued with the interpretation of the differentially expressed genes (DEGs) mapped and annotated to functional pathways, thus to speculate the defence mechanism of *S. aureus* to NAg and Ag⁺.

6.3. Experimental Methods

6.3.1. Preparation of antibacterial agents

Silver nanoparticle (NAg, $d_{\text{TEM}} = \sim 2$ nm, 6.6 wt% Ag homogenously dispersed on 30 nm TiO₂ support^{12,97}) was synthesised by Flame Spray Pyrolysis (FSP) at University of New South Wales (UNSW) and silver nitrate (AgNO₃; Ajax Finechem) was used as ionic silver (Ag⁺) source. NAg stock was always prepared fresh in Cation-adjusted Mueller-Hinton Broth (Ca-MHB; BD, Australia) prior to each experiment whereas AgNO₃ stock were dissolved in autoclaved water and stored in room temperature. To ensure a homogeneous suspension, NAg suspension was ultra-sonicated (Vibra-cell, Sonics & Materials, Inc.) at 50% output control for 20 s prior to use. Experiments with NAg and Ag⁺ were performed under dark conditions to render the TiO₂ support of the NAg particles photocatalytically inactive and to prevent Ag⁺ reduction to Ag⁰, respectively⁹⁷.

6.3.2. Bacterial strains

Bacterial strains used in this study were the NAg- and Ag⁺-resistant strain (henceforth known as the NAg-r and Ag-r strain, respectively) obtained from the resistance development study presented in Chapter 3. The selected strains were sampled from the passaging culture shown in blue circle and diamond in **Figure 3.4A** for NAg-r and Ag-r strains, respectively. The study also used *S. aureus* ATCC25923 (henceforth known as the wild-type (WT) strain) as a control.

6.3.3. Growth responses following NAg or Ag⁺ exposure

The growth responses of the WT, NAg-r, and Ag-r strain in the absence and presence of NAg was performed as explained previously (see Chapter 5 Section 5.3.2).

6.3.4. Sample preparation for RNA extraction

A single colony of the bacterial strain was inoculated and grown overnight in a 5 mL Ca-MHB at 37°C and 200 rpm. The overnight culture was sub-cultured to a 25 mL MHB with starting OD₆₀₀ = ~0.05 for 2 h at 37°C 200 rpm to reach mid-exponential phase (OD₆₀₀ = ~0.4). The culture was treated with NAg or Ag⁺ for 30 min at a specified concentration based on their respective minimum inhibitory concentrations (MICs) to the WT strain (**Table 6.1**). For NAg exposure, the NAg-r strain was exposed to 0.5x and 1.5xMIC whereas the Ag-r strain was exposed to 0.5x and 3xMIC of Ag⁺. Different factors of MIC for the high exposure condition (1.5xMIC and 3xMIC for NAg and Ag⁺, respectively) were used such that the systems would have equivalent silver content (4.5 µg Ag/mL). Untreated bacterial culture was conducted in parallel to serve as a control. These experiments were performed in three biological replicates.

Table 6.1 Treatment conditions used in transcriptomic study for the wild-type (WT), NAg-resistant (NAg-r) and Ag⁺-resistant (Ag-r) strains

Treatment conditions	Bacterial strains		
	WT	NAg-r	Ag-r
NAg treatment			
Low NAg concentration (0.5xMIC, low-NAg)	1.5 µg Ag/mL	1.5 µg Ag /mL	-
High NAg concentration (1.5xMIC, high-NAg)	-	4.5 µg Ag /mL	-
Ag⁺ treatment			
Low Ag ⁺ concentration (0.5xMIC, low-Ag)	0.75 µg Ag /mL	-	0.75 µg Ag /mL
High Ag ⁺ concentration (3xMIC, high-Ag)	-	-	4.5 µg Ag /mL

6.3.5. RNA extraction, RNA quality control, RNA sequencing and read mapping, and RNA-seq data analysis

RNA extraction, RNA quality control, RNA sequencing and read mapping, as well as RNA-seq data analysis were performed as previously explained in Chapter 5 Section 5.3.4 to Section 5.3.7).

6.4. Results and Discussion

6.4.1. Growth responses following NAg and Ag⁺ exposure

Prior to the whole transcriptomic study, the growth rate of the wild-type (WT), NAg- (NAg-r) and Ag⁺-resistant (Ag-r) strains in the absence and presence of either NAg or Ag⁺ were compared. The study on the bacterial growth comparison was to ensure that the exposure to NAg or Ag⁺ does not alter the growth responses of the silver resistant

strain when compared to the untreated respective resistant strain and to the WT strain. Hence, the genes expression in the transcriptomic study would represent the expression due to the silver exposure, not due to the growth alteration.

In the presence of the antibacterial agent, this study exposed the silver resistant strain to a low and high concentration of the antibacterial agent. The low and high concentration represents the concentration below and above the MIC of the respective antibacterial agent to the WT strain, respectively. Both of the exposure concentrations are sub-inhibitory concentrations for the resistant strains. The concentration differences aimed to investigate gene expressions at different exposure conditions that potentially play a role as the defence mechanisms of the resistant strain.

The growth profile of the NAg-resistant (NAg-r) strain at low NAg concentration (0.5xMIC of NAg, 1.5 µg Ag/mL) was shown to be comparable to that of the untreated (NT) WT and NAg-r strains (**Figure 6.1**), noted from the growth rate (0.56 1/h) when compared to that of WT NT (0.54 1/h) and NAg-r NT (0.63 1/h). A similar result was observed in the growth profile of Ag⁺-resistant (Ag-r) strain at low Ag⁺ concentration (0.5xMIC of Ag⁺, 0.75 µg Ag/mL) with a growth rate of 0.62 1/h (**Figure 6.2**). This was comparable to the growth rate of Ag-r NT and WT NT (0.59 1/h, and 0.54 1/h, respectively). Therefore, 1.5 µg Ag/mL and 0.75 µg Ag/mL were used in the following studies as the low NAg and Ag⁺ concentration, respectively.

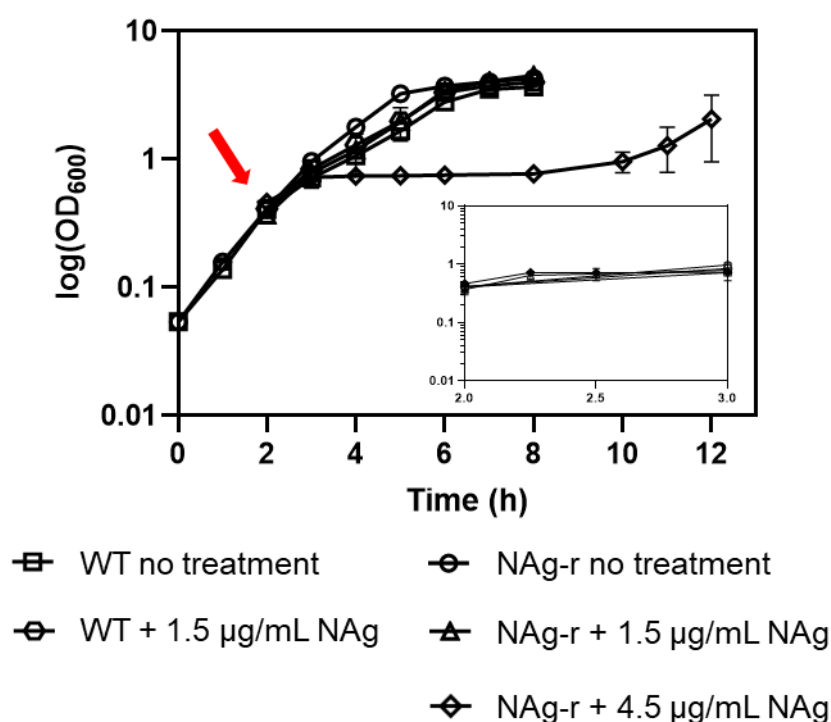


Figure 6.1. Growth curve of the wild-type (WT) and NAg-resistant (NAg-r) strains in the absence (square and circle, respectively) and presence of different NAg concentrations as measured by the optical density at 600 nm. The NAg concentrations represent low NAg

concentration (0.5xMIC, 1.5 $\mu\text{g Ag/ml}$; hexagon and triangle for the WT and the NAg-r strain, respectively) and high NAg concentration (1.5xMIC, 4.5 $\mu\text{g Ag/mL}$; diamond). The red arrow denotes the time when NAg was added to the culture. The inset shows an expansion of range between 2.0 – 3.0 h to display comparable growth between treated and untreated WT and the NAg-r strain (symbols are overlapped). RNA was extracted at the 2.5 h. Each data point represents the mean of at least two biological replicates and the error bars represent standard error of measurement (SEM).

At the high silver concentration (1.5xMIC, 4.5 $\mu\text{g Ag/mL}$ for NAg exposure and 3xMIC, 4.5 $\mu\text{g Ag/mL}$ for Ag⁺ exposure), both NAg-r and Ag-r strain displayed an extended lag phase (see **Figure 6.1** and **Figure 6.2**, respectively). For the Ag-r strain, the study observed that exposure to 4.5 $\mu\text{g Ag/mL}$ of Ag⁺ resulted in a declined growth between the hours of 4 and 6 (**Figure 6.2**). The growth profiles were however, still comparable to the untreated WT and the untreated respective resistant strains within the 30 min of exposure (see **Figure 6.1** and **Figure 6.2** inset). Therefore, 4.5 $\mu\text{g Ag/mL}$ was deemed acceptable for use in subsequent experiments as the high exposure concentration for both NAg and Ag⁺ (within the 30 min exposure prior RNA extraction) since the change in growth rate was minimal.

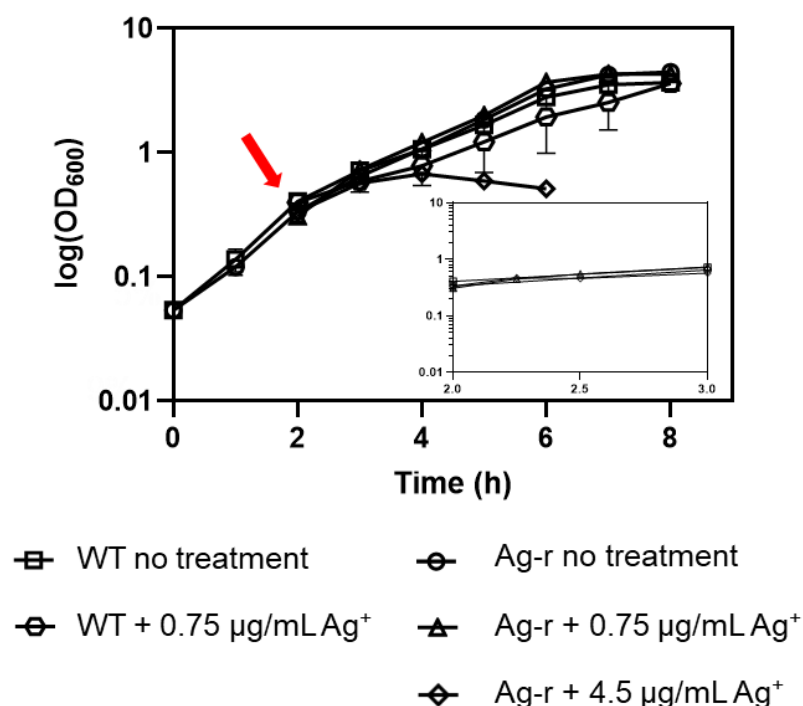


Figure 6.2. Growth curve of the wild-type (WT) and Ag⁺-resistant (Ag-r) strains in the absence (square and circle, respectively) and presence of different Ag⁺ concentrations as measured by the optical density at 600 nm. The Ag⁺ concentrations represent low Ag⁺ concentration (0.5xMIC, 0.75 $\mu\text{g Ag/ml}$; hexagon and triangle for the WT and the Ag-r strain, respectively) and high Ag⁺ concentration (3xMIC, 4.5 $\mu\text{g Ag/mL}$; diamond). The red arrow denotes the time when Ag⁺ was added to the culture. The inset shows an expansion of range between 2.0 – 3.0 h to display comparable growth between treated and untreated WT and the Ag-r strain (symbols are overlapped). RNA was extracted at the 2.5 h. Each data point represents the mean of at least two biological replicates and the error bars represent standard error of measurement (SEM).

6.4.2. Variation analysis between samples

To investigate the variation between samples, principal component analysis (PCA) was performed by Dr. Xianghui Liu and Dr. Xinyi Zhu at SCELSE (see **Figure 6.3**). As briefly mentioned in Chapter 5, PCA is commonly used to simplify a complex and large data set, such as gene expression profiles, to examine the samples' degree of variation¹⁹⁴. In this study, the two principal components (PCs) were plotted and coloured according to the gene expression profile of 9 treatment groups and its biological replicates.

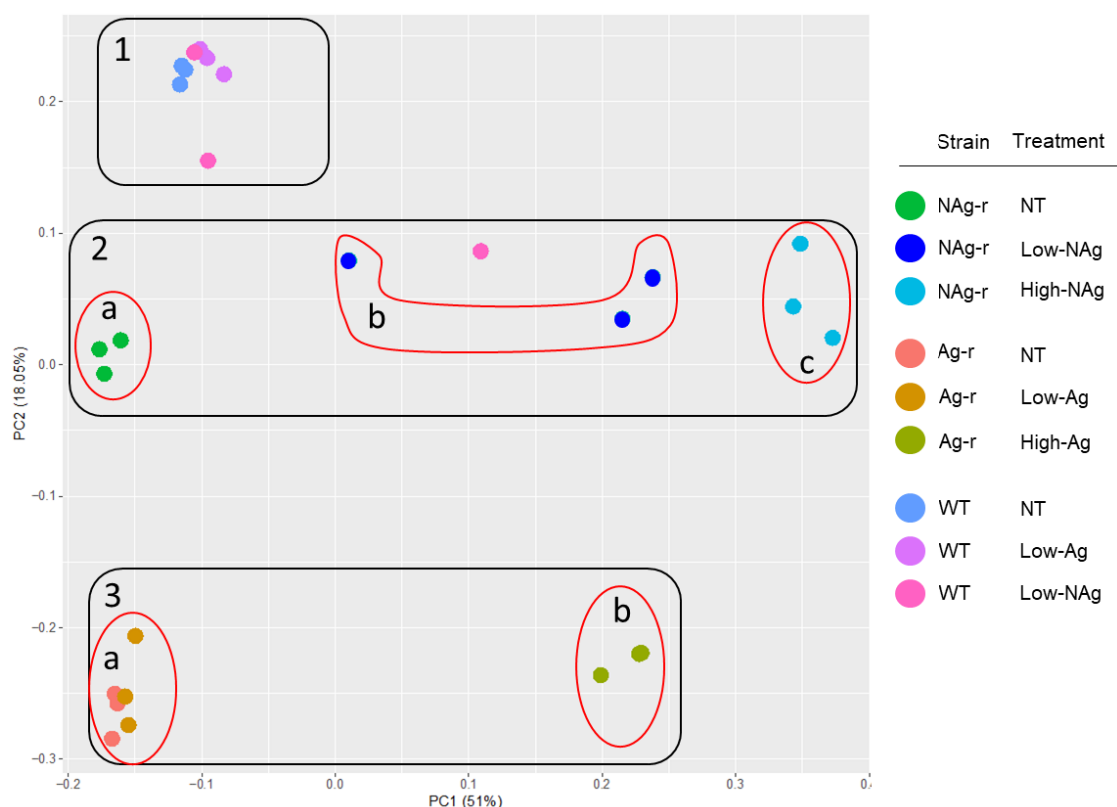


Figure 6.3. Principal component analysis (PCA) plot of the gene expression profile of 9 treatment groups and its biological replicates. The colour and the circle represent the treatment groups and the biological replicates, respectively. NAg-r: NAg-resistant strain, Ag-r: Ag⁺-resistant strain, WT: Wild-type strain, NT: no treatment, low-NAg: low concentration of NAg, high-NAg: high concentration of NAg, low-Ag: low concentration of Ag⁺, high-Ag: high concentration of Ag⁺.

The PCA results displayed three separated positions for three bacterial strains (box 1, 2, and 3 in **Figure 6.3** for WT, NAg-r, and Ag-r strain, respectively). It was interesting that even at the cellular physiological level (absence of silver), the resistant strains displayed different gene expression profiles to the WT, which might be due to the prior prolonged silver exposure that lead to their genome mutations and resistance traits. This will be discussed in more detail below.

For the NAg-r strain, three distinct groups was shown throughout the x-axis (see box 2 a, b, and c in **Figure 6.3**), denoting high variability (51%). The results suggest that the

treatment of NAg-r strain at low or high NAg concentration, when compared to the the NAg-r NT samples, significantly affected the strain's gene expression profiles.

For Ag-r strain, the Ag-r low-Ag samples were shown to have high similarity to the Ag-r NT samples, as noted from the position of these two treatment groups in the PCA plot (see box 3 a in **Figure 6.3**). This result implies that there was minimal variability between the profile of untreated Ag-r strain and the Ag-r strain exposed to low Ag⁺ concentration. Despite this, the study observed that the Ag-r high-Ag samples were located in a distant position from the Ag-r NT and Ag-r low-Ag samples (box 3 b in **Figure 6.3**), indicating that only the addition of Ag⁺ at high concentration could provide significant impact to the gene expression profiles.

It was shown that in almost all samples, the biological replicates for each of the treatment condition had minimal variation, indicating good quality data. However, this study observed that one of NAg-r low-NAg biological replicates was distantly placed from the other two biological replicates (box 2 b in **Figure 6.3**), denoting a high variation on the x-axis (51%) and may cause an issue in the downstream analysis. Similarly to the WT low-NAg samples (Chapter 5), this datum was included in the RNA-seq data analysis and interpretation due to time constraint to repeat the experiments, nevertheless, it is with a caution that follow up studies are required to validate the results.

6.4.3. Comparison of differentially expressed genes in the NAg- and Ag⁺-resistant strains

To investigate the defence mechanism to NAg and Ag⁺, the gene expression of the NAg-r and Ag-r strain in the absence and presence of silver was measured and analysed. Firstly, in the absence of the respective antibacterial agent, gene expressions were compared to the untreated wild-type (WT) strain, as to understand the physiological adaptation traits developed in the resistant strain (see Section 6.4.4). Next, the NAg-r and Ag-r strain were treated with low or high NAg or Ag⁺ concentrations for 30 min, and compared to the untreated respective resistant strain as the study aimed to understand the resistance responses of the resistant strain in the presence of silver (see Section 6.4.5). It is important to note that following a prolonged silver exposure, the resistant strain displayed an increase in MIC (Chapter 3) and changes in bacterial genome (Chapter 4) when compared to the WT strain, indicating a phenotypic- and genomic-resistance characteristic, respectively. The total expressed genes number followed the cut off threshold of log₂ fold change > 2 and p-adjusted value < 0.05 (**Table 6.2** and **Table 6.3** for NAg and Ag⁺ resistance study, respectively).

In the absence of NAg, the RNA-seq analysis saw in total 94 differentially expressed genes (DEGs) with 61 and 33 genes being upregulated and downregulated by the NAg-r strain, respectively, relative to the untreated WT (**Table 6.2**). The upregulation was seen primarily in genes involved in the nucleotide metabolism whilst distinct downregulation was seen in genes involved in cell envelope metabolism. This gene expression profile represents the physiological adaptation of the NAg-r strain, as a result of prior prolonged exposure to NAg.

Table 6.2. Total genes differentially expressed in different NAg treatments in the NAg-resistant strain

	Upregulated	Downregulated	Total
NAg-r NT vs WT NT	61	33	94
NAg-r low-NAg vs NAg-r NT	129	157	286
NAg-r high-NAg vs NAg-r NT	282	337	619

NT: no treatment; low-NAg: low NAg concentration (0.5x NAg MIC); high-NAg: high NAg concentration (1.5x NAg MIC); WT: wild-type strain; NAg-r: NAg-resistant strain.

Further, the NAg-r strain was exposed to a low NAg concentration (low-NAg), and high NAg concentration (high-NAg). The low- and high-NAg represented 0.5x NAg MIC (1.5 µg Ag/mL) and 1.5x NAg MIC (4.5 µg Ag/mL) of the wild-type (WT). Interestingly, at both of these exposure conditions, the NAg-r strain downregulated more than half of the total DEGs for the respective exposure condition (~55% for either low- or high-NAg) (**Table 6.2**). The majority of these DEGs were associated with nucleotide metabolism, translation, transporters, and virulence factors. Interestingly, the bacteria also upregulated a large number of oxidative stress genes. Both of these upregulation and downregulation profiles could suggest the defence mechanism of the NAg-r strain to survive NAg toxicity. Detailed discussion on the pathways is provided in Section 6.4.4.2 and Section 6.4.5.2 for the physiological adaptation and the resistance responses in the presence of NAg, respectively.

The investigation of Ag⁺ resistance mechanisms was carried out by subjecting the Ag⁺-resistant (Ag-r) strain to different Ag⁺ treatments, which include untreated (NT), low Ag⁺ concentration (low-Ag), and high Ag⁺ concentration (high-Ag). The low- and high-Ag represented 0.5x Ag⁺ MIC (0.75 µg Ag/mL) and 3x Ag⁺ MIC (4.5 µg Ag/mL) of the wild-type (WT) strain, respectively.

Table 6.3. Total genes differentially expressed in different Ag⁺ treatments in the Ag⁺-resistant strain

Pair-wise comparison	Upregulated	Downregulated	Total
Ag-r NT vs WT NT	74	66	140
Ag-r low-Ag vs Ag-r NT	0	0	0
Ag-r high-Ag vs Ag-r NT	161	240	401

NT: no treatment; low-Ag: low Ag⁺ concentration (0.5xMIC, 0.75 µg/mL); high-Ag: high Ag⁺ concentration (3xMIC, 4.5 µg/mL); WT: wild-type strain; Ag-r: Ag⁺-resistant strain.

In the absence of Ag⁺, 74 and 66 DEGs were seen to be upregulated and downregulated relative to the untreated WT strain (**Table 6.3**). This genes expression profile represents the physiological adaptation of Ag-r upon prolonged exposure to Ag⁺, which include the upregulation of nucleotide metabolism and translations genes as well as the downregulation of genes involved in cell envelope metabolism.

Interestingly, upon exposure to low Ag⁺ concentration, the Ag-r strain did not exhibit any significant differences in gene expression when compared to the untreated Ag-r strain whereas at high Ag⁺ concentration, the exposure condition resulted in the downregulation of roughly 60% of the total DEGs (240 and 161 genes for downregulation and upregulation, respectively) (**Table 6.3**). The result imply that the adaptation in the Ag-r strain upon prolonged exposure to Ag⁺ potentially transforms the strain's capability to manage Ag⁺ mild toxicity. However, this was not observed when the NAg-r strain was exposed to the low NAg concentration (**Table 6.2**), whereby the study observed the upregulation and downregulation of 129 and 157 genes, respectively. This indicates that different toxicity features between NAg and Ag⁺ may influence resistance responses. It was thought that the NAg solid particulate may contribute to this difference because if NAg toxicity only originates from soluble silver, as some have thought²⁰, it would be expected that the transcriptomic profiles of the NAg-r observed at low-NAg and the Ag-r observed at low-Ag would be similar, *i.e.* no differentially expressed genes at the NAg-r low-NAg samples. The resistance response of the Ag-r strain in the presence of Ag⁺ were, therefore, interpreted from the expression shown by the strain exposed at high Ag⁺ concentration.

It is important to note that the investigation on the NAg-r and Ag-r strain in the absence or the presence of the respective silver compound is aimed to not only understand the individual resistance mechanism to the respective silver species but also to distinguish the unique resistance mechanisms between NAg and Ag⁺. The latter was considered as studies have previously reported that the entity of NAg could not be separated from Ag⁺ as the soluble ionic silver is released from the nanoparticle *via* oxidative dissolution^{18,43}. Nevertheless, unlike Ag⁺, NAg antibacterial activity is also governed by the solid particle⁹⁹ and its physicochemical properties, such as size²⁵, shape^{28,29} and presence of surface functional groups^{7,25}. Therefore, in the following sections, the comparison between NAg and Ag⁺ resistance mechanism was first presented followed by the defence mechanisms of the NAg-r strain as this was the focus of the study. The Ag⁺ resistance mechanism would also be highlighted where relevant and appropriate.

This whole transcriptomic study *via* RNA sequencing is an initial study that capture the snapshot of genes expression and, in this case, to understand the global defence

mechanisms to silver toxicity. As such, it is therefore important to validate the interpretation deduced from the data. Quantitative polymerase chain reaction (qPCR) could be performed firstly in general to confirm the level of targeted genes expression, followed by studies specific to the pathways affected. Due to time constraint, these validation studies have not been performed. Suggested pathway-specific studies, nevertheless, are provided after each biological interpretation.

6.4.4. Physiological adaptations of the NAg- and Ag⁺-resistant strain

6.4.4.1. The comparison between NAg- and Ag⁺-resistant strain

To understand the relationship between the physiological adaptations of the NAg-r and Ag-r strain upon prolonged exposure to its respective antibacterial agent, this study compared their transcriptomic profiles. These profiles were acquired following RNA sequencing and analysis of the silver resistant strains relative to the WT in the absence of silver, *i.e* NAg-r NT vs WT NT and Ag-r NT vs WT NT. Briefly, the physiological adaptation study was performed following 50 days exposure of *S. aureus* to NAg or Ag⁺ (see Chapter 3). The resistant strains have shown the capability to grow at higher NAg or Ag⁺ dosage compared to the WT strain (see Chapter 3).

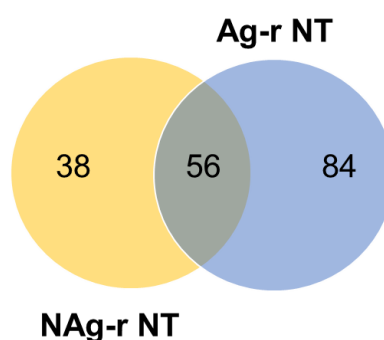


Figure 6.4. Venn diagram representing the number of genes expressed from untreated (NT) NAg-resistant (NAg-r) strain and Ag⁺-resistant (Ag-r) strain when compared to the untreated wild-type (WT) strain.

It was shown that both strains shared the expression of 56 genes (**Figure 6.4**), which include genes associated with nucleotide metabolism, cell envelope metabolism, and translation genes, with an overall comparable expression level (**Figure 6.5**). This is exemplified with the expression of cell envelope metabolism genes (*cap5A-P*) that were downregulated to the same degree in both strains (**Figure 6.5**). A similar expression was observed in other genes such as nucleotide metabolism genes (*purA*, *purKCSQLFMNHD*, *guaC* for purine system and *carAB-pyrFE* for *pyr* system) and translation-associated genes (*rpsN*, *rplX*, *rpmC*, and *rplV*) that were upregulated. It was expected to find similarities between the gene expression profiles, reflecting the physiological adaptation of NAg-r and Ag-r strains as both NAg and Ag⁺ share

intracellular presence of soluble silver ions that contribute to the toxicity^{63,112}, which will therefore, result in predicted shared mechanisms for defence. The soluble silver ions of the NAg is likely the outcome of leaching process, due to oxidative dissolution of the particle surface¹⁸ and this process has also been hypothesised to occur inside the cells, thereby referring to the Trojan horse-type of toxicity mechanisms⁶².

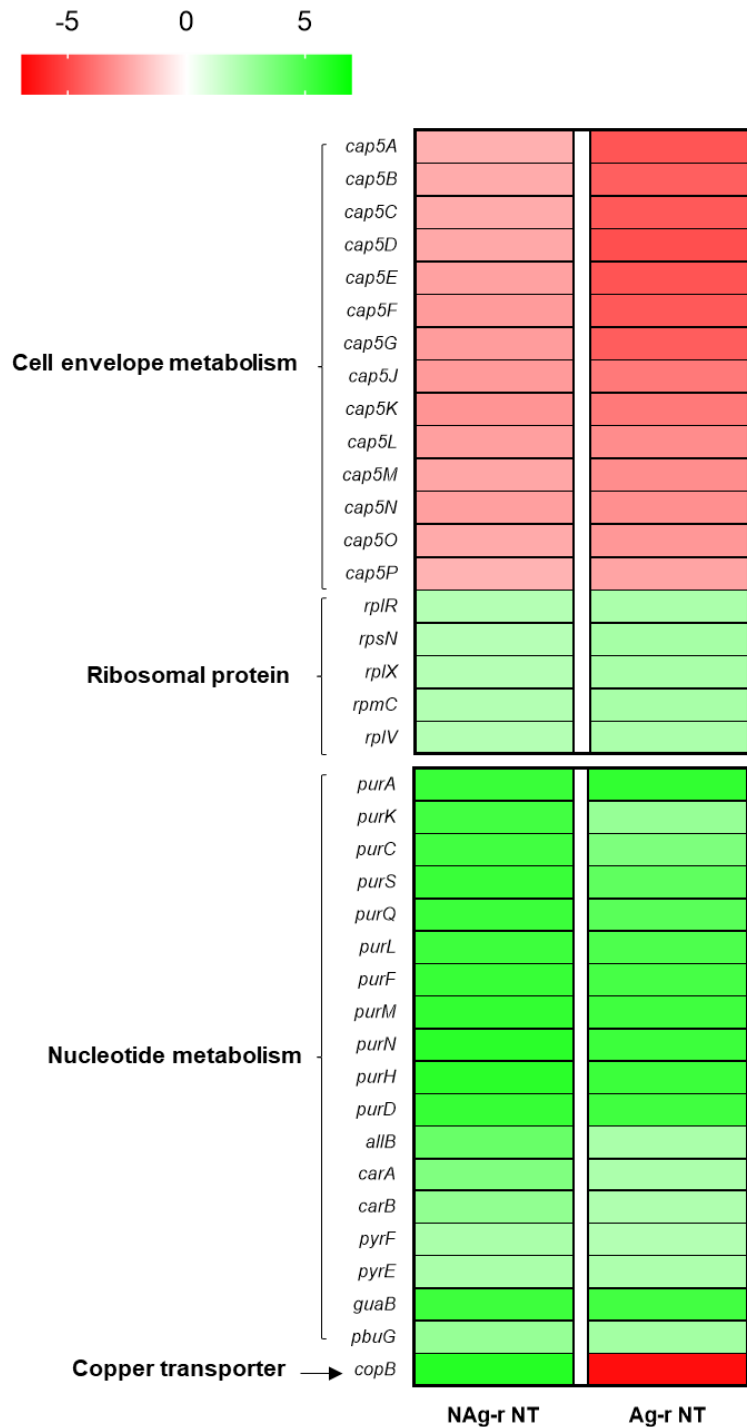


Figure 6.5. Heat map representing the level of expression in some of the common differentially expressed genes in the untreated (NT) NAg-resistant (NAg-r) strain and the Ag⁺-resistant (Ag-r) strain.

Interestingly, among the 56 genes shared between the untreated NAg-r and Ag-r strain (**Figure 6.4**), expression of one gene (*copB*, a copper efflux gene²²²) showed opposite level of expression (**Figure 6.5**). In the untreated NAg-r, this gene was highly upregulated whereas in the untreated Ag-r strain, the opposite was observed. Similarly, other metal transporters gene (*cadA*) was seen to be highly downregulated in the untreated Ag-r, however was not detected in the NAg-r strain transcriptomic profile (**Table S6.2**). This finding implies that the Ag-r strain may 'shut down' metal transport proteins in the absence of Ag⁺. The underlying mechanism and reason to this phenomenon are currently unknown. Nevertheless, a confirmation *via* qPCR would be required to validate the expression level.

The data herein also saw 38 and 84 DEGs that uniquely belonged to the transcriptomic profile of NAg-r and the Ag-r strain, respectively (**Figure 6.4**). Genomic changes observed in the respective resistant strain (Chapter 4) may be accounted for the differences that in turn, could potentially affect genes expression directly or indirectly.

In the NAg-r strain transcriptomic data (38 in **Figure 6.4**), the transcriptomic data detected the upregulation of denitrification genes (*narI**JHG* and *nirDB*). Unique expression was also seen in an additional 5 purine synthesis genes (*purECQ* and *purB*) for the NAg-r NT than those of the Ag-r NT by ~5 log2 fold change (see **Table S6.1**) that may be facilitated by the *purR* mutation (TCT-to-TAT mutation at the amino acid position 169) in the NAg-r strain (Chapter 4). *PurR*, gene encodes the purine operon repressor protein in *S. aureus*, functions to negatively regulate the purine operon by binding to the DNA and blocking RNA polymerase from performing transcription¹⁴⁴. The identified single nucleotide variation that was located within the PRPP binding site of the protein was hypothesised to reduce the binding affinity of the protein with the DNA¹⁴³, or in other words, prevent the repressor protein from performing its function. The enrichment of whole purine operon (*purA*, *purEKCSQLFMNHD*, and *purB*) observed in the NAg-r strain, with respect to the WT strain, therefore supports this hypothesis. Consistent with the findings herein, a previous study saw an enhancement of whole purine nucleotide biosynthesis genes from *B. subtilis* and *S. aureus* Δ *purR*, implying that the non-functional PurR increases the expression of whole purine operon^{229–231}.

From RNA transcript data uniquely seen in the Ag-r strain (84 in **Figure 6.4**), this study detected the upregulation of 19 more ribosomal protein genes than that of the NAg-r NT. In addition to that, the transcript data showed the upregulation of cysteine synthesis as well as cysteine and methionine uptake genes (*cysK*, *metB*, *tauBC*, *metNP*, *nlpA*, and *tcyABC*, see **Table S6.2**). Similar to the unique DEGs of the NAg-r

strain, the unique responses of the Ag-r strain may result from the mutation in *cymR* (GGT-to-GAT mutation) (Chapter 4). CymR is a cysteine metabolism regulator that represses the cysteine metabolism and cysteine-related transporter genes¹⁷⁰. The findings herein (**Table S6.2**) are consistent with an earlier study that reported the upregulation of these genes in *S. aureus* $\Delta cymR$ relative to *S. aureus* parental strain¹⁷⁰, indicating that the mutation may result in a non-functional CymR protein in the Ag-r strain.

It was hypothesised previously that the mutation in *cymR* may prevent cysteine intake due to cysteine-mediated oxidative stress (Chapter 4). Since there is little work on the functional analysis of *cymR*, the effect of non-synonymous mutation in *cymR* was hypothesised from that of *tcyA*, a gene encoding an L-cystine binding protein. This study previously hypothesised that due to a shifted reading frame that resulted in early termination of protein synthesis, TcyA protein was likely to be non-functional, thus preventing cysteine uptake. Nevertheless, the transcriptomic data shown here supports a different role of the mutation in *cymR* where a likely non-functional CymR results in increased cysteine uptake.

In the following sections, the biological interpretation of the transcriptomic profiles to understand the physiological adaptation exhibited in the NAg-r strain is presented (Section 6.4.4.2) as this is the main focus of the study. Nevertheless, this study also discusses the particular physiological traits identified in the Ag-r strain (Section 6.4.4.3). Similar to Chapter 5, the discussion in this chapter focusses on the interpretation of the pathways, rather than a single gene, because changes within a pathway were thought to provide more significant contribution to the understanding of the defence mechanism.

6.4.4.2. Physiological adaptations of the NAg-resistant strain

This section focused on the physiological adaptations of the NAg-resistant (NAg-r) strain that displayed resistant characteristic upon a prolonged exposure to NAg and minimum inhibitory concentration (MIC) determination (see Chapter 3). The NAg MIC of the NAg-r strain was 5-7 fold higher than that of the wild-type (WT) strain. From these findings, further investigation on the physiological traits of NAg-r *via* transcriptomic study was thought capable to advance the knowledge of the bacterial adaptation to the nanoparticle. The transcriptomic analysis was performed using BioCyc Omics Dashboard¹⁹¹, NCBI database¹⁹³ and manual interpretation using published literatures.

In the absence of NAg, the functional pathway mapping and analysis revealed 13 pathways associated with the DEGs (38 + 56 in **Figure 6.4**) in NAg-r NT compared to WT NT (see **Figure 6.6A**). The majority distribution of the total DEGs (approximately 20%) were associated with purine synthesis in nucleotide metabolism. Interestingly, most genes in this pathway were seen to be highly upregulated (log2 fold change > 5.0 with respect to the WT NT) (**Figure 6.6B and C, Table S6.1**). Also among the highly upregulated genes were genes associated with denitrification pathway, that approximately takes up 6% of the total DEGs. Another distinct results were observed from cell envelope metabolism as all the genes associated here were downregulated by 2.0 – 3.0 log2 fold.

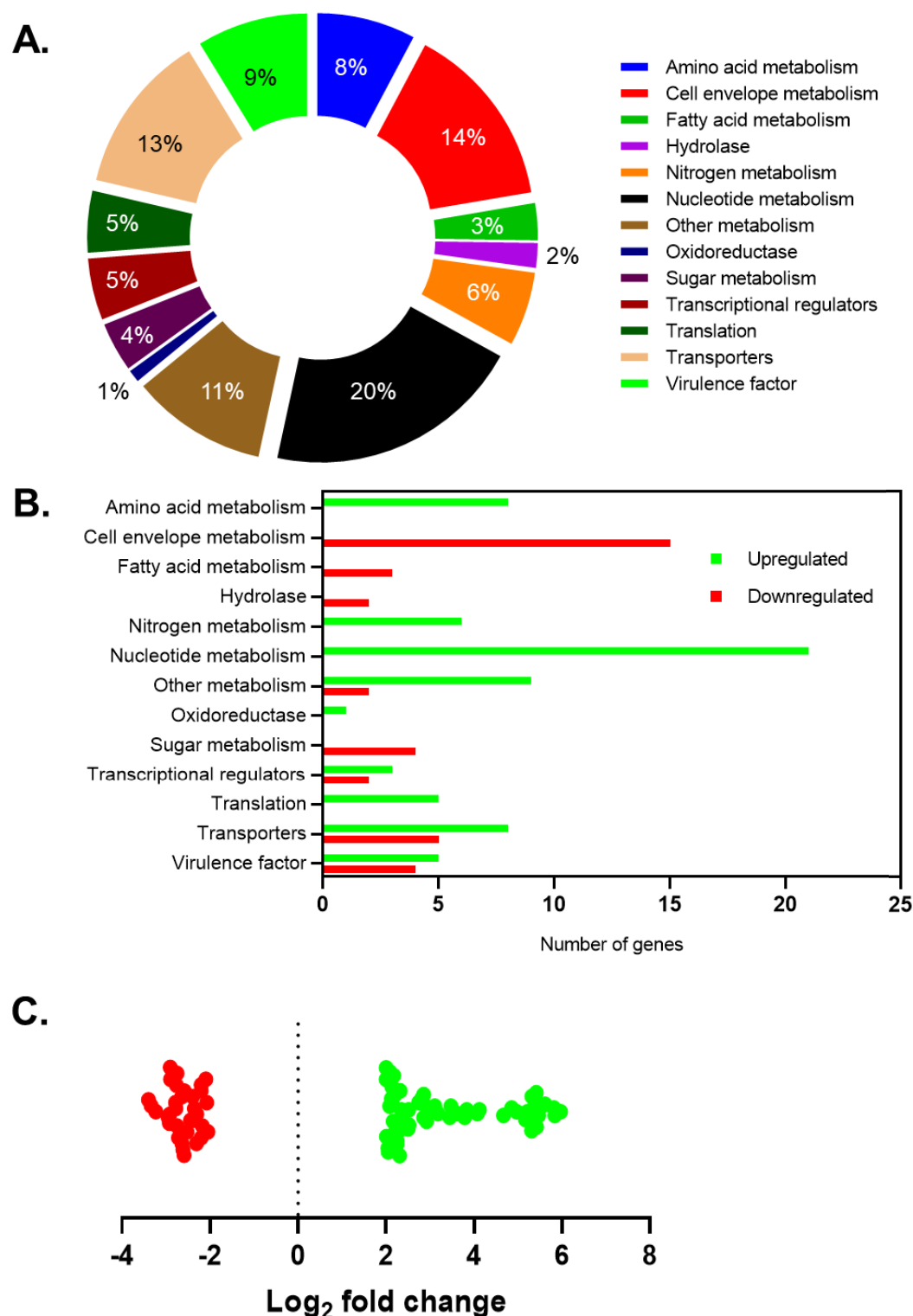


Figure 6.6. The proportion (A), number of upregulated and downregulated (B) differentially expressed genes (DEGs) based on the functional pathway, and dot plot (C) representing the distribution of DEGs based on their log₂ fold change in the untreated NAg-resistant strain with respect to the WT strain of *S. aureus*. The functional pathways were classified from BioCyc Omics Dashboard, NCBI database, and manual interpretation.

To start with, this study hypothesised that the physiological adaptations of the NAg-r strain upon previous NAg prolonged exposure (see Chapter 3 for prolonged exposure to NAg) **may facilitate its fitness recovery in the absence of NAg**.

Supporting this hypothesis the observed upregulation of **nucleotide metabolism** genes, particularly in purine and pyrimidine nucleotides biosynthesis genes, in the untreated NAg-r strain as relative to the untreated WT. These nucleotides are precursors for deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) synthesis, essential biological molecules that carries the genetic and hereditary information for cellular processes in living organisms⁵¹. Extensive damage to DNA or RNA will disrupt normal cell functioning including growth and hence, can be lethal. In addition, the nucleotides have important roles in the generation of chemical energy (adenosine triphosphate (ATP), guanosine triphosphate (GTP), cytosine triphosphate (CTP), thymine triphosphate (TTP), and uridine triphosphate (UTP)) for cellular processes¹⁹².

The transcription data showed the upregulation of 14 genes (*purA*, *purEKCSQLFMNHD*, *purB*, and *guaC*) by approximately 2.3 – 6.0 log₂ fold-change (relative to the WT NT, see **Table S6.1**). The *pur* genes (*purEKCSQLFMNHD*) encode enzymes that catalyse a series of steps to generate inosine monophosphate (IMP), the first nucleotide intermediate in the *de novo* purine synthesis pathway, and ultimately leading to the production of adenosine monophosphate (AMP) catalysed by enzymes encoded by *purAB* or guanosine monophosphate (GMP) which *guaC* encode GMP reductase that catalyses the removal of H⁺ from IMP (see **Figure 6.7** for the synthesis pathway).

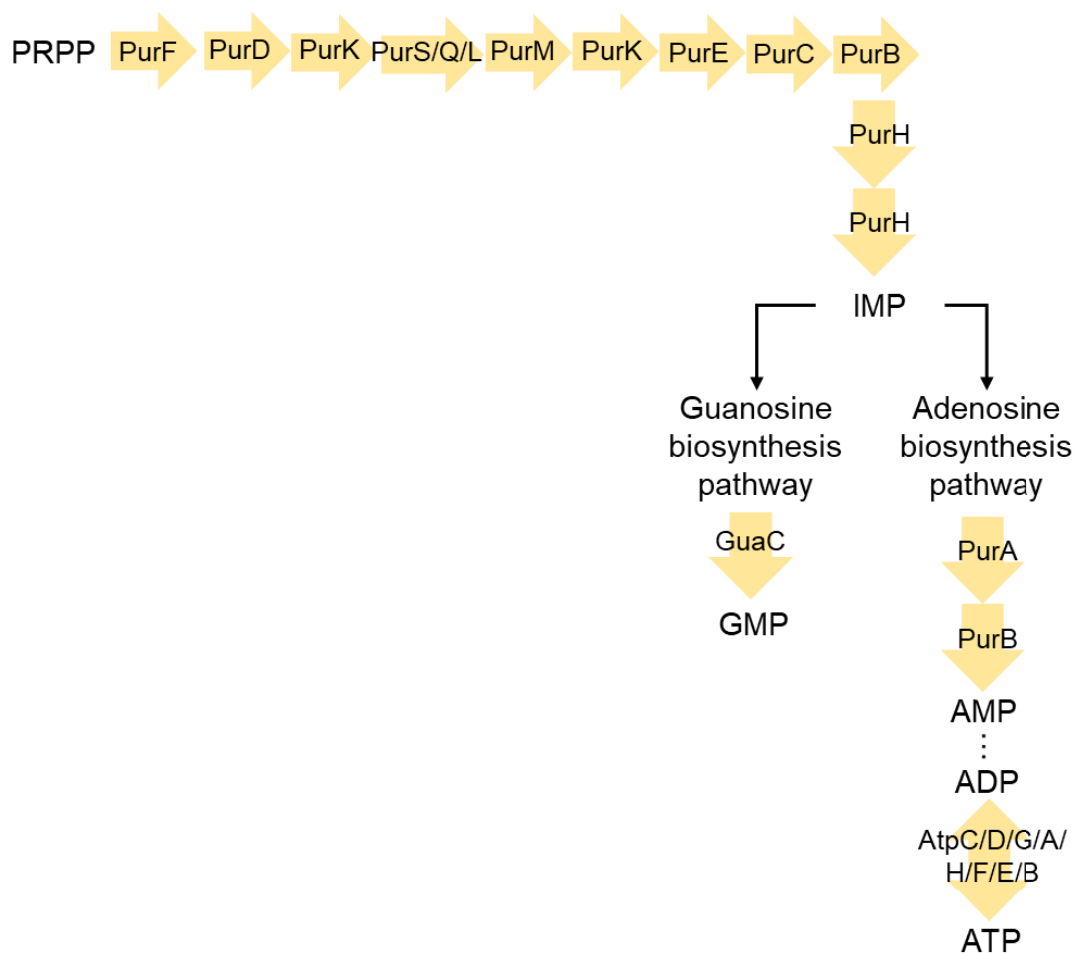


Figure 6.7. *De novo* purine nucleotide synthesis pathway in *S. aureus*. The pathway shows the transformation of phosphoribosyl pyrophosphate (PRPP) to inosine monophosphate (IMP) in 11 steps, catalysed by enzymes encoded by the *pur* operon genes (*purEKCSQLFMNHD*). IMP can be further transformed to adenine or guanine *via* adenosine or guanosine biosynthesis pathway, respectively. In adenosine biosynthesis pathway, IMP is converted to adenosine monophosphate (AMP) by enzymes encoded by *purA* and *purB*, resulting in the generation of adenosine triphosphate (ATP) by ATP synthase, encoded by *atpCDGAHFEB*. Guanosine monophosphate (GMP), on the other hand, can be generated by GMP reductase (encoded by *guaC*) from IMP. Arrows coloured in yellow represent enzymes encoded by genes discussed in this study (Section 6.4.4.2 and 6.4.5.2.).

Apart from purine, the study also observed upregulation of 7 out of 8 genes from pyrimidine biosynthesis operon (*uraA-pyrBC-carAB-pyrFE*) as well as the bifunctional pyrimidine operon regulator and uracil phosphoribosyltransferase (*pyrR*, located upstream of the operon) approximately by 2.3 – 5.0 log₂ fold (relative to WT NT, see **Table S6.1**). *Pyr* operon participates in the uridine monophosphate (UMP) synthesis process, the first nucleotide intermediate in *de novo* pyrimidine biosynthesis, ultimately resulting in the production of uridine triphosphate (UTP) or cytidine triphosphate (CTP) (see **Figure 6.8** for the synthesis pathway). Note that apart from the UMP synthesis genes (*pyrBC-carAB-pyrFE*), the upregulation was also seen in *uraA*, a uracil transporter.

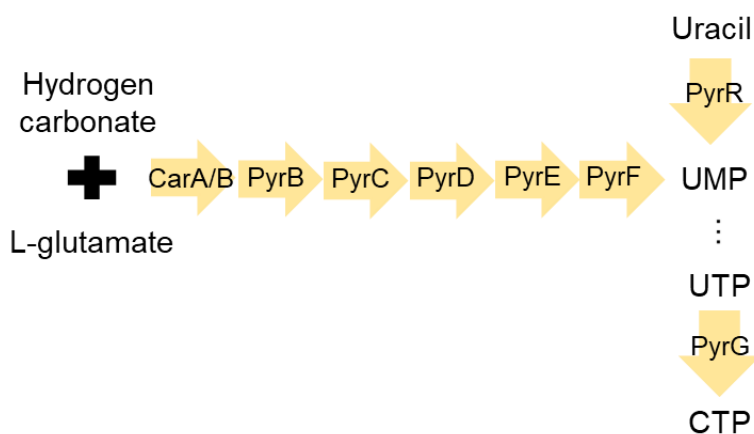


Figure 6.8. De novo pyrimidine nucleotide synthesis pathway in *S. aureus*. The pathway requires L-glutamate and hydrogen carbonate to generate uracil monophosphate (UMP), which is catalysed by enzymes encoded by pyrimidine biosynthesis operon (*pyrBC-carAB-pyrFE*) and *pyrD* through 6 reaction steps. UMP can also be synthesised from uracil via an enzymes encoded by *pyrR*. UMP will be further transformed to uridine triphosphate (UTP) and cytidine triphosphate (CTP). Arrows coloured in yellow represent enzymes encoded by genes discussed in this study (Section 6.4.4.2 and 6.4.5.2).

Many research efforts have reported increased expression of purine and pyrimidine synthesis genes in antibiotic-resistant *S. aureus* strains, being associated with their resistance traits. A study by Mongodin *et al.* observed an elevated expression (3 – 30 fold change) of purine synthesis genes (*purA*, *purEKCSQLFMNHD*, and *purB*) in a vancomycin-resistant *S. aureus* strain (VRSA)²³². Interestingly, as also observed in the current study (see explanation in Section 6.4.4.1 and Chapter 4), the work also found mutations in the *purR* gene, in this case, at the nucleotide position 140 (that results in single amino acid changes in the protein, from isoleucine to lysine). The link between the *pur* gene to the development of adaptation traits is also shown by Yee *et al.*, reporting that the deletion of *purB* and *purD* in methicillin-resistant *S. aureus* (MRSA) resulted in the loss of the bacterium's persistence characteristic to rifampicin and gentamicin²³³, implying a role for these purine synthesis genes in endurance to antibiotics. Note that the persistence phenotype was restored upon re-introduction of the deleted genes. A metabolomics analysis in another study detected an increased concentrations of IMP, GMP, and UMP in daptomycin-resistant *S. aureus* when compared to the susceptible counterpart¹⁴⁵.

This study also detected the upregulation by ~2.0 log₂ fold change (relative to the WT NT, see **Table S6.1**), of genes encode **ribosomal proteins**. These proteins form part of the ribosome, a macromolecular systems in cells that synthesise the proteins from their mRNA transcripts, the so-called translation process. Together with ribosomal RNA (rRNA), the ribosomal proteins compose the large or small subunits of ribosome.

The transcriptomic data also showed an interesting upregulation of **nitrogen metabolism pathway genes** by 2.8 – 5.3 log₂ fold change (relative to WT NT, **Table S6.1**). These genes encode the enzymes respiratory nitrate reductase (*narGHIJ*) and nitrite reductase genes (*nirBD*) as well as a transporter (*narT*). In *S. aureus*, *narGHIJ*, encodes the alpha, beta, gamma, and delta chain of nitrate reductase, known to catalyse the reduction of nitrate (NO₃⁻) to nitrite (NO₂⁻). Nitrite is then further reduced to ammonia catalysed by nitrite reductase large and small subunit, encoded by *nirBD* (see **Figure 6.9**)²³⁴. Ammonia is an important moiety, used in many cellular processes as nitrogen source for protein and nucleotide, the latter includes the synthesis of IMP and UMP whereby nitrogen moieties are present almost all of the intermediates²³⁵. Therefore, metabolic changes in nitrogen metabolism may be in direct or indirect response to the observed changes in purine and pyrimidine synthesis genes.



Figure 6.9. Nitrate reduction to ammonia pathway in *S. aureus*.

Therefore, collectively with the observed upregulation of purine biosynthesis genes (*purA*, *purEKCSQFLFMNHD*, *purB*, and *guaC*), pyrimidine biosynthesis genes (*uraA*-*pyrBC-carAB-pyrFE*), the ribosomal protein genes, as well as the denitrification genes, it was thought that these physiological adaptation responses (as a result of the prolonged exposure to NAg) by the NAg-r strain were intended to increase the bacterium's fitness when growing in the absence of NAg. Many studies have shown that antibiotic-resistant bacteria are often 'penalized' with a fitness cost as the mutations that grant the resistance characteristics occurs in genes involved in physiologically important biological processes²³⁶. One example is the reported lower growth rate of multi-drug resistant *E. coli* when grown in the absence of antibiotics²³⁷. Nevertheless, studies have also suggested that these antibiotic-resistant bacteria potentially activate a compensatory adaptation to cope with the fitness cost and subsequently, to grow well in the absence of antibiotics^{236,238}, the latter was observed herein with the NAg-r strain showing a slightly better growth (higher growth rate) when compared to that of the WT strain in the absence of NAg (see **Figure 6.1**).

DNA synthesis is one of the likely routes for the increased purine and pyrimidine nucleotides level. Therefore, future studies could consider the measurement of DNA replication rate by quantifying and analysing the gene expression ratio of the origin (*oriC*) to the terminus (*ter*) of the NAg-r and the WT strain *via* qRT-PCR, as has been

performed by an earlier study²³⁹. Additionally, it might be interesting to measure the denitrification rate of the NAg-r strain using a block acetylene method²⁴⁰.

Next, this study also observed changes in the expression of 14 genes associated with **cell envelope metabolism pathway**, in particular, the capsular polysaccharide biosynthesis (*cap5* cluster). Out of the 16 *cap* genes in the cluster, 14 genes (*cap5A* to *cap5P*, except *cap5HI*) were downregulated by 2.1 – 3.0 log₂ fold in the NAg-r strain relative to the WT NT (**Table S6.1**). Capsular polysaccharide is a high molecular weight polysaccharide that attached to the cell surface and is one of the essential features in *S. aureus* virulence and defence against host invasion as it facilitates the bacterium's survival during phagocytosis in the host^{241,242}. Earlier transcriptomic study on *S. aureus* (susceptible) biofilm also saw that NAg exposure at sub-MIC concentration downregulated the expression of capsular polysaccharide biosynthesis genes¹⁸³, as detected here on the planktonic *S. aureus*.

The *cap* cluster encodes enzymes that synthesise the capsular polysaccharides²⁴³. The synthesis process utilizes undecaprenyl-phosphate-D-N-acetylglucosamine (UDP-GlcNAc) as the starting precursor and lipid I as lipid carrier (**Figure 6.10**). This precursor undergoes a three-staged process simultaneously in the cytoplasm, the conversion of UDP-GlcNAc to UDP-N-acetyl-D-fucosamine (UDP-D-FucNAc), UDP-N-acetyl-L-fucosamine (UDP-L-FucNAc), and UDP-N-acetyl-D-mannosaminuronic acid (UDP-D-ManNAcA) (see **Figure 6.10** for the synthesis pathway). These processes are thought to be regulated by Cap5A-C²⁴³.

UDP-D-GlcNAc is not only required for capsular polysaccharide synthesis but is also a precursor for cell wall peptidoglycan, wall teichoic acid (WTA) and lipoteichoic acid (LTA) synthesis^{168,217}, the three fundamental components of cell surface that provide cell protection and survival for survival, cell integrity for shape as well as having important roles in cell division^{217,244}. The peptidoglycan synthesis also requires lipid I as one of the intermediates. Interestingly, this study identified a mutation in *uppS* (see Chapter 4 **Table S4.1**), encoding undecaprenyl diphosphate synthase (UPPS) enzyme that catalyses the formation of undecaprenyl dipyrophosphate (UPP), a precursor for lipid I formation¹⁶⁶ (see **Figure 6.10** box for lipid I synthesis). It was hypothesised that the mutation may reduce the level of UPPS¹⁶⁷, and consequently, of lipid I.

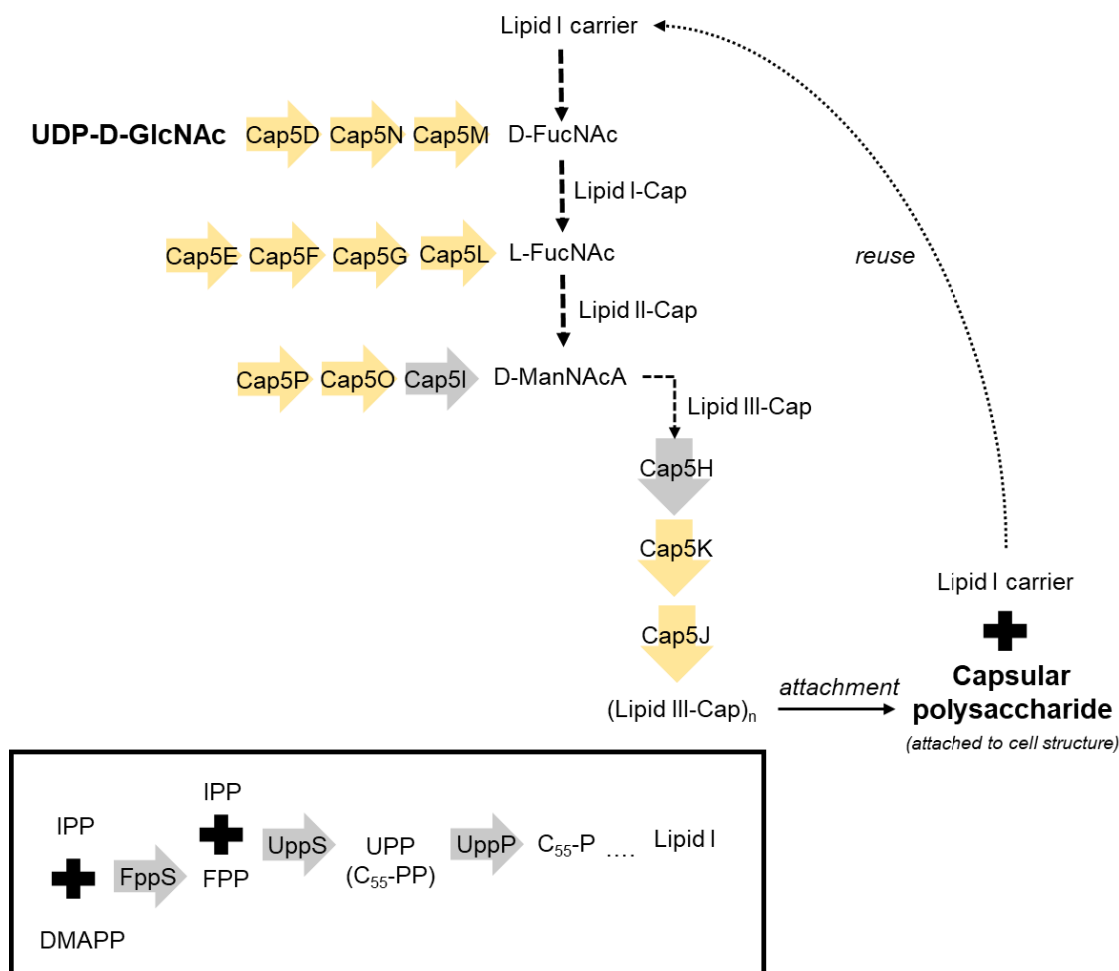


Figure 6.10. Capsular polysaccharide synthesis from UDP-D-GlcNAc and lipid I synthesis (inside a box) pathway in *S. aureus*. A three-staged process to convert UDP-D-GlcNAc to D-FucNAc, L-FucNAc, and D-ManNAcA is catalysed by enzymes or transferred by protein carriers encoded by *cap5DEFGILMNOP* the *cap5* cluster genes. Lipid I acts as a lipid carrier that transfer and join the intermediate molecules prior translocation and polymerization by enzymes encoded by *cap5HKJ*. Upon the attachment of the lipid-capsular polysaccharides to the cell structure, lipid I molecule is released to be reused. Inside the box: the early steps of lipid I synthesis are catalysed by enzymes encoded by *fppS*, *uppS*, and *uppP*, converting IPP and DMAPP to C₅₅-P that eventually leads to the generation of lipid I. Arrows coloured in yellow are enzymes or protein carriers encoded by genes detected in the transcriptomic data in this study (Section 6.4.4.2) whereas greyed arrows represent enzymes discussed here. Lipid I-Cap: Lipid I–UDP-D-FucNAc, Lipid II-Cap: Lipid I–(UDP-D-FucNAc)–(UDP-L-FucNAc), Lipid III-Cap: Lipid I–(UDP-D-FucNAc)–(UDP-L-FucNAc)–(UDP-D-ManNAcA), UDP-D-GlcNAc: undecaprenyl-phosphate-D-N-acetylglucosamine, UDP-D-FucNAc: UDP-N-acetyl-D-fucosamine, UDP-D-ManNAcA: UDP-N-acetyl-D-mannosaminuronic acid, IPP: isopentenyl diphosphate, DMAPP: dimethylallyl diphosphate, FPP: farnesyl diphosphate, UPP: undecaprenyl diphosphate.

Taken together, it was thought the downregulation of capsular polysaccharide biosynthesis genes could be one of the means for the NAg-r strain to allocate UDP-D-GlcNAc and lipid I, or even cellular energy molecules, for a cell protection feature. Studies have reported cell wall damages in *S. aureus* upon physical interaction of NAg with the cell wall (visible in the electron transmission micrographs)^{8,54}. Recently, a study by Gunawan *et al.* detected the disintegration of glycan strands in peptidoglycan as well as the destruction of the entire lipoteichoic acid molecule, including its D-ala side

branch (both cell wall components were isolated from the Gram-positive *B. subtilis*), following exposure to NAg⁹⁸. Therefore, it would be reasonable to deduce that the bacterium changes its expression of cellular machinery genes, in this case, the downregulation of the capsular polysaccharide genes, to prioritize the availability of precursors for rebuilding of the cell wall components.

The abundance level of capsule polysaccharide in the cell has been reported to influence bacteria's susceptibility to other antibacterial agents. Hanson and Neely, for example, observed an increased resistance of *Streptococcus iniae* to lysozyme and bacitracin when the bacterium capsule polysaccharide level was low²⁴⁴. Lysozyme is an enzyme that hydrolyses β -4-glycosidic bond between N-acetylmuramic acid and N-acetyl-glucosamine in peptidoglycan (part of the glycan strands) while bacitracin inhibits the dephosphorylation of UPP during peptidoglycan synthesis. However, the opposite effect (an increased susceptibility) was observed with ampicillin and methicillin²⁴⁴, a β -lactam antibiotic, whilst there was no effect with the glycopeptide antibiotic, vancomycin²⁴⁵. In the case of low UPPS level, Lee and Helmann demonstrated that this condition causes *B. subtilis* to be more susceptible to methicillin and bacitracin¹⁶⁸. In agreement to these previous studies, the NAg-r strain has demonstrated an increased resistance to lysostaphin, a glycylglycine endopeptidase enzyme that cleaves the pentaglycine bridge in *S. aureus* peptidoglycan, and an increased susceptibility in methicillin when compared to the WT strain (see Chapter 3), therefore suggesting changes in peptidoglycan structure or production in the NAg-r strain.

Earlier studies has synthesised and utilized fluorescent D-amino acid (FDAA) to label sites of nascent peptidoglycan synthesis in bacteria^{246,247}. To confirm the allocation of UDP-D-GlcNAc and lipid I to peptidoglycan synthesis, the aforementioned technique could be implemented to the NAg-r and the WT strain to subsequently quantify the peptidoglycan presence between the resistant and the susceptible strain or examine live bacterial growth. Additionally, the cell wall thickness of the NAg-r and the WT strain could be quantitatively compared under an electron microscope.

This findings herein reported the adaptation of the NAg-r strain upon the prolonged exposure to increasing NAg concentration (Chapter 3). Up to this stage, the NAg-r strain displayed significant alteration in the expression of various metabolisms genes in the absence of NAg, e.g. nucleotide, nitrogen metabolism, and cell envelope metabolism, when compared to the untreated WT strain. It was thought that these adaptation mechanisms are the consequence of a fitness cost to the NAg-r strain upon prior prolonged NAg exposure as well as the cell's effort to allocate precursors and

energy. However, due to time constraint, studies to validate these hypotheses have not been performed yet.

To gain a better understanding on the resistance mechanism, the study was advanced to investigation of the NAg-r strain responses in the presence of NAg. The detailed explanation on the biological interpretation of the transcriptomic data were presented in Section 6.4.5.

6.4.4.3. Physiological adaptations of the Ag⁺-resistant strain

The physiological traits of the Ag-r strain was investigated by examining the transcriptomic profile of the untreated Ag-r strain relative to the WT strain (**Figure S6.1**). Upon prolonged exposure to Ag⁺, the Ag-r strain was identified to have a ~3 fold MIC increase (from 1.5 µg/mL to ~5 µg/mL, see Chapter 3).

The transcriptomic analysis revealed common DEGs between the untreated Ag-r strain and the NAg-r strain, *e.g.* in the upregulation and downregulation of genes associated with nucleotide metabolism (purine and pyrimidine biosynthesis genes) and cell envelope metabolism (capsular polysaccharide biosynthesis genes) pathways, respectively (**Figure S6.1**). Detailed explanation on these pathways are presented in Section 6.4.4.2.

In addition to those metabolism, a distinct physiological adaptation of the Ag-r strain was seen within **cysteine metabolism and transporters** pathway. The study observed 2.5 – 10.3 log₂ fold change relative to the WT NT in 11 genes encoding the cysteine synthase (*cysK*) and cystathionine gamma-lyase (*metB*) enzyme, sulfonate uptake transporters (*tauB* and *tauC*), methionine uptake transporters (*metN*, *metP*, and *nlpA*), and L-cystine uptake transporters (*tcyABC*) (**Table S6.2**). In fact, more than half of these genes were identified to be among the highly upregulated genes (> 6.0 log₂ fold change relative to WT NT) (**Figure S6.1**). These enzymes catalyses the formation of L-cysteine from O-acetylserine or L-cystathionine and the transporters facilitate the uptake of some sulphur-based amino acids or molecules^{170,248}.

The amino acid cysteine is required as a precursor to important molecules such as ROS scavenger thioredoxin. Due to the presence of thiol (-SH) in its side chain, cysteine plays a role in the oxidation-reduction (redox) reaction that often occur in thioredoxin, enabling the scavenger to reduce cellular oxygen radicals⁶⁵. Cysteine is also a precursor for the synthesis of Fe-S cluster, as earlier described, is one of the major cellular targets of NAg. The cluster are present in many important proteins and enzymes such as aconitase and dehydratase for the latter^{200,204}. The herein observed genomic mutation in *cymR* gene that encodes a putative operon regulator (repressor)

protein CymR (Chapter 4), along with the detected increase expression of cysteine intake and synthesis, was likely the bacterium's adaptation mechanism in response to Ag⁺ interaction with cysteine-containing molecules^{65,80}.

The cellular cysteine level in the Ag-r strain can be measured with an amino acid analyser for a follow-up study, thus to be compared with that of the WT strain. Additionally, it may be interesting to investigate the protein expression of CymR in the Ag-r and the WT strain using targeted proteomic study.

In summary, the study herein showed that the Ag-r strain upregulated purine and pyrimidine biosynthesis genes while at the same time, downregulated capsular polysaccharide biosynthesis genes relative to the WT strain, similar to the expression profile of NAg-r strain. Not observed in the NAg-r strain, the Ag-r strain also upregulated cysteine metabolism and transporters genes, which could associate with detected single nucleotide mutation in the cysteine metabolism regulator *cymR* gene in the Ag-r strain (Chapter 4). All of these physiological adaptations are likely the bacterium's attempt to recover its fitness after prolonged exposure to Ag⁺, to allocate essential precursor and energy molecules for optimum bacterial growth. This is consistent with the observed comparable growth of the Ag-r strain when compared to the WT strain in the absence of Ag⁺ (**Figure 6.2**).

6.4.5. Resistance responses of the NAg- and Ag⁺-resistant strain in the presence of silver

To investigate the resistance responses, the NAg-r strain was exposed to NAg at a low NAg concentration (low-NAg, 1.5 µg Ag/mL) and high NAg concentration (high-NAg, 4.5 µg Ag/mL) for 30 min prior to RNA extraction and whole transcriptomic analysis. At the same time, the Ag-r strain was also exposed to low Ag⁺ concentration (low-Ag, 0.75 µg Ag/mL) and high Ag⁺ concentration (high-Ag, 4.5 µg Ag/mL) for 30 min.

6.4.5.1. The comparison between NAg- and Ag⁺-resistant strain

Firstly, the study compared the DEGs in NAg-r strain when exposed to high-NAg to those of in Ag-r strain when exposed to high-Ag. Note that the DEGs for each of the silver-resistant strain were first deduced from the comparison of the silver-exposed system to its respective non-treatment (NT) system. For example, for the NAg-r strain, the DEGs were deduced from NAg-r exposed to high-NAg relative to NAg-r NT.

The transcription data showed that, at the equivalent Ag exposure concentration (4.5 µg Ag/mL) the NAg-r strain was associated with a larger set of DEGs in comparison to the Ag-r strain (**Figure 6.11**). This could be attributed to the additional toxicity

mechanisms that may originate from the undissolved NAg particle. In agreement, a previous transcriptomic study reported greater number of DEGs upon exposure of *E. coli* (susceptible to NAg and Ag⁺) to NAg, when compared to Ag⁺⁷².

Of these responses, there were 315 common DEGs shared between the NAg-r high-NAg and Ag-r high-Ag (**Figure 6.11**), which represent approximately 50% of the NAg-r strain's total DEG and over 75% of the Ag-r strain's total DEGs. Among these DEGs, iron uptake and oxidative stress genes were upregulated and pyrimidine biosynthesis as well as ribosomal protein synthesis genes were downregulated. The results herein, again, suggest that despite their unique toxicity mechanisms, NAg and Ag⁺ share common source of toxicity, most likely the soluble ionic silver, and therefore, the defence mechanisms against it.

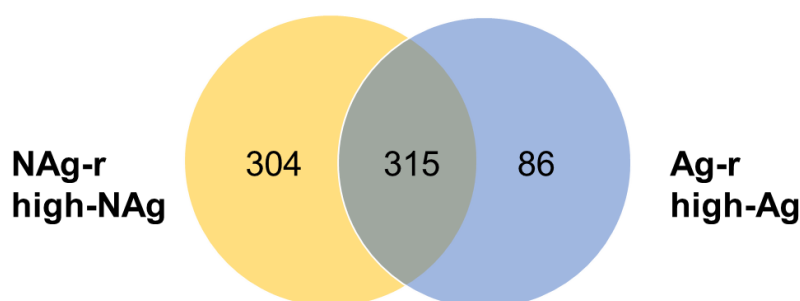


Figure 6.11. Venn diagram representing the number of significantly expressed genes in the NAg-r high-NAg and Ag-r high-Ag samples. NAg-r: NAg-resistant strain, Ag-r: Ag⁺-resistant strain; high-NAg: high NAg concentration (4.5 µg Ag/mL), high-Ag: high Ag⁺ concentration (4.5 µg Ag/mL).

There were 304 unique responses found for the NAg-r strain and 86 DEGs for the Ag-r strain (**Figure 6.11**). For the NAg-r strain, these DEGs were associated with cysteine metabolism and transporters, denitrification, ATP synthesis, Fe-S assembly, and DNA repair genes. In contrast, the highlight for the Ag-r unique DEGs was in the downregulation of the purine synthesis genes. These differences, apart from the likely effects of the additional solid particulate toxicity contribution imposed on the NAg-r strain, could also result from the unique genomic mutations acquired by the NAg-r and Ag-r strains following their respective prolonged silver exposure (Chapter 4).

This study did not detect DEGs for the purine biosynthesis genes (PurR-regulated genes: *purA*, *purEKCSQFLMNHD*, and *purB*) in the NAg-r strain when exposed to high NAg (relative to the NAg-r NT, see **Table 6.2** for the pair-wise comparison and **Table S6.5** for the DEGs). This finding indeed further validates our hypothesis that the *purR* gene mutation, which was detected in the NAg-r strain, is a factor for the upregulation of the purine operon seen with the physiological adaptation of NAg-r, the latter deduced from the transcript data of NAg-r NT vs the WT NT (Section 6.4.4.2).

The transcription data herein also support the identified mutation in cysteine metabolism regulator (*cymR*) in the Ag-r strain (Chapter 4 and Section 6.4.4.3). No DEGs were detected for the cysteine synthesis and transporter genes (*cysK*, *metB*, *tauBC*, *metN*, *metP*, *nlpA*, *tcyABC* and *tcyP*) that are regulated by CymR in the Ag-r strain when exposed to high Ag⁺ concentration (relative to Ag-r NT, see **Table 6.3** for the pair-wise comparison and **Table S6.6** for the DEGs). Interestingly, however, these DEGs were detected in the NAg-r strain when exposed to high NAg concentration (2.2 – 4.4 log₂ fold upregulation, relative to NAg-r NT, **Table S6.5**). Recalling earlier findings with the genomic studies (Chapter 4), this study did not detect any mutation with the *cymR* gene in the NAg-r strain.

At this stage, this study has indicated, to a certain extent, a shared, common defence mechanisms of *S. aureus* to NAg and Ag⁺. Considering the proportion of the shared DEGs (**Figure 6.11**) from the similar transcriptomic profiles (**Figure 6.14** and **Figure S6.2**) for NAg-r and Ag-r strain, it is reasonable to suggest that the bacterium's resistance responses to Ag⁺ do not differ significantly from those of the NAg. Therefore, in the next section, the main focus will be on the biological interpretation of cellular pathways identified in the resistance responses to NAg.

6.4.5.2. Resistance responses of the NAg-resistant *S. aureus* strains in the presence of NAg

The study determined the DEGs (log₂ fold change > 2.0 and p-adjusted value < 0.05) by comparing gene expressions in the NAg-r strain when exposed to the low and high NAg concentration, relative to the the WT when exposed at low NAg concentration (**Figure 6.12**).

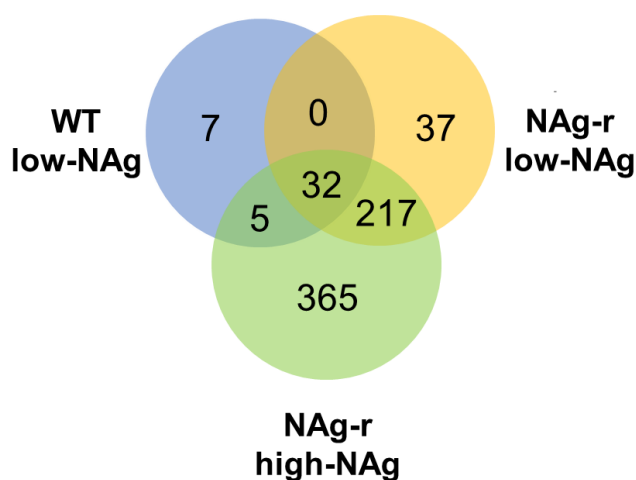


Figure 6.12. Venn diagram representing the number of significantly expressed genes from different strains and NAg exposure. WT: wild-type strain, NAg-r: NAg-resistant strain; low-NAg: low NAg concentration, high-NAg: high NAg concentration.

Shared between the WT low-NAg, NAg-r low-NAg and NAg-r high-NAg were 32 DEGs, which include the upregulation of siderophore biosynthesis genes (*sbnA-H*), oxidative stress-related genes (*ahpFC*), DNA repair genes (*uvrB*), as well as metal transporters (*copA* and *copZ*) (see **Figure 6.13** and **Table S6.3**). These set of genes are considered as the 'general toxicity responses' in *S. aureus* regardless of the bacterium's resistance level. The detailed explanation on these genes was presented in Chapter 5 and will not be further analysed in this chapter.

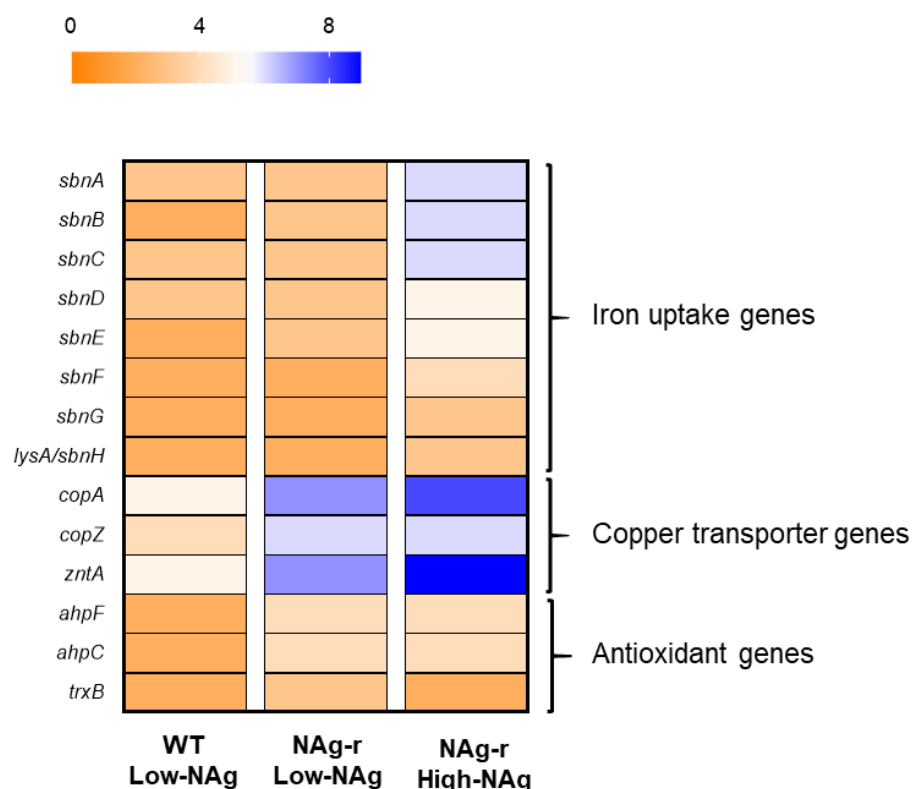


Figure 6.13. Heat map representing the level of expression of the common DEGs observed when comparing the gene expression in the wild-type strain (WT) when exposed to low NAg concentration (low-NAg), the NAg-resistant (NAg-r) strain when exposed to low-NAg and NAg-r strain when exposed high NAg concentration (high-NAg).

The next focus is the biological interpretation from 619 DEGs in the NAg-r low-NAg and high-NAg (37 + 217 + 365 in **Figure 6.12**) and of these, 217 transcripts (~85% of total DEGs in the NAg-r low-NAg) were shared between the two NAg exposures. An interesting observation for these shared DEGs (217 in **Figure 6.12**), the changes in the gene expression levels were more pronounced in the high NAg exposure system (**Figure 6.13**). For example, if one gene was upregulated in the low exposure condition, then the fold-change of the upregulation was higher in the high exposure condition. Similarly, if one gene was downregulated in the low exposure condition, then the fold-change of the downregulation was more significant in the high exposure condition (**Table S6.5**). This was most likely due to the high NAg exposure exhibiting higher toxicity levels, and therefore, the more pronounced defence mechanisms by the

bacterium. It is also apparent that the high exposure condition was associated with more DEGs, possibly affecting more cellular pathways for protection effects, indicated by the additional 365 DEGs (**Figure 6.12**) seen in the NAg-r strain high NAg.

Herein, we begin the discussion on the resistance responses of the NAg-r strain when in the presence of NAg starts by first describing the cellular pathways affected at low NAg exposure and subsequently, at high NAg exposure. The functional pathway mapping of the transcriptomic data and gene annotation was carried out using BioCyc Omics Dashboard¹⁹¹, NCBI database¹⁹³, and manually using published literatures.

At the low NAg exposure, the 254 DEGs (217 + 37 in **Figure 6.12**) observed (excluding the previously described 32 shared DEGs for WT low-NAg, NAg-r low-NAg, and NAg-r high-NAg) were predominantly associated with the translation pathway, comprising 21% from the 254 DEGs, **Figure 6.14A**, and these were mostly downregulated ribosomal protein genes (**Figure 6.14B**). The second most affected pathway was the transporter pathway, comprising 14% of the total DEGs (**Figure 6.14A**). The transcriptomic data interestingly showed the most significant downregulation with the nitrogen metabolism pathway ($< -5.0 \log_2$ fold change relative to the NAg-r NT, **Table S6.5**), for example, the nitrate transporter gene (*narT*) was downregulated by $-6.5 \log_2$ fold change relative to NAg-r NT (see **Figure 6.14C** and **Table S6.5**). Many genes in other pathways, such as nucleotide metabolism and virulence factors, were also downregulated (96% and 76% from the total DEGs in the respective pathway, respectively, **Figure 6.14B**).

In the case of NAg-r high-NAg, there were 365 DEGs (**Figure 6.12**) that solely detected in this exposure condition. The largest proportion (17% of 365 DEGs) was associated with the transporter genes, and the majority of these genes were downregulated (**Figure 6.15A** and **B**). Predominantly downregulated genes were observed in nucleotide metabolism pathway, approximately 93% from the total DEGs in this pathway. On the other hand, the findings herein showed upregulation in cysteine metabolism and transporter pathway and interestingly, all genes associated in this pathway were upregulated (**Figure 6.15B**). This study also observed upregulation of genes associated with the oxidative stress pathway, particularly in iron-sulphur (Fe-S) assembly genes (see **Table S6.5**).

In the following, the potential roles of the observed changes in the expression levels of many cellular pathways on the bacterium's defence mechanism when in the presence of NAg at low and high concentration, in other words, how the bacterium changes its metabolic state to confer resistance responses when in the presence of NAg.

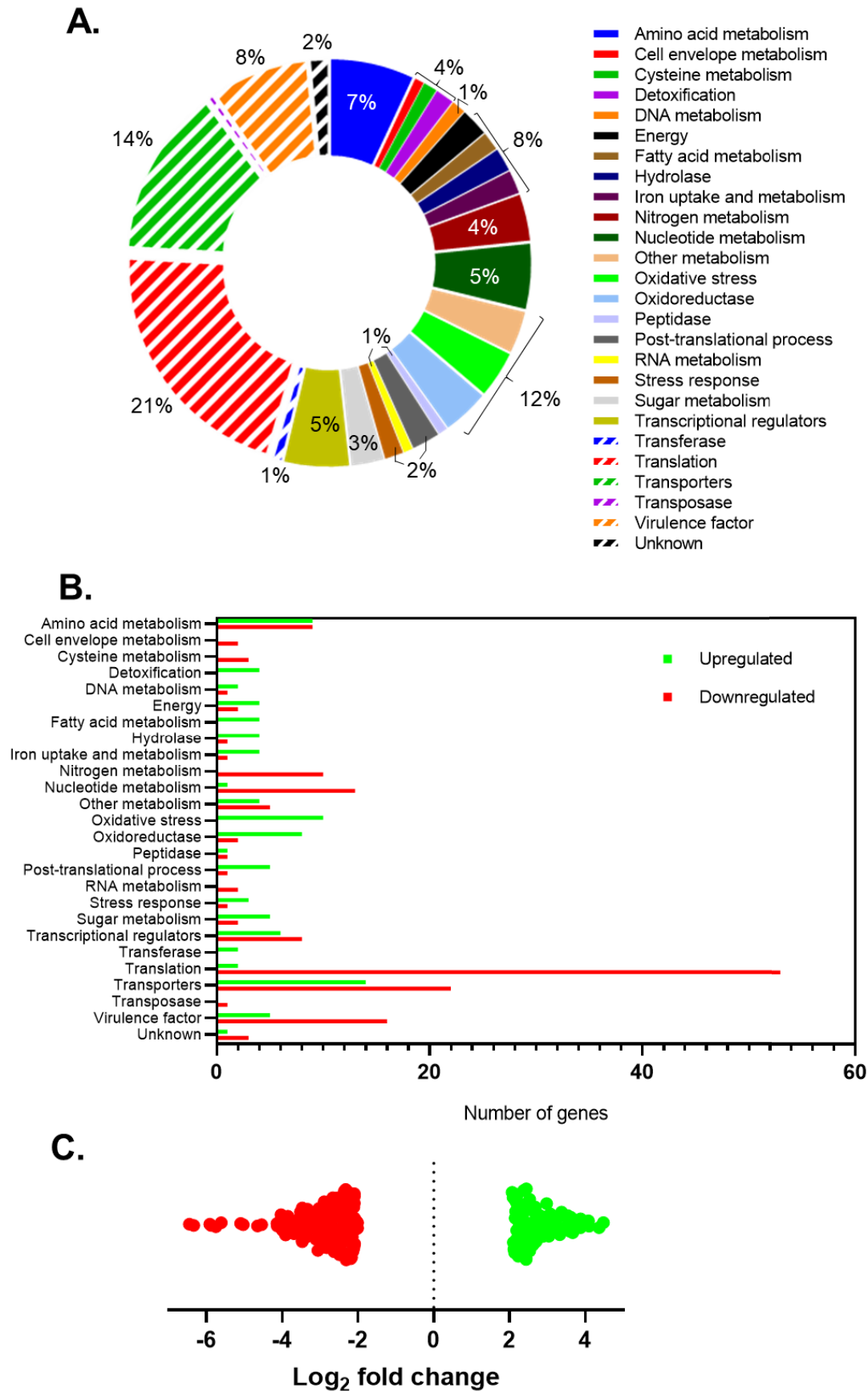


Figure 6.14. The percentage proportion of functional pathways affected (A), the number of upregulated and downregulated DEGs (B), and the distribution of log2 fold change (C) in the NAg-resistant strain exposed to low NAg concentration (1.5 µg Ag/mL) when compared to the untreated NAg-resistant strain. The transcriptomic profiles was excluded from the general toxicity responses (32 in **Figure 6.12**). Total genes shown in this figure are 254 (217 + 37 genes in **Figure 6.12**). The functional pathway mapping and gene annotation was conducted *via* BioCyc Omics Dashboard, NCBI database, and manually using published literatures.

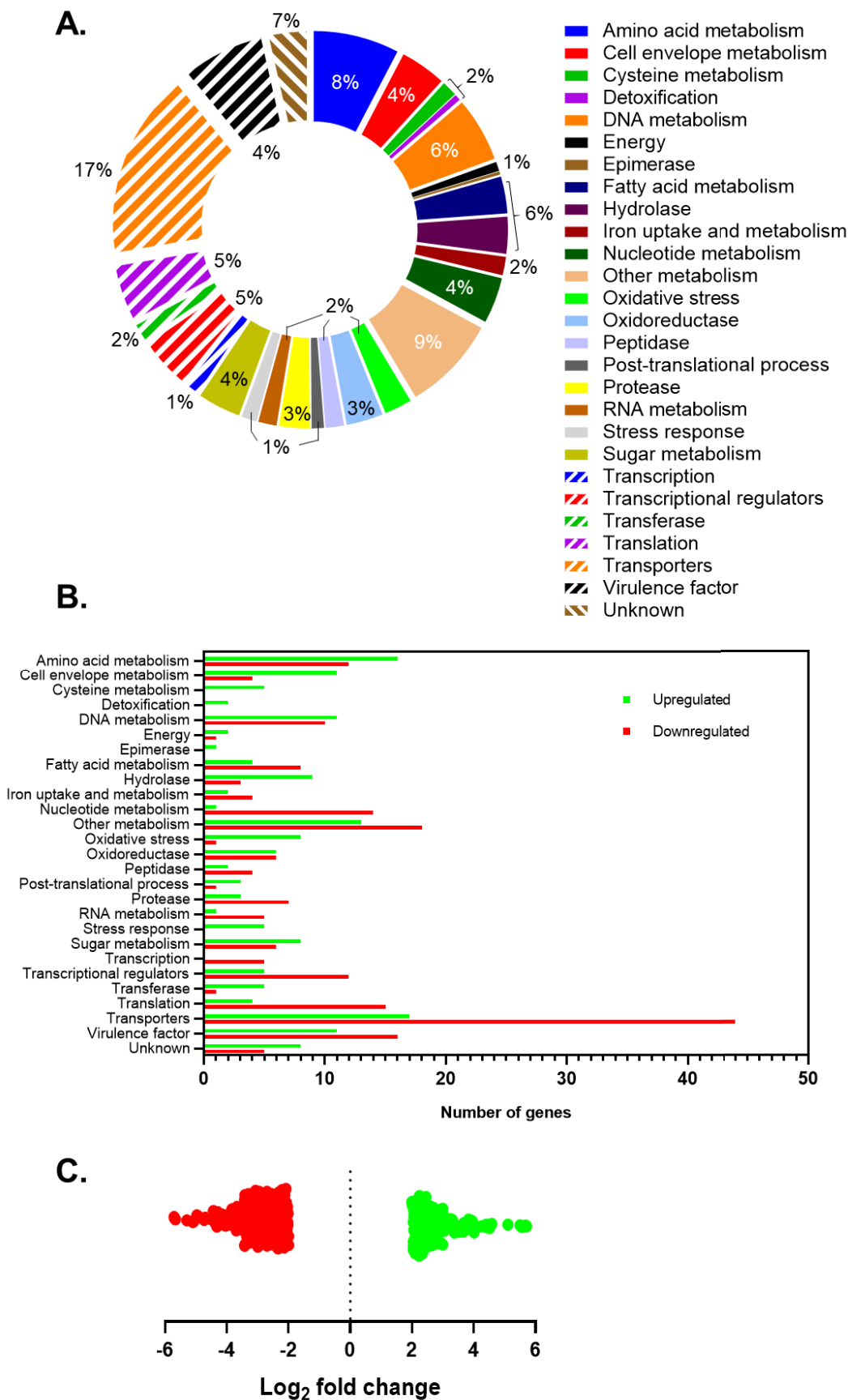


Figure 6.15. The percentage proportion of functional pathways affected (A), the number of upregulated and downregulated DEGs (B), and the distribution of log₂ fold change (C) in the

NAg-resistant strain exposed to high NAg concentration (4.5 µg Ag/mL) when compared to the untreated NAg-resistant strain. The transcriptomic profile was excluded from the general toxicity responses (32 in **Figure 6.12**) and the genes shared with NAg-r low-NAg (217 in **Figure 6.12**). Total genes shown in this figure are 365 (see **Figure 6.12**). The functional pathway mapping and gene annotation was conducted *via* BioCyc Omics Dashboard, NCBI database, and manually using published literatures.

Firstly, for the nucleotide metabolism pathway, the study detected DEGs genes associated with **the downstream of purine metabolism** (after IMP synthesis, see **Figure 6.7**). For example, the five genes *atpCDGAH* that encode 5 subunits in ATP synthase F₁ component (the subunit epsilon, beta, gamma, alpha, and delta, respectively) were downregulated by 2.1 – 2.2 log₂ fold change (**Table S6.5**) in the NAg-r low-NAg relative to the NAg-r NT. Interestingly, when the NAg-r strain was exposed to high NAg concentration, this study observed downregulation of more ATP synthase genes, this time *atpFEB* that encode ATP synthase F₀ component subunit b, c, and a, respectively, by 2.1 – 4.3 log₂ fold change (relative to NAg-r NT, **Figure 6.16** and **Table S6.5**), and therefore, downregulation of the entire synthesis systems for ATP synthase. Consisting of catalytic core (F₁) and a membrane proton channel (F₀), ATP synthase is an enzyme catalysing the conversion of ADP to ATP, and vice versa, facilitated by the proton pump across bacterial plasma membrane²⁴⁹. Although related with purine metabolism, it was suggested that, in *S. aureus*, *atpCDGAH* and *atpFEB* are not regulated by PurR, but by Rex, a redox-responsive repressor protein, instead^{250,251}. This study also observed the downregulation of **pyrimidine synthesis genes** in both NAg-r low-NAg and NAg-r high-NAg, which interestingly, were found on similar genes and comparable extent of 2.3 – 5.8 log₂ fold (relative to NAg-r NT, **Figure 6.16** and **Table S6.5**). The downregulated genes *uraA-pyrBC-carAB-pyrF* and *pyrD* that encode enzymes involved in the UMP synthesis steps as well as *pyrG* that encodes CTP synthase enzyme, the latter catalyses the conversion of UTP and L-glutamine to CTP (see **Figure 6.8**).

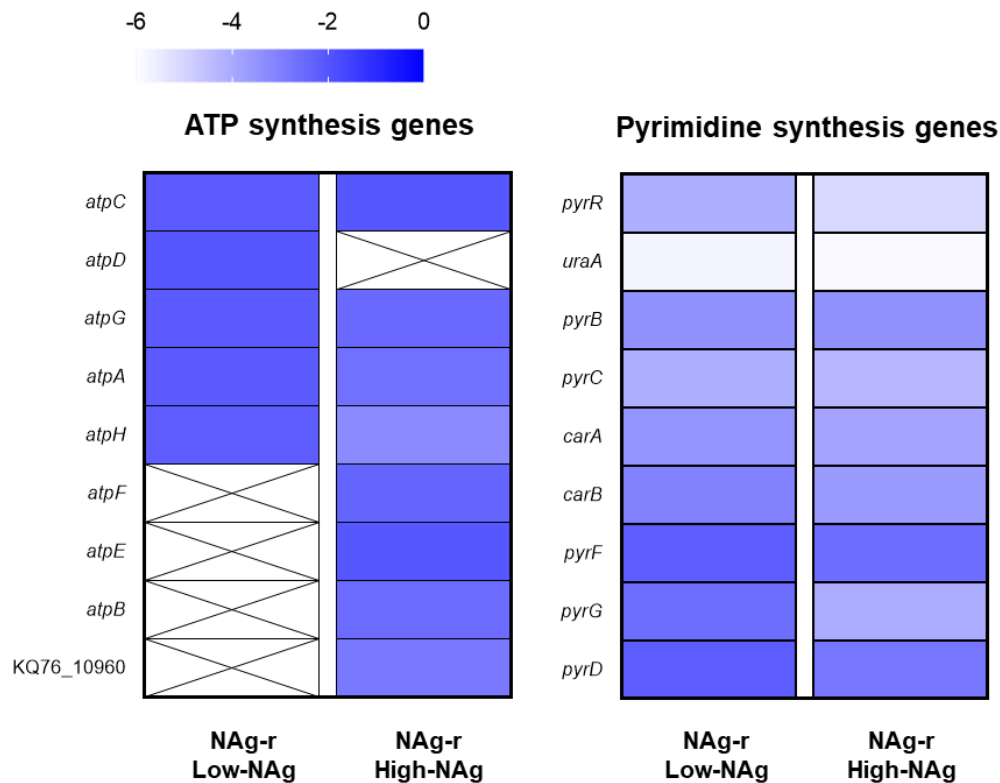


Figure 6.16. Heat map representing the level of expression of ATP synthesis and pyrimidine synthesis genes observed in the NAg-resistant (NAg-r) strain when exposed to low NAg concentration (low-NAg) and NAg-r strain when exposed to high NAg concentration (high-NAg). X indicates that genes were not significantly expressed in the specified exposure condition.

The transcriptomic data also showed the downregulation of about 50 ribosomal protein genes involved in the **translation** process. Exposing the NAg-r strain to low NAg concentration resulted in 2.1 – 4.0 log₂ fold downregulation of the gene expression (relative to NAg-r NT, see **Table S6.5**). Interestingly, at high NAg exposure, a more significant downregulation were observed on the same genes, this time by 2.8 – 6.4 log₂ fold (**Table S6.5**). Downregulation was also seen in genes encoding tRNA synthetase, translation initiation and elongation factor, and peptide chain release, by 2.0 – 5.7 log₂ fold in both the NAg-r low-NAg and high-NAg (**Table S6.5**).

This study identified most significant downregulation on **nitrogen metabolism pathway** (**Table S6.5**). These genes, *narGHIJ* that encode nitrate reductase and *nirBD* that encode nitrite reductase (the two enzymes convert nitrate to nitrite, then nitrite to ammonia, respectively) as well as *narT* that encode nitrate transporter, were downregulated by 5.0 – 6.3 log₂ fold, when compared to the untreated NAg-r strain (**Table S6.5** and **Figure 6.9**). Also seen was the downregulation of the oxygen-sensing regulator (*nreABC*) that encode the protein regulator for the nitrate and nitrite reductase by 2.5 – 2.8 log₂ fold²³⁴. Other studies have reported downregulation of these nitrogen metabolism genes in *P. aeruginosa*, both in its planktonic or biofilm forms upon exposure to sub-inhibitory NAg concentrations^{184,252}.

As metabolic routes are known to be very complex and the routes may be inter correlated between one to another, the changes in the metabolic states of the NAg-r strain when exposed to NAg may have been direct or indirect responses to NAg. At this stage, it is not clear as with the roles of the downregulation of the aforementioned metabolic genes in the bacterium's defence mechanisms. It is possible that the downregulation is affecting bacterium's metabolism with an intended goal to protect the cell or to maintain growth in the presence of silver. This would probably be the case for a comparable growth profile between the NAg-r strain in the presence or absence of NAg (see **Figure 6.1** inset). Studies have also shown that downregulation of metabolic pathways, such as purine and pyrimidine synthesis, as well as the depletion of ATP level are associated with the emergence of *S. aureus* sub-population that is resilient to stress^{253–255}.

Future studies can consider conducting a global metabolomics study to investigate the metabolic pathways affected by NAg exposure. Additionally, ribosome profiling method could be considered to identify and quantify protein affected during NAg exposure.

Apart from the downregulated cellular pathways, the study also observed considerable number of upregulated genes, in particular those that associate with oxidative stress responses, in the NAg-r strain when exposed to low and high NAg (relative to the NAg-r NT). This included the ROS scavenger, iron-sulphur (Fe-S) assembly, and cysteine synthesis and uptake genes. These oxidative stress-related cellular mechanisms did not seem to be affected in the physiological adaptation responses of the bacterium (due to the prolonged NAg exposure) (described in Section 6.4.4.2). Therefore, it was hypothesised that these **oxidative stress pathways have major roles in the mechanisms of defence to NAg toxicity**, that is, when in the presence of NAg.

First, the study detected an upregulation of 3.5 and 3.7 log₂ fold in genes that encode the **ROS scavenger enzymes** superoxide dismutase (*sodA*) and catalase (*katA*), respectively (**Table S6.5**) in NAg-r strain when exposed to low NAg concentration (relative to NAg-r NT). Superoxide dismutase catalyzes dismutation reaction of superoxide radical ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), while catalase subsequently converts H_2O_2 to water and oxygen^{256,257}. The transcript level of other ROS scavenger moieties, the thioredoxin- and thioredoxin reductase-related genes, also increased by 2.8 – 3.6 log₂ fold in the NAg-r strain at both low and high NAg exposure (relative to NAg-r NT, **Table S6.5**). Thioredoxin and thioredoxin reductase are a two-component enzyme present in Gram-positive bacteria, capable of scavenging reactive oxygen species (ROS). The enzyme converts the harmful radicals to water *via* oxidation-reduction (redox) reactions that involve the enzyme's disulphide bond (converted to

thiol in the reaction)^{214,215}. Interestingly, for the NAg-r high-NAg system, the transcript data showed upregulation of additional thioredoxin-related genes by 2.1 – 4.1 log2 fold, **Figure 6.17** and **Table S6.5**). This upregulation of additional oxidative stress-associated genes was most likely the bacterium's 'second tier' complementary defence mechanism in response to the higher NAg toxicity.

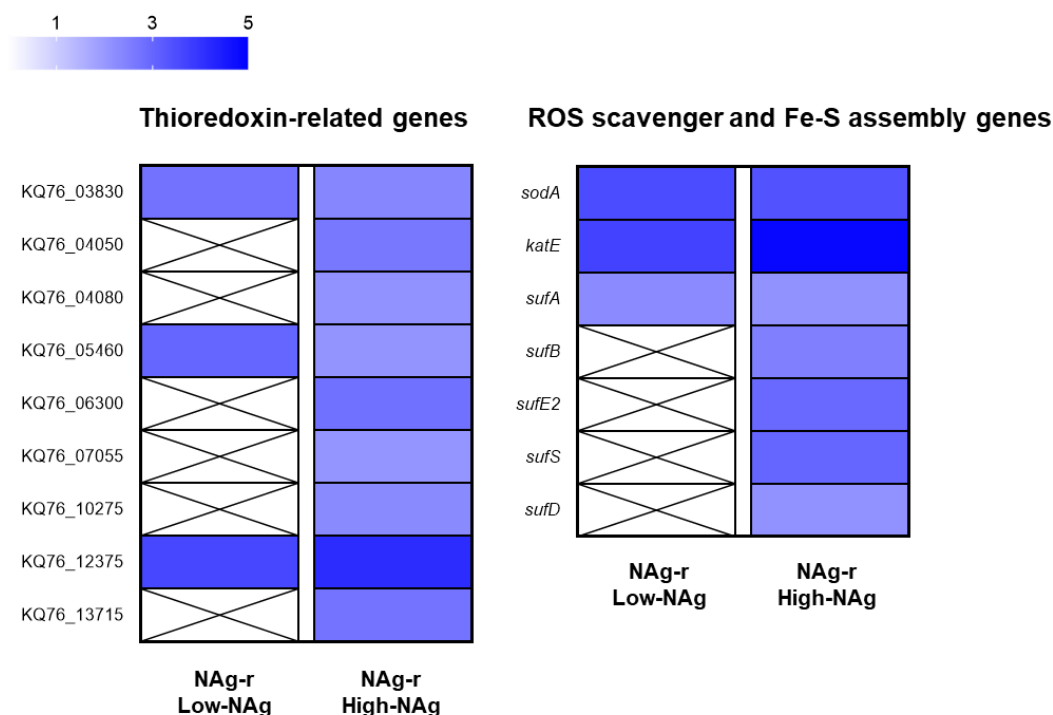


Figure 6.17. Heat map representing the level of expression of thioredoxin-related, ROS scavenger, and iron-sulphur (Fe-S) assembly genes observed in the NAg-resistant (NAg-r) strain when exposed to low NAg concentration (low-NAg) and high NAg concentration (high-NAg). X indicates that the genes were not significantly expressed in the specified exposure condition.

Next, the study observed upregulation of one of the protein in **iron-sulphur (Fe-S) assembly** encoded by *sufA* in the NAg-r at low NAg exposure (**Table S6.5**). Interestingly, upon exposure to high NAg concentration, the NAg-r strain not only upregulated the expression of *sufA*, but also additional genes in the *suf* system (*sufDSEB*) that complement *sufA* (**Figure 6.17** and **Table S6.5**). Suf system has been known as the only molecular system that synthesizes Fe-S cluster in *S. aureus* and other Gram-positive bacteria^{200,258,259}. SufS is a cysteine desulphurase enzyme that removes sulphur from the amino acid L-cysteine to obtain an intermediate, S⁰, a sulphur atom, and in the process generating the by-product, L-alanine. S⁰ is then transferred to the Fe-S molecular scaffold SufBCD possibly by SufE. Iron (Fe²⁺) is reported to be added into the Fe-S cluster assembly process by SufD. Following the cluster synthesis, SufA transports the cluster to an apoprotein or to another target structure^{200,258}.

Detailed explanation on NAg attack on Fe-S cluster has been presented in Chapter 5 Section 5.4.4. Briefly, studies have reported that Fe-S cluster is one of the major targets of NAg in bacteria⁷⁵. The leached silver ion from the nanoparticle, due to its high affinity to the cysteine's thiol (-SH) group in the Fe-S cluster, has been indicated to displace the iron in the cluster, releasing the Fenton-active Fe²⁺ into the cytoplasm^{79,80}. Additionally, the Fe-S cluster is prone to the NAg-induced generation of cellular oxygen radical, in particular the superoxide radical, that has been reported to disrupt the cluster, which again, releases Fe²⁺ into the cytoplasm⁷⁵. Iron-sulfur clusters are present in many proteins. In *S. aureus*, these clusters can be found for example, in the energy producing tricarboxylic acid (TCA) cycle succinate dehydrogenase and aconitase enzymes^{200,258}. The targeting of the clusters by NAg could inhibit the enzymes activity.

The assembly of the Fe-S cluster through the Suf system requires soluble iron and cysteine as substrates^{200,258}. Indeed, as earlier described, this study also detected an upregulation of staphyloferrin B biosynthesis genes that involve in **iron acquisition** (in the NAg-r strain in relative to the wild-type strain, see Chapter 5 and explanation in **Figure 6.13**). It is thought that staphyloferrin B form soluble complexes with the insoluble iron (Fe³⁺), the latter generated as a result of NAg targeting on the Fe-S cluster (the released Fe²⁺ from the cluster is oxidized to Fe³⁺ in the Fenton reaction), to 'feed' the Fe³⁺ as Fe³⁺-staphyloferrin B soluble complexes into the Suf system for the assembly of the Fe-S cluster (see **Figure 6.18**).

This study also observed the upregulation of genes in the **cysteine metabolism** pathway. These genes encode transporters and enzymes that work together to generate cysteine from a number of substrates. When exposed to high NAg concentration, the NAg-r strain upregulated L-cysteine and homocysteine synthesis and metabolism genes (2.4 – 4.4 log2 fold change, relative to the NAg-r NT) encoding the homoserine O-acetyltransferase (*met2*), cysteine synthase (*cysK*), serine acetyltransferase (*cysE*), cystathionine beta-lyase (*metC*) and cystathionine gamma-lyase (*metC*). In addition, cysteine (*tcyABC* and *tcyP*) and methionine uptake genes (*metN*, *metP*, and *nlpA*) were also upregulated by 2.1 – 5.1 log2 fold (**Table S6.5**).

In addition to the Fe-S cluster, cysteine, as earlier mentioned, also forms the active site of the thioredoxin^{31,65,74}. The observed upregulation of the cysteine metabolism genes is therefore consistent with the earlier described upregulation of thioredoxin-related genes in the NAg-r strain upon exposure to NAg (**Figure 6.17**). A previous study has reported upregulation of genes associated with the Fe-S assembly, iron uptake, as well as cysteine biosynthesis in *E. coli* upon exposure to NAg⁷². Other study observed upregulation of ROS scavenger genes in *P. aeruginosa* upon exposure to NAg^{64,184}.

These studies, although being carried out on NAg susceptible strains, are consistent with the defensive responses observed in our study.

In summary, herein the study detected the upregulation of ROS scavenger, Fe-S cluster assembly, iron acquisition, as well as cysteine metabolism and uptake genes in the NAg-resistant *S. aureus*, most likely as defence mechanisms of the bacterium when in the presence of the nanoparticle. This is indeed consistent with the generation of cellular oxygen radicals as one of the major toxicity paradigms of NAg. Future work can compare the cellular oxidative stress in the NAg-r strain with the wild-type strain during exposure to NAg. Measurements can be carried out using the electron paramagnetic resonance (EPR) spectroscopy with cyclic hydroxylamine spin probing technique. This technique has been shown by Gunawan *et al.* to be capable of identifying cellular ROS in bacteria⁹⁸. Additionally, the deletion of the *suf* genes and/or ROS scavenger genes in the NAg-r strain could be performed to elucidate their roles in mediating the resistance characteristics.

Next, the **DNA repair** mechanism is also thought to be one of the defence responses exhibited by the NAg-r strain when exposed to NAg. It has been known that bacteria switch on their SOS responses in the form of DNA repair mechanism when encountering endogenous or exogenous stress that disrupts their genome integrity *e.g.* starvation, antibiotic, or oxidative stress²⁶⁰. This hypothesis is supported by the herein observed upregulation of the SOS response regulators: *recA* and *lexA* genes by 2.2 and 2.3 log₂ fold, respectively and the LexA-regulated SOS genes: excinuclease subunit ABC (*uvrABC*), DNA polymerase IV (*dinP/dinB*), and DNA polymerase V (*umuC*) by 2.6 – 5.7 log₂ fold in the NAg-r high NAg (relative to the NAg-r NT, see **Table S6.5**). No changes were observed in the expression levels of these DNA repair genes, except for the *dinP/dinB*, in the NAg-r low NAg (relative to the NAg-r NT), which suggest less DNA damages on the latter strain.

In the absence of stress, LexA binds to the promoter of the SOS genes that facilitate DNA repair, repressing the transcription process. However, when the level of single-stranded DNAs (ssDNAs) increased, a sign of replication distress, the cells induce an initial SOS response by having RecA, a nucleoprotein, to form a protein-ssDNAs complex that subsequently induces LexA self-released from binding on the promoter of the SOS genes and activates the transcription of the SOS genes. Upon obtaining repaired DNAs, the newly synthesized LexA will re-bind to the promotor of the SOS genes^{260,261}. Therefore, it is not unusual to observe both RecA and LexA being synthesized at the same time²⁶⁰.

When LexA self-released from the promoter, SOS proteins encoded by *uvrABC* gene for excinuclease subunit ABC, *dinP/dinB* for DNA polymerase IV, and *umuC* for DNA polymerase V could thus be transcribed. The three-component excinuclease protein functions collectively to excise nucleotides at DNA lesions and leaving a gap for DNA polymerase I to fill in with the nucleotide^{261,262}. Similar to DNA polymerase I, DNA polymerase IV and V work as 'translesion' polymerases repairing DNA damages, however, often at lower accuracy^{260,263}. DNA polymerase IV and V are thought to serve as the last resort polymerases for DNA repair mechanism^{260,263}.

The increased expression of the SOS response/DNA repair genes have been previously reported, in *P. aeruginosa* when exposed to NAg^{184,252}, as well as, in *S. aureus* when exposed to the antibiotic ciprofloxacin²⁶⁴. Studies have indeed shown that NAg targets DNA, including in *S. aureus*, most likely due to its affinity to the phosphorus and sulphur moieties in bacterial DNA^{55,67}. Moreover, studies have also deduced that oxygen radicals generated in the presence of NAg (or those that are naturally present in cells as by-products of aerobic metabolism) can disrupt DNA bases²⁶⁵.

Apart from the qPCR analysis to confirm the levels of expression (mRNA transcripts) of the SOS/DNA repair genes, future work can also include studies to confirm the transcription of the genes in the NAg-r strain when in the presence of NAg. For example, with the use of fluorescence-tagged *lexA* and *recA* promoters, we can quantify the fluorescence levels when in the presence of NAg relative to the no antimicrobial culture (for negative control) and UV-treated culture (UV induces cell stress, for positive control)²⁶⁶. Future work can also compare the presence of cellular oxygen radicals in the NAg-r strain and wild-type strain when in the presence of NAg.

Up to this stage, this study has shown that, in addition to the general toxicity responses (see explanation for **Figure 6.13** and Chapter 5), the NAg-r strain exhibited oxidative stress-associated defence by upregulating the expression of the ROS scavenger and Fe-S cluster assembly genes. In agreement with the latter, the study also observed the upregulation of cysteine metabolism genes to synthesize cysteine, suggesting cysteine demand to assemble the targeted Fe-S cluster. Ultimately, the upregulation of the bacterial SOS response/DNA repair genes was also thought to function as one of the defence mechanisms of the NAg-r strain. It was also noticed that exposure of the resistant strain to NAg affected many metabolic pathways in the bacterium (**Table S6.5**). Considering the known inter-correlation of metabolic pathways in bacteria, it was hypothesised that NAg-induced changes on one metabolic pathway can subsequently affect many more metabolic pathways. For example, the purine and pyrimidine

nucleotide metabolisms are closely related to histidine, alanine, and arginine amino acid metabolisms. Studies have also shown the correlation between purine synthesis or metal homeostasis regulators with virulence factors in *S. aureus* (virulence factor is a feature in pathogenic bacteria that facilitates infection in host and causes diseases)^{267,268}.

Future work can also include a whole metabolomic study (identification and quantification of cell metabolites, including small organic molecules like amino acids and nucleotides). This will not only validate the findings on the defence responses, both the physiological adaptation and resistance characteristics to NAg, but will also shed light on the inter-correlation of the identified cellular pathways. This study also suggest future work on the Sigma B (σ^B), a protein that regulates general stress response genes in *S. aureus*¹⁶⁹. Many studies, including by Bischoff *et al.*, have correlated the σ^B protein activity to the observed changes in the expression of capsular polysaccharide biosynthesis, amino acid metabolism (valine, isoleucine, and leucine), and toxin synthesis genes in *S. aureus*²⁶⁹, all of which were indeed, observed as well in this study. Future studies can elucidate the potential role of σ^B in the NAg defence mechanisms, for example, by deleting the *sigB* gene in the NAg-r strain (the gene encodes σ^B protein) and subsequently, comparing the NAg MIC for the NAg-r $\Delta sigB$ (deleted *sigB*) with that of the NAg-r strain.



Figure 6.18. A proposed model of NAg-resistant *S. aureus* responses to NAg toxicity based on the findings herein. NAg has been shown to disrupt respiratory chain and subsequently release reactive oxygen species (ROS). Along with the solid particulate and soluble silver ion, the toxic bioavailable silver may attack iron-sulphur (Fe-S) cluster, therefore releasing soluble Fe²⁺. In the presence of oxygen, Fe²⁺ undergoes Fenton reaction, being oxidized to insoluble Fe³⁺ and at the same time, generating ROS from H₂O₂. These episodes lead to soluble iron-lacking condition and additional ROS formation to further damage the cell. To survive, it was proposed that the cell may repair 1) iron-sulphur (Fe-S) cluster *via* Suf system. This system requires L-cysteine and soluble Fe²⁺ as precursors. Consequently, the cell may 2) synthesise cysteine and transport cysteine and methionine in. Briefly, cysteine can be synthesized *via* the acetylation of serine by serine acetyltransferase (CysE), generating O-acetyl-serine. Cysteine synthase (CysK) subsequently catalyses the reaction of O-acetyl-serine and hydrogen sulphide to form L-cysteine. Note that the hydrogen sulphide, in this case, be generated from the conversion of homoserine, catalysed by homoserine O-acetyltransferase (Met2). Another pathway of cysteine synthesis is through the two-step conversion of homocysteine with L-cystathione as intermediate. Cystathione beta-synthase (MetC) and cystathionine gamma-synthase (MetB) are the two enzymes catalysing these two-step reaction, yielding L-cysteine. In addition, due to the iron-lacking condition, it is likely that the cell 3) synthesise staphyloferrin B *via* *sbn* gene cluster to form a soluble Fe³⁺-staphyloferrin B to be used by the Suf system. At the same time, 4) the ROS scavenger, including thioredoxin, may attempt to deplete ROS directly and 5) copper transporter to pump Ag⁺ out. To note, detailed explanation on staphyloferrin B synthesis and copper transporter were presented in Chapter 5 whilst the remaining were presented in this chapter. Some figures were adapted from ^{75,196,197,207}. WTA: wall teichoic acid, LTA: lipoteichoic acid, Fe-S: Iron-sulphur cluster, OPS: O-phospho-L-serine, ACEGA: N-(1-amino-1-carboxy-2-ethyl)-glutamic acid, α-KG: α-ketoglutarate, L-Dap: L-2,3-diamopropionic acid.

6.5. Conclusion

To conclude, the whole transcriptomic study in this chapter has revealed an altered gene expression in the NAg- (NAg-r) and Ag⁺-resistant (Ag-r) *S. aureus* that provides an insight into the defence mechanisms against the toxicity of the respective silver compound.

The study found that the NAg-r strain exhibits a physiological adaptation responses to cope with the fitness cost as a result of the prolonged exposure to the nanoparticle. The strain increased the expression levels of specific nucleotide synthesis as well as decreased the expression levels of cell envelope genes to maintain fitness by redistributing precursors and energy molecules to important cellular processes for bacterial growth. Similar adaptation responses were identified in the Ag-r strain, in this case, due to the prior prolonged exposure to Ag⁺. It is important to note that these unique physiological adaptation responses manifest only when in the absence of the silver antimicrobials and are likely to stem from the observed gene mutations, in particular the purine nucleotide and cysteine metabolisms genes. The resistant strains exhibited a 'step-up' defence responses when in the presence of the silver antimicrobials.

The exposure of the NAg-r strain to low and high NAg concentrations saw increased in the expression levels of specific metabolic genes, including the iron acquisition and cysteine synthesis genes, most likely for the assembly of the iron-sulphur (Fe-S) cluster, one of the main NAg targets in bacteria. Not seen in its physiological adaptation, the NAg-r strain also increased the expression of the ROS scavenger protein synthesis genes, most likely to defend against the cellular oxidative stress, also a well-known toxicity paradigms of the nanoparticle. These gene upregulations were also seen with the Ag-r strain, in particular at high Ag⁺ concentration, however, interestingly, the NAg-r strain upregulated more oxidative stress-related genes than the Ag-r strain, possibly due to the higher levels of oxidative stress inflicted by the nanoparticle at equivalent silver contents, as have been reported in some studies.

Equally important, the findings also suggest that the potential mechanisms of defence to NAg toxicity herein observed with *S. aureus* are likely to be relevant to different species of bacteria, although there could be additional mechanisms involved with other bacteria. This was deduced from the common responses identified from the genetically-diverse NAg-susceptible bacteria, e.g. iron uptake and Fe-S cluster assembly, of which responses stem from different molecular system, yet, likely with similar functions. This study also acknowledges the toxicity of soluble silver (Ag⁺ ions)

that leached from NAg particle as well as the additional toxicity effect that potentially originate from the solid particulate.

As the first study to elucidate the defence mechanisms of the NAg-r and Ag-r strain to the toxicity of their respective silver compound, follow up studies are necessary to validate the proposed mechanisms of defence. With many promising leads that the transcriptomic study generated, confirming the hypotheses would advance the knowledge of NAg resistance in bacteria, subsequently mediate the risk of resistance occurrence and increase the effectiveness of long term NAg usage.

6.6. Supplementary Information

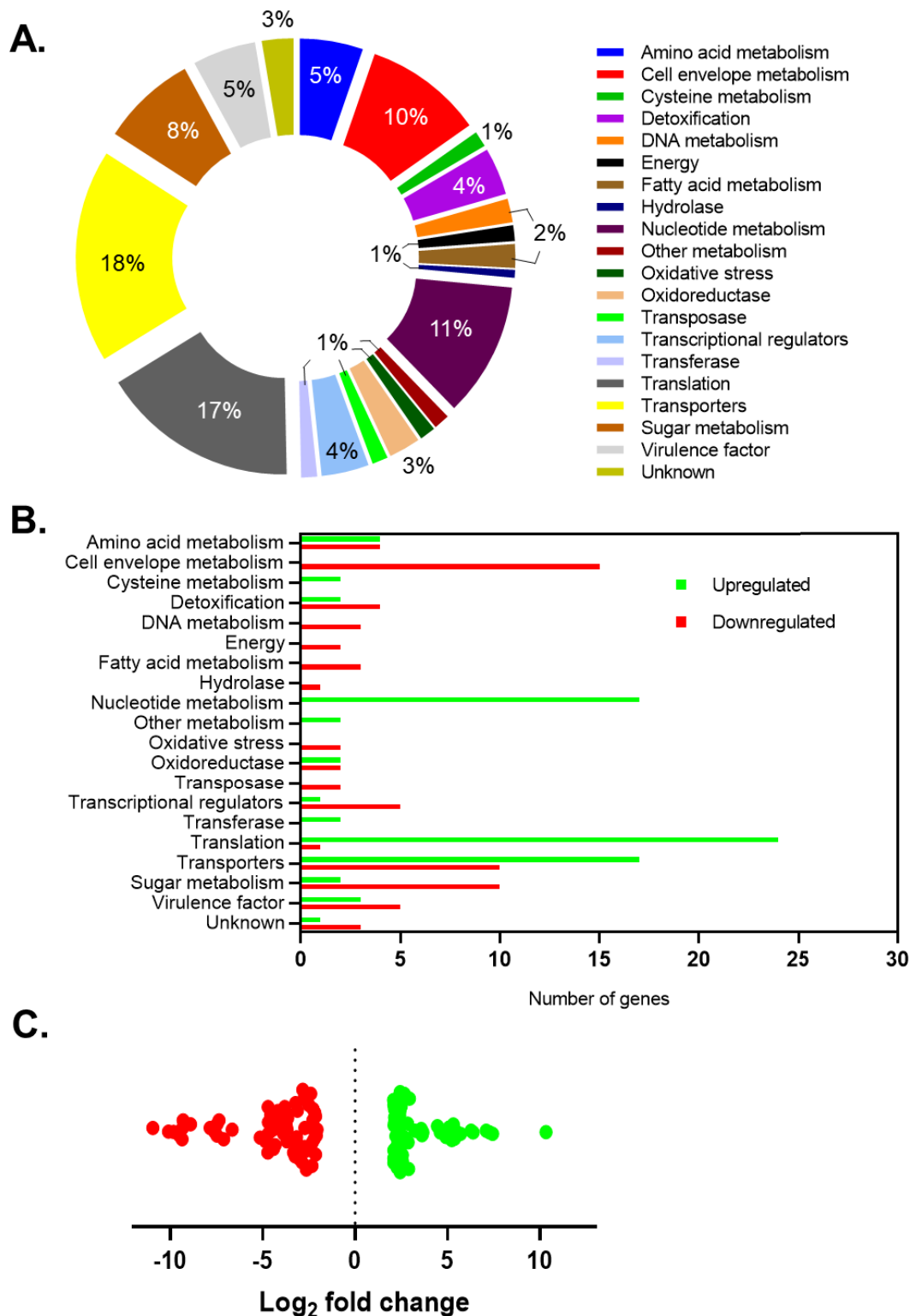


Figure S6.1. The proportion (A), number of upregulated and downregulated (B) differentially expressed genes (DEGs) based on the functional pathway, and dot plot (C) representing the distribution of DEGs based on their log₂ fold change in the untreated Ag⁺-resistant strain with respect to the WT strain of *S. aureus*. The functional pathways were classified from BioCyc Omics Dashboard, NCBI database, and manual interpretation.

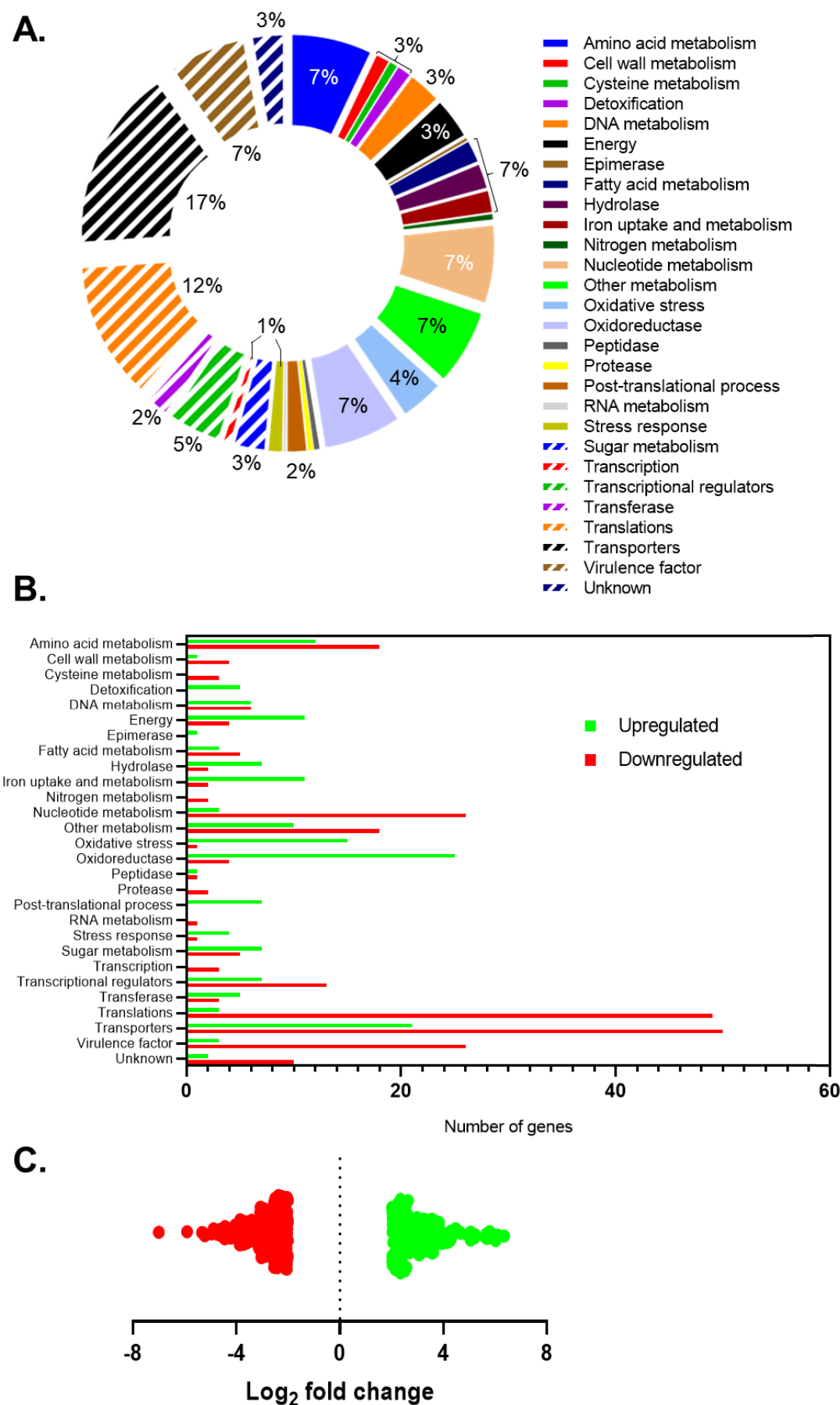


Figure S6.2. The proportion (A), number of upregulated and downregulated (B) differentially expressed genes (DEGs) based on the functional pathway, and dot plot (C) representing the distribution of DEGs based on their log₂ fold change in the Ag⁺-resistant strain exposed to extreme inhibitory Ag⁺ concentration (4.5 µg Ag/mL) when compared to the untreated Ag⁺-resistant strain. The functional pathways were classified from BioCyc Omics Dashboard, NCBI database, and manual interpretation.

Table S6.1. List and functional groups of differentially expressed genes in the untreated NAg-resistant *S. aureus* strains (NAg-r NT) when compared to the untreated wild-type strain (WT NT). Highlighted tables are the genes or pathways discussed in the main text.

Locus tag	Log2 fold change	Annotations	Gene
Amino acid metabolism			
<i>Isoleucine</i>			
KQ76_07125	2.34	serine/threonine dehydratase	<i>ilvA</i>
<i>L-Tryptophan</i>			
KQ76_06750	2.21	Anthranilate synthase component I	<i>trpE</i>
<i>L-alanine</i>			
KQ76_07130	2.13	Alanine dehydrogenase	<i>ald</i>
<i>L-glutamine</i>			
KQ76_05765	3.47	Carbamoyl-phosphate synthase small chain	<i>carA</i>
KQ76_05770	3.00	Carbamoyl-phosphate synthase large chain	<i>carB</i>
<i>L-histidine</i>			
KQ76_12135	2.25	Imidazolonepropionase	<i>hutI</i>
KQ76_12140	2.13	Urocanate hydratase	<i>hutU</i>
<i>L-serine</i>			
KQ76_07125	2.34	serine/threonine dehydratase	<i>ilvA</i>
Cell envelope metabolism			
<i>Teichoic acid biosynthesis</i>			
KQ76_00565	-2.10	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
<i>Capsular polysaccharide biosynthesis</i>			
KQ76_00490	-2.18	capsular polysaccharide biosynthesis protein	<i>cap5A</i>

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KQ76_00495	-2.31	Capsular polysaccharide biosynthesis protein	<i>cap5B</i>
KQ76_00500	-2.32	Capsular polysaccharide biosynthesis protein	<i>cap5C</i>
KQ76_00505	-2.41	Polysaccharide biosynthesis protein	<i>cap5D</i>
KQ76_00510	-2.60	Capsular polysaccharide biosynthesis protein	<i>cap5E</i>
KQ76_00515	-2.75	Capsular polysaccharide biosynthesis enzyme	<i>cap5F</i>
KQ76_00520	-2.72	UDP-N-acetylglucosamine 2-epimerase	<i>cap5G</i>
KQ76_00535	-2.79	Capsular polysaccharide biosynthesis protein	<i>cap5J</i>
KQ76_00540	-2.96	Capsular polysaccharide biosynthesis protein	<i>cap5K</i>
KQ76_00545	-2.64	glycosyltransferase	<i>cap5L</i>
KQ76_00550	-2.45	capsular polysaccharide biosynthesis protein	<i>cap5M</i>
KQ76_00555	-2.65	Capsular polysaccharide biosynthesis protein	<i>cap5N</i>
KQ76_00560	-2.36	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>cap5O</i>
KQ76_00565	-2.10	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
Nitrogen metabolism			
KQ76_12490	2.82	Respiratory nitrate reductase gamma chain	<i>narI</i>
KQ76_12495	5.31	Respiratory nitrate reductase delta chain	<i>narJ</i>
KQ76_12500	4.86	Respiratory nitrate reductase beta chain	<i>narH / narY</i>
KQ76_12505	3.11	Respiratory nitrate reductase alpha chain	<i>narG</i>
KQ76_12520	2.86	Nitrite reductase [NAD(P)H] large subunit	<i>nirB</i>
KQ76_12515	3.84	Nitrite reductase [NAD(P)H] small subunit	<i>nirD</i>
Fatty acid metabolism			
<i>Sphingomyelin metabolism (FA/lipid synthesis)</i>			
KQ76_10330	-2.79	Sphingomyelinase	NA
<i>Glycerolipid metabolism</i>			
KQ76_01375	-2.62	Lipase precursor	<i>estA</i>

<i>Fermentation to short-chain fatty acids</i>			
KQ76_00885	-2.21	3-oxoacid CoA-transferase	<i>ydiF</i>
Hydrolase			
KQ76_03975	-2.21	Thermonuclease	<i>yncB</i>
KQ76_13210	-2.93	Hydrolase, HAD superfamily	<i>cof</i>
Nucleotide metabolism			
<i>Purine</i>			
KQ76_00085	5.42	Adenylosuccinate synthetase	<i>purA</i>
KQ76_05035	4.67	5-(carboxyamino) imidazole ribonucleotide mutase	<i>purE</i>
KQ76_05040	5.17	5-(carboxyamino) imidazole ribonucleotide synthase	<i>purK</i>
KQ76_05045	5.21	phosphoribosylaminoimidazolesuccinocarboxamide synthase	<i>purC</i>
KQ76_05050	5.42	Phosphoribosylformylglycinamide synthase, PurS subunit	<i>purS</i>
KQ76_05055	5.38	phosphoribosylformylglycinamide synthase, PurQ subunit	<i>purQ</i>
KQ76_05060	5.32	phosphoribosylformylglycinamide synthase, PurL subunit	<i>purL</i>
KQ76_05065	5.47	Amidophosphoribosyltransferase	<i>purF</i>
KQ76_05070	5.62	Phosphoribosylformylglycinamide cyclo-ligase	<i>purM</i>
KQ76_05075	5.83	Phosphoribosylglycinamide formyltransferase	<i>purN</i>
KQ76_05080	5.82	IMP cyclohydrolase / Phosphoribosylaminoimidazolecarboxamide formyltransferase	<i>purH</i>
KQ76_05085	5.50	Phosphoribosylamine-glycine ligase	<i>purD</i>
KQ76_10160	2.45	Adenylosuccinate lyase	<i>purB</i>
KQ76_06580	5.31	GMP reductase	<i>guaC</i>
<i>Pyrimidine</i>			

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KQ76_05745	3.18	bifunctional pyrimidine operon transcriptional regulator	<i>pyrR</i>
KQ76_05755	3.62	Aspartate carbamoyltransferase	<i>pyrB</i>
KQ76_05760	4.12	Dihydroorotase	<i>pyrC</i>
KQ76_05765	3.47	Carbamoyl-phosphate synthase small subunit	<i>carA</i>
KQ76_05770	3.00	Carbamoyl-phosphate synthase large subunit	<i>carB</i>
KQ76_05775	2.32	Orotidine 5'-phosphate decarboxylase	<i>pyrF</i>
KQ76_05780	2.31	Orotate phosphoribosyltransferase	<i>pyrE</i>
KQ76_05750	4.97	Uracil permease @ Uracil:proton symporter UraA	<i>uraA</i>
Other metabolism			
<i>Flavin biosynthesis</i>			
KQ76_09190	2.35	6,7-dimethyl-8-ribityllumazine synthase	<i>ribE</i>
KQ76_09195	2.53	Riboflavin biosynthesis protein RibBA	<i>ribBA</i>
KQ76_09200	2.50	Riboflavin synthase	<i>ribC</i>
KQ76_09205	2.20	Riboflavin biosynthesis protein RibD	<i>ribD</i>
<i>Prophyrin compound biosynthesis</i>			
KQ76_12510	4.08	Uroporphyrinogen-III methyltransferase	<i>cysG</i>
<i>Cobalamin biosynthesis</i>			
KQ76_12525	2.75	cobalamin biosynthesis protein CbiX	<i>cbiX</i>
<i>Glutaryl-CoA degradation</i>			
KQ76_00870	-2.08	3-hydroxyacyl-CoA dehydrogenase	<i>fadB, caiD</i>
KQ76_00875	-2.35	Acyl-CoA dehydrogenase, long-chain specific	<i>caiA</i>
<i>Generation of free radical in anaerobic</i>			
KQ76_00830	2.00	Pyruvate formate-lyase activating enzyme	<i>pflA</i>
<i>Urea cycle (inorganic nutrients)</i>			
KQ76_05765	3.47	Carbamoyl-phosphate synthase small subunit	<i>carA</i>

KQ76_05770	3.00	Carbamoyl-phosphate synthase large subunit	<i>carB</i>
Oxidoreductase			
KQ76_07145	3.45	Sulfite reductase subunit alpha	<i>piuB?</i>
Sugar metabolism			
<i>UDP-sugar biosynthesis</i>			
KQ76_00510	-2.60	UDP-glucose 4-epimerase	<i>cap5E</i>
KQ76_00555	-2.65	UDP-glucose 4-epimerase	<i>cap5N</i>
KQ76_00560	-2.36	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>cap5O</i>
KQ76_00565	-2.10	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
Transcriptional regulators			
KQ76_12475	2.12	DNA-binding response regulator	<i>citB</i>
KQ76_12480	2.16	sensor histidine kinase	<i>COG4585</i>
KQ76_06315	2.09	Glutamine synthetase repressor	<i>soxR</i>
KQ76_10790	-2.93	Osmosensitive K ⁺ channel histidine kinase KdpD	<i>kdpD</i>
KQ76_10795	-2.53	DNA-binding response regulator KdpE	<i>kdpE</i>
Translation			
<i>Ribosome synthesis</i>			
KQ76_11620	2.04	50S ribosomal protein L18	<i>rpIR</i>
KQ76_11635	2.00	30S ribosomal protein S14	<i>rpsN</i>
KQ76_11645	2.01	50S ribosomal protein L24	<i>rpIX</i>
KQ76_11660	2.08	50S ribosomal protein L29	<i>rpmC</i>
KQ76_11675	2.05	50S ribosomal protein L22	<i>rpIV</i>
Transporters			

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<i>Amino acid</i>			
KQ76_07120	2.11	Serine/threonine exchanger	<i>steT</i>
KQ76_01370	2.92	Predicted histidine uptake transporter	<i>yjiH</i>
<i>Carbohydrate</i>			
KQ76_12410	-3.24	PTS system, sucrose-specific IIBC component	<i>ptsG1,ptsG2</i>
KQ76_00765	-2.59	Maltose/maltodextrin transport ATP-binding protein Malk	<i>malk</i>
<i>Inorganic ion</i>			
KQ76_14375	5.97	Copper-translocating P-type ATPase B	<i>copB</i>
KQ76_12460	3.79	Nitrate/nitrite transporter NarT	<i>narT</i>
<i>PTS alpha-glucoside transporter</i>			
KQ76_12100	-2.77	PTS alpha-glucoside transporter subunit IIBC	<i>ptsG1,ptsG2</i>
KQ76_13275	-2.21	PTS system, glucose-specific EIICBA	<i>ptsG1,ptsG2, nagE</i>
<i>Other transporters</i>			
KQ76_10555	2.93	Ammonium transporter family	<i>amtB</i>
KQ76_12810	-2.05	Choline ABC transport system, permease protein	<i>opuBB</i>
<i>Membrane protein</i>			
KQ76_13435	2.91	Membrane protein	<i>pfoR</i>
<i>Transporter</i>			
KQ76_11715	2.86	Hypoxanthine/guanine permease PbuG	<i>pbuG</i>
Virulence factor			
<i>Exotoxin</i>			
KQ76_09505	2.35	Exotoxin, phage associated	NA
KQ76_09510	2.25	Enterotoxin	NA
KQ76_09515	2.18	Exotoxin, phage associated	NA
KQ76_09520	2.52	Exotoxin, phage associated	NA

<i>Secretion system</i>			
KQ76_06590	2.37	secretion protein	<i>ykwD</i>
<i>Toxin</i>			
KQ76_12645	-2.90	Cytolytic pore-forming protein S component Gamma-hemolysin HlgC	<i>hlgC</i>
KQ76_12650	-2.75	Cytolytic pore-forming protein F component Gamma-hemolysin HlgB	<i>hlgB</i>
KQ76_11505	-2.91	Truncated cell surface protein map-w / toxin	NA
<i>General virulence factor</i>			
KQ76_05625	-3.40	Hemolytic protein	NA
Unknown			
KQ76_04830	-3.34	Conjugal transfer protein	NA

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Table S6.2. List and functional groups of differentially expressed genes in the untreated Ag⁺-resistant *S. aureus* strains (Ag-r NT) when compared to the untreated wild-type strain (WT NT). Highlighted tables are the genes or pathways discussed in the main text.

Locus tag	Log2 fold change	Annotations	Gene
Amino acid metabolism			
<i>Histidine</i>			
KQ76_14045	-2.63	ATP phosphoribosyltransferase regulatory subunit	<i>hisZ</i>
<i>Isoleucine</i>			
KQ76_10625	2.04	Dihydroxy-acid dehydratase (EC 4.2.1.9)	<i>IlvD</i>
<i>Valine</i>			
KQ76_10625	2.04	Dihydroxy-acid dehydratase (EC 4.2.1.9)	<i>IlvD</i>
<i>Glycine</i>			
KQ76_13645	-2.17	Choline dehydrogenase	<i>betA</i>
KQ76_13650	-2.13	Betaine aldehyde dehydrogenase	<i>adhE</i>
<i>L-lysine</i>			
KQ76_10340	-2.27	succinyl-diaminopimelate desuccinylase	NA
<i>L-glutamine</i>			
KQ76_05765	2.26	carbamoyl phosphate synthase small subunit	<i>carA</i>
KQ76_05770	2.18	carbamoyl phosphate synthase large subunit	<i>carB</i>
Cell envelope metabolism			
<i>Teichoic acid biosynthesis</i>			
KQ76_00565	-2.50	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
<i>Capsular polysaccharide</i>			
KQ76_00490	-4.69	capsular polysaccharide biosynthesis protein	<i>cap5A</i>
KQ76_00495	-4.41	Capsular polysaccharide biosynthesis protein	<i>cap5B</i>
KQ76_00500	-4.56	Capsular polysaccharide biosynthesis protein	<i>cap5C</i>
KQ76_00505	-4.84	Polysaccharide biosynthesis protein	<i>cap5D</i>
KQ76_00510	-4.70	Capsular polysaccharide biosynthesis protein	<i>cap5E</i>
KQ76_00515	-4.56	Capsular polysaccharide biosynthesis enzyme	<i>cap5F</i>
KQ76_00520	-4.47	UDP-N-acetylglucosamine 2-epimerase (non-hydrolyzing)	<i>cap5G</i>
KQ76_00535	-3.68	Capsular polysaccharide biosynthesis protein	<i>cap5J</i>

KQ76_00540	-3.69	Capsular polysaccharide biosynthesis protein	<i>cap5K</i>
KQ76_00545	-3.16	glycosyltransferase	<i>cap5L</i>
KQ76_00550	-3.14	capsular polysaccharide biosynthesis protein	<i>cap5M</i>
KQ76_00555	-3.10	Capsular polysaccharide biosynthesis protein	<i>cap5N</i>
KQ76_00560	-2.87	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>cap5O</i>
KQ76_00565	-2.50	UDP-N-acetylglucosamine 2-epimerase (non-hydrolyzing)	<i>cap5P</i>
Cysteine metabolism			
KQ76_02075	7.38	Cysteine synthase	<i>cysK</i>
KQ76_02080	10.27	Cystathionine gamma-synthase	<i>metB/mccB</i>
Detoxification			
<i>Arsenate</i>			
KQ76_09215	3.59	Arsenical resistance operon repressor	<i>arsR</i>
KQ76_09220	3.52	Arsenite/antimonite:H ⁺ antiporter ArsB	<i>arsB</i>
KQ76_14240	-7.36	ArsR family transcriptional regulator (plasmid)	<i>arsR</i>
KQ76_14265	-7.46	ArsR family transcriptional regulator (plasmid)	<i>arsR</i>
KQ76_14270	-9.36	arsenical efflux pump membrane protein	<i>arsB</i>
KQ76_14275	-8.90	protein ArsC	<i>arsC</i>
DNA metabolism			
KQ76_14300	-7.11	recombinase	NA
KQ76_14305	-7.84	recombinase	NA
KQ76_14320	-9.32	Replication protein Rep	COG5527
Energy			
<i>Fermentation to short-chain fatty-acids</i>			
KQ76_00865	-5.13	acetyl-CoA acetyltransferase	<i>paaJ</i>
KQ76_00885	-3.80	Propionate CoA-transferase	<i>ydiF</i>
Fatty acid metabolism			
<i>Sphingomyeline metabolism</i>			
KQ76_01375	-3.05	lipase 2	<i>estA</i>

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KQ76_10330	-4.17	sphingomyelin phosphodiesterase	NA
KQ76_13995	-2.39	Triacylglycerol lipase	estA
Hydrolase			
KQ76_03975	-2.05	thermonuclease	YncB
Nucleotide metabolism			
<i>Purine</i>			
KQ76_00085	5.64	Adenylosuccinate synthetase	purA
KQ76_05040	2.88	5-(carboxyamino) imidazole ribonucleotide synthase	purK
KQ76_05045	3.56	phosphoribosylaminoimidazolesuccinocarboxamide synthase	purC
KQ76_05050	4.39	Phosphoribosylformylglycinamide synthase, PurS subunit	purS
KQ76_05055	4.55	phosphoribosylformylglycinamide synthase, PurQ subunit	purQ
KQ76_05060	4.86	phosphoribosylformylglycinamide synthase, PurL subunit	purL
KQ76_05065	5.06	Amidophosphoribosyltransferase	purF
KQ76_05070	5.25	Phosphoribosylformylglycinamide cyclo-ligase	purM
KQ76_05075	5.33	Phosphoribosylglycinamide formyltransferase	purN
KQ76_05080	5.37	IMP cyclohydrolase / Phosphoribosylaminoimidazolecarboxamide formyltransferase	purH
KQ76_05085	5.23	Phosphoribosylamine-glycine ligase	purD
KQ76_06580	5.17	GMP reductase	guaC
<i>Pyrimidine</i>			
KQ76_05760	2.33	Dihydroorotase	allB
KQ76_05765	2.26	Carbamoyl-phosphate synthase small subunit	carA
KQ76_05770	2.18	Carbamoyl-phosphate synthase large subunit	carB
KQ76_05775	2.07	Orotidine 5'-phosphate decarboxylase	pyrF
KQ76_05780	2.24	Orotate phosphoribosyltransferase	pyrE
Other metabolism			
<i>Urea cycle</i>			
KQ76_05765	2.26	carbamoyl phosphate synthase small subunit	carA
KQ76_05770	2.18	carbamoyl phosphate synthase large subunit	carB
Oxidative stress			
<i>Bacillithiol</i>			
KQ76_05640	-4.72	bacillithiol biosynthesis cysteine-adding enzyme BshC	bshC

<i>Peroredoxin activity</i>			
KQ76_12955	-2.60	carboxymuconolactone decarboxylase family protein	<i>yciW</i>
Oxidoreductase			
KQ76_00785	-3.33	NADH-dependent dehydrogenase	<i>mviM</i>
KQ76_00790	-3.65	NADH-dependent dehydrogenase	<i>mviM</i>
KQ76_07145	3.60	sulfite reductase subunit alpha	<i>piuB</i>
KQ76_10560	3.19	oxidoreductase	<i>tusA</i>
Transposase			
KQ76_14250	-7.59	IS6 family transposase ISSau6	<i>Rve</i>
KQ76_14370	-7.46	Transposase	<i>pinE</i>
Transcriptional regulators			
KQ76_00710	-2.64	MurR/RpiR family transcriptional regulator	<i>rpiR</i>
KQ76_10790	-2.72	Osmosensitive K ⁺ channel histidine kinase KdpD	<i>kpdD</i>
KQ76_10795	-2.73	DNA-binding response regulator KdpE	<i>kpdE</i>
KQ76_13090	2.62	GntR family transcriptional regulator	<i>gntR</i>
KQ76_14325	-9.30	Transcriptional regulator, Xre family	<i>NA</i>
KQ76_14360	-10.94	LysR family transcriptional regulator	<i>lysR</i>
Transferase			
KQ76_02170	2.07	Acetyltransferase, GNAT family	<i>yhbS</i>
KQ76_13605	2.64	Aminotransferase	<i>gabT</i>
Translation			
<i>Ribosome protein</i>			
KQ76_11565	2.08	50S ribosomal protein L17	<i>rplQ</i>
KQ76_11575	2.02	30S ribosomal protein S11	<i>rpsK</i>
KQ76_11605	2.07	50S ribosomal protein L15	<i>rplO</i>
KQ76_11610	2.26	50S ribosomal protein L30	<i>rpmD</i>
KQ76_11615	2.36	30S ribosomal protein S5	<i>rpsE</i>
KQ76_11620	2.29	50S ribosomal protein L18	<i>rplR</i>

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KQ76_11625	2.38	50S ribosomal protein L6	<i>rplF</i>
KQ76_11630	2.32	30S ribosomal protein S8	<i>rpsH</i>
KQ76_11635	2.44	30S ribosomal protein S14	<i>rpsN</i>
KQ76_11640	2.40	50S ribosomal protein L5	<i>rplE</i>
KQ76_11645	2.35	50S ribosomal protein L24	<i>rplX</i>
KQ76_11650	2.38	50S ribosomal protein L14	<i>rplN</i>
KQ76_11655	2.34	30S ribosomal protein S17	<i>rpsQ</i>
KQ76_11660	2.37	50S ribosomal protein L29	<i>rpmC</i>
KQ76_11665	2.33	50S ribosomal protein L16	<i>rplP</i>
KQ76_11670	2.30	30S ribosomal protein S3	<i>rpsC</i>
KQ76_11675	2.30	50S ribosomal protein L22	<i>rplV</i>
KQ76_11680	2.31	30S ribosomal protein S19	<i>rpsS</i>
KQ76_11685	2.17	50S ribosomal protein L2	<i>rplB</i>
KQ76_11690	2.03	50S ribosomal protein L23	<i>rplW</i>
KQ76_11695	2.04	50S ribosomal protein L4	<i>rplD</i>
KQ76_01610	2.18	30S ribosomal protein S6	<i>rpsF</i>
KQ76_01615	2.17	single-stranded DNA-binding protein	<i>ssb</i>
KQ76_01620	2.30	30S ribosomal protein S18	<i>rpsR</i>
<i>Translation</i>			
KQ76_10515	-2.09	RNAIII (delta-hemolysin)	NA
Transporters			
<i>Amino acid</i>			
KQ76_12585	2.48	L-cystine ABC transporter, ATP-binding protein TcyC	<i>tcyC</i>
KQ76_12590	2.36	L-cystine ABC transporter, permease protein TcyB	<i>tcyB</i>
KQ76_12595	2.39	L-cystine ABC transporter, substrate-binding protein TcyA	<i>tcyA</i>
KQ76_00600	4.89	sulfonate ABC transporter ATP-binding protein	<i>tauB</i>
KQ76_00610	5.02	sulfonate ABC transporter permease	<i>tauC</i>
KQ76_01810	6.21	L-cystine uptake protein TcyP, sodium:anion symporter family	<i>tcyP</i>
KQ76_02085	7.22	methionine ABC transporter ATP-binding protein	<i>metN</i>
KQ76_02090	7.03	methionine ABC transporter permease	<i>metP</i>

KQ76_02095	6.33	methionine ABC transporter substrate-binding protein	<i>nlpA</i>
<i>Carbohydrate transport</i>			
KQ76_00690	2.34	PTS glucose EIICBA component	<i>ptsG1,ptsG2,nagE</i>
KQ76_00705	-2.27	PTS transporter subunit IIBC	<i>ptsG1,ptsG2</i>
KQ76_00775	-2.13	arabinogalactan ABC transporter permease	<i>ugpA</i>
KQ76_00780	-2.82	Maltose ABC transporter permease	<i>malG</i>
KQ76_13275	2.78	PTS system, glucose-specific EIICBA	<i>ptsG1,ptsG2,nagE</i>
KQ76_13845	-3.12	PTS mannose transporter subunit IIABC	<i>frwC,frwB,ptsN</i>
<i>ABC transporters</i>			
KQ76_00730	2.22	ABC transporter ATP-binding protein	<i>gsiA</i>
KQ76_00740	2.61	peptide ABC transporter permease	<i>dppC</i>
<i>Membrane protein</i>			
KQ76_13435	2.81	Membrane protein	<i>pfoR</i>
KQ76_14365	-9.50	Putative membrane protein YeiH	<i>yeiH</i>
<i>Metal</i>			
KQ76_14235	-9.81	Cadmium-transporting ATPase	<i>cadA</i>
KQ76_14375	-6.65	copper-transporting P-type ATPase B	<i>copB</i>
<i>Other</i>			
KQ76_11715	2.50	Hypoxanthine/guanine permease PbuG	<i>pbuG</i>
KQ76_12810	-2.18	choline ABC transporter permease	<i>opuBB</i>
KQ76_12815	-2.32	glycine/betaine ABC transporter ATP-binding protein	<i>opuBA</i>
KQ76_12245	2.17	multidrug MFS transporter	<i>araJ</i>
KQ76_12250	2.05	multidrug efflux protein	<i>emrA</i>
KQ76_13690	-2.28	Citrate/H ⁺ symporter of CitMHS family	<i>citM</i>
Sugar metabolism			
<i>UDP-sugar biosynthesis</i>			
KQ76_01330	2.84	carbohydrate kinase,carbohydrate kinase	<i>rbsK</i>
KQ76_00510	-4.70	UDP-glucose 4-epimerase	<i>cap5E</i>

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KQ76_00555	-3.10	Capsular polysaccharide synthesis enzyme	<i>cap5N</i>
KQ76_00560	-2.87	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>cap5O</i>
KQ76_00565	-2.50	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
<i>Mannose</i>			
KQ76_13850	-3.24	mannose-6-phosphate isomerase	<i>manA</i>
<i>D-gluconate</i>			
KQ76_13085	2.51	gluconate kinase	<i>xylB</i>
<i>Glutaryl-CoA degradation</i>			
KQ76_00865	-5.13	acetyl-CoA acetyltransferase	<i>paaJ</i>
KQ76_00870	-4.69	3-hydroxyacyl-CoA dehydrogenase	<i>fadB, caiD</i>
KQ76_00875	-4.33	glutaryl-CoA dehydrogenase	<i>caiA</i>
<i>Secondary metabolite degradation</i>			
KQ76_00700	-2.32	N-acetylmuramic acid-6-phosphate etherase	<i>murQ</i>
<i>Other</i>			
KQ76_00795	-3.87	xylose isomerase	<i>ycjR</i>
Virulence factor			
<i>Toxin</i>			
KQ76_05555	-3.71	Cytolytic pore-forming protein => Alpha-hemolysin	<i>NA</i>
KQ76_11505	-4.37	Truncated cell surface protein map-w	<i>NA</i>
KQ76_12645	-3.22	Cytolytic pore-forming protein S component => Gamma-hemolysin HlgC	<i>hlgC</i>
KQ76_12650	-3.64	Cytolytic pore-forming protein F component => Gamma-hemolysin HlgB	<i>hlgB</i>
<i>Virulence factor</i>			
KQ76_05625	-4.09	hemolytic protein	<i>NA</i>
<i>Secretion protein</i>			
KQ76_06590	2.38	secretion protein	<i>ykwD</i>
<i>Phage</i>			
KQ76_07505	2.15	head-tail adaptor protein	<i>NA</i>
<i>Exotoxin</i>			
KQ76_09505	2.05	Exotoxin, phage associated	<i>NA</i>
Unknown			

KQ76_04470	-2.22	Similar to cell surface protein Map-w	NA
KQ76_10320	-2.75	protein map	NA
KQ76_11965	2.03	CHAP domain-containing protein	COG3942
KQ76_14280	-10.09	lactococcin 972 family bacteriocin	NA

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Table S6.3. List and the functional groups of the overlapped differentially expressed genes of NAg-resistant (NAg-r) *S. aureus* strain exposed to low (low-NAg, 1.5 µg Ag/mL) and high NAg concentration (high-NAg, 4.5 µg Ag/mL) and the wild-type strain (WT) exposed at low NAg concentration (low-NAg, 1.5 µg Ag/mL).

Locus Tag	Log 2 fold change			Annotations	Gene
	WT low-NAg vs WT NT	NAg-r low-NAg vs NAg-r NT	NAg-r high-NAg vs NAg-r NT		
Amino acid metabolism					
Lysine					
KQ76_00355	2.50	2.70	3.48	Diaminopimelate decarboxylase	lysA/sbnH
Cysteine metabolism					
KQ76_02080	2.79	2.08	2.77	Cystathionine gamma-synthase	metC
Cell envelope					
Cell wall organization					
KQ76_01130	2.31	2.69	3.70	Glycyl-glycine endopeptidase LytM precursor	lytM
Detoxification					
KQ76_09220	2.10	2.81	2.73	Arsenical efflux pump membrane protein	arsB
KQ76_14270	3.00	3.94	3.94	Arsenical pump membrane protein	arsB
KQ76_14275	2.89	4.11	4.62	Protein ArsC	wzb
DNA metabolism					
DNA repair					
KQ76_02535	2.02	4.40	7.10	Excinuclease ABC subunit B	uvrB
Epimerase					
KQ76_12780	2.08	3.22	2.93	Epimerase	wcaG
Iron uptake and metabolism					
KQ76_00320	3.25	3.70	6.14	Siderophore biosynthesis protein	sbnA

KQ76_00325	2.50	3.89	6.71	2,3-diaminopropionate biosynthesis protein	<i>sbnB</i>
KQ76_00330	3.22	3.47	6.04	Siderophore biosynthesis protein	<i>sbnC</i>
KQ76_00335	3.07	3.51	5.37	Siderophore biosynthesis protein	<i>sbnD</i>
KQ76_00340	2.67	3.28	5.05	Siderophore biosynthesis protein	<i>sbnE</i>
KQ76_00345	2.30	2.77	4.15	Siderophore biosynthesis protein	<i>sbnF</i>
KQ76_00350	2.17	2.61	3.69	Siderophore biosynthesis protein SbnG	<i>sbnG</i>
KQ76_00355	2.50	2.70	3.48	Diaminopimelate decarboxylase	<i>lysA/sbnH</i>
Other metabolism					
KQ76_01125	2.57	4.07	5.38	Choloylglycine hydrolase	<i>yxel</i>
KQ76_12525	-2.24	-6.09	-5.89	cobalamin biosynthesis protein CbiX	<i>cbiX</i>
Oxidative stress					
KQ76_01795	2.04	4.32	4.48	Alkyl hydroperoxide reductase protein F	<i>ahpF</i>
KQ76_01800	2.05	4.25	4.49	Alkyl hydroperoxide reductase protein C	<i>ahpC</i>
KQ76_04050	2.08	3.62	2.63	thiol reductase thioredoxin	
Oxidoreductase					
KQ76_01395	2.12	3.25	2.82	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
KQ76_01805	2.13	4.32	3.88	NADPH-dependent oxidoreductase	<i>nfnB</i>
KQ76_01885	2.03	4.05	3.92	NAD(P)-dependent oxidoreductase	<i>ybjT</i>
KQ76_04340	2.16	4.23	3.94	NADH-dependent flavin oxidoreductase	<i>fadH</i>
KQ76_08465	2.22	4.29	3.86	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
Post-translational process					
KQ76_01400	2.02	3.00	2.64	Glycine cleavage system protein H	<i>gcvH</i>
Transcriptional regulators					

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KQ76_14240	5.32	7.77	9.55	Transcriptional regulator	<i>arsR</i>
Translation					
<i>Ribosomal protein</i>					
KQ76_06575	2.61	2.99	4.53	30S ribosomal protein S14p	<i>rpsN</i>
Transporters					
<i>Metal</i>					
KQ76_13375	5.07	7.85	8.03	Copper-translocating P-type ATPase	<i>copA</i>
KQ76_13380	4.68	6.68	6.85	Copper(I) chaperone CopZ	<i>copZ</i>
KQ76_14235	5.08	7.46	9.45	Cadmium-transporting ATPase	<i>cadA</i>
<i>Other</i>					
KQ76_00600	2.90	3.23	3.18	Sulfonate ABC transporter ATP-binding protein	<i>tauB</i>

Table S6.4. List and the functional groups of the overlapped differentially expressed genes of NAg-resistant (NAg-r) *S. aureus* strain at high NAg concentration (high-NAg, 4.5 µg Ag/mL) and the wild-type strain (WT) at low NAg concentration (low-NAg, 1.5 µg Ag/mL).

Locus Tag	Log2 fold change		Annotations	Gene
	WT low-NAg vs WT NT	NAg-r high-NAg vs NAg-r NT		
Amino acid metabolism				
L-isoleucine				
KQ76_11515	2.41	3.81	Acetolactate synthase, catabolic	ilvB / alsS
Cysteine metabolism				
KQ76_02075	2.35	2.30	cysteine synthase	cysK
Energy				
Fermentation of pyruvate				
KQ76_11510	2.65	4.16	Alpha-acetolactate decarboxylase	alsD
KQ76_11515	2.41	3.81	Acetolactate synthase, catabolic	ilvB / alsS
Oxidoreductase				
KQ76_04285	3.05	5.53	pyridine nucleotide-disulfide oxidoreductase	NA
Transporters				
KQ76_00610	2.73	2.74	sulfonate ABC transporter permease	tauC

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Table S6.5. List and the functional groups of the differentially expressed genes of NAg-resistant (NAg-r) *S. aureus* strain at low (low NAg, 1.5 µg Ag/mL) and high NAg concentration (high-NAg, 4.5 µg Ag/mL) exclude the genes expressed by the wild-type strain at low NAg concentration (low-NAg, 1.5 µg Ag/mL). Highlighted tables are the genes or pathways discussed in the main text.

Locus tag	Log2 fold change		Annotations	Gene
	NAg-r low-Ag vs NAg-r NT	NAg-r high-Ag vs NAg-r NT		
Amino acid metabolism				
Arginine				
KQ76_00265		4.52	N-acyl-L-amino acid amidohydrolase	abgB
KQ76_05590	-2.26		ornithine carbamoyltransferase	argF
KQ76_00650		-2.73	N-acetylglutamate kinase	argB
KQ76_08390		-2.35	Allophanate hydrolase 2 subunit 2	DUR1B
KQ76_08395		-2.66	Allophanate hydrolase 2 subunit 1	DUR1A
L-lysine				
KQ76_06880		2.70	Aspartate-semialdehyde dehydrogenase	asd
KQ76_06885		3.18	4-hydroxy-tetrahydrodipicolinate synthase	dapA
KQ76_06890	2.19	3.60	4-hydroxy-tetrahydrodipicolinate reductase	dapB
KQ76_06895	2.37	3.57	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate N-acetyltransferase	dapD
KQ76_10340		-3.43	succinyl-diaminopimelate desuccinylase	NA
KQ76_10345	2.10	4.69	succinyl-diaminopimelate desuccinylase	argE
L-serine				
KQ76_07125	-3.11	-3.93	Serine/threonine dehydratase	ilvA
L-alanine				
KQ76_07130	-3.40	-3.92	Alanine dehydrogenase	ald
KQ76_08880		-2.29	Alanine dehydrogenase	ald
Methionine				
KQ76_01565		3.72	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	metE
KQ76_09290	-2.09	2.83	S-adenosylmethionine synthetase	metK
L-asparagine				
KQ76_07350		-3.35	L-asparaginase	ansA

KQ76_10115	-2.19	-2.90	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	<i>gatB</i>
KQ76_10120	-2.03	-4.09	glutamyl-tRNA(Gln) amidotransferase subunit A	<i>gatA</i>
<i>L-isoleucine</i>				
KQ76_06875		2.57	Aspartate kinase	<i>lysC</i>
KQ76_07125	-3.11	-3.93	Serine/threonine dehydratase	<i>ilvA</i>
KQ76_10630		5.62	Acetolactate synthase large subunit	<i>ilvB</i>
KQ76_10635		5.56	Acetolactate synthase small subunit	<i>ilvH</i>
KQ76_10640	2.65	6.27	Ketol-acid reductoisomerase	<i>ilvC</i>
KQ76_10665	2.10	4.61	L-threonine dehydratase biosynthetic	<i>ilvA</i>
<i>L-leucine</i>				
KQ76_10645	3.00	7.00	2-isopropylmalate synthase	<i>leuA</i>
KQ76_10650	2.44	5.81	3-isopropylmalate dehydrogenase	<i>leuB</i>
KQ76_10655	2.61	5.90	3-isopropylmalate dehydratase large subunit	<i>leuC</i>
KQ76_10660	2.28	5.67	3-isopropylmalate dehydratase small subunit	<i>leuD</i>
<i>L-histidine</i>				
KQ76_12140	-2.09		Urocanate hydratase	<i>hutU</i>
KQ76_12155		2.98	Formiminoglutamase	<i>speB</i>
KQ76_14005		2.37	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP diphosphatase	<i>hisI1,hisI2</i>
KQ76_14015		2.11	1-(5-phosphoribosyl)-5-((5- phosphoribosylamino)methylideneamino)imidazole -4- carboxamide isomerase	<i>hisA</i>
KQ76_14045	-4.54	-2.64	ATP phosphoribosyltransferase regulatory subunit	<i>hisZ</i>
<i>Glycine</i>				
KQ76_13645		-5.66	Choline dehydrogenase	<i>betA</i>
KQ76_13650		-4.75	Betaine aldehyde dehydrogenase	<i>adhE</i>
<i>Glutamine</i>				
KQ76_06320	-2.77		Glutamine synthetase type I	<i>glnA</i>
<i>Glutamate</i>				

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KQ76_02140		3.66	Glutamate synthase beta subunit	<i>gltD</i>
KQ76_06755		-2.97	glutamine amidotransferase / anthranilate synthase subunit II	<i>pabA</i>
<i>Tryptophan</i>				
KQ76_06750		-3.61	Anthranilate synthase, aminase component	<i>trpE</i>
KQ76_00185		2.07	2-amino-3-carboxymuconate-6-semialdehyde decarboxylase	<i>NA</i>
KQ76_10200		-2.63	Prephenate dehydratase	<i>pheA2</i>
<i>Threonine</i>				
KQ76_06535		2.71	Threonine synthase	<i>thrC</i>
KQ76_06540		2.38	Homoserine kinase	<i>thrB</i>
<i>Tyrosine</i>				
KQ76_06740		-3.47	Prephenate dehydrogenase	<i>tyrA</i>
<i>Proline</i>				
KQ76_13360		2.07	L-glutamate gamma-semialdehyde dehydrogenase	<i>adhE</i>
<i>L-valine</i>				
KQ76_10625		4.02	Dihydroxy-acid dehydratase	<i>ilvD</i>
KQ76_10640	2.65	6.27	Ketol-acid reductoisomerase	<i>ilvC</i>
Cell envelope metabolism				
<i>General cell wall-related</i>				
KQ76_04795	-2.50	-5.10	Poly(glycerol-phosphate) alpha-glucosyltransferase	<i>rfaB</i>
KQ76_04970		2.63	mannosyl-glycoprotein endo-beta-N-acetylglucosamidase	<i>lytD,cwlA</i>
KQ76_05665		-2.15	Phospho-N-acetylmuramoyl-pentapeptide-transferase	<i>rfe</i>
KQ76_07365		2.11	Elastin binding protein	<i>ebpS</i>
KQ76_13430		-4.43	transglycosylase IsaA	<i>isaA</i>
<i>Capsular polysaccharide biosynthesis</i>				
KQ76_00495		2.18	Capsular polysaccharide synthesis enzyme	<i>cap5B</i>
KQ76_00500		2.05	Capsular polysaccharide synthesis enzyme	<i>cap5C</i>
KQ76_00505		2.33	Capsular polysaccharide biosynthesis protein	<i>cap5D</i>
KQ76_00510		2.51	UDP-glucose 4-epimerase	<i>cap5E</i>
KQ76_00515		2.55	Capsular polysaccharide synthesis enzyme	<i>cap5F</i>
KQ76_00520		2.31	UDP-N-acetyl-L-fucosamine synthase	<i>cap5G</i>

KQ76_00555		2.55	Capsular polysaccharide synthesis enzyme	<i>cap5N</i>
KQ76_00560		2.27	UDP-N-acetyl-D-mannosamine dehydrogenase	<i>cap5O</i>
KQ76_00565		2.18	UDP-N-acetylglucosamine 2-epimerase	<i>cap5P</i>
KQ76_13955	-2.69		Capsular biosynthesis protein Cap8C	<i>cap8C</i>
Cysteine metabolism				
<i>L-cysteine</i>				
KQ76_00060		2.83	Homoserine O-acetyltransferase	<i>met2</i>
KQ76_02390		2.82	Cysteine synthase	<i>cysK</i>
KQ76_02570		2.55	Serine acetyltransferase	<i>cysE</i>
KQ76_13320		2.18	Methylated-DNA--protein-cysteine methyltransferase	<i>adaB</i>
<i>Homocysteine/cystathione synthesis</i>				
KQ76_01565		3.72	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase	<i>metE</i>
KQ76_01570		3.92	bifunctional 5,10-methylenetetrahydrofolate reductase / homocysteine S-methyltransferase	<i>metH1,metF</i>
KQ76_01575		4.42	Cystathionine beta-lyase	<i>metC</i>
KQ76_01580		2.37	Cystathionine gamma-synthase	<i>metC</i>
<i>Inhibitor of cysteine protease</i>				
KQ76_10165	-2.97	-5.44	Staphopain A precursor	NA
KQ76_10170	-2.59	-3.94	staphostatin A	NA
Detoxification				
<i>Arsenate</i>				
KQ76_09215		2.46	Arsenical resistance operon repressor	<i>arsR</i>
<i>Methylglyoxal</i>				
KQ76_01465	2.07	3.08	Glyoxalase	<i>gloA</i>
KQ76_07875	2.37		glyoxalase	<i>gloA</i>
KQ76_09280	2.85	2.63	glyoxal reductase	<i>ARA1</i>
KQ76_13170	2.12	4.08	Glyoxalase	<i>gloA</i>
KQ76_13185		2.78	glyoxalase	<i>catE</i>

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DNA metabolism				
Foreign DNA uptake/transformation				
KQ76_03740	-3.13		Competence protein ComF homolog	<i>comFC</i>
KQ76_08290		2.53	Competence protein ComE 2	<i>comE</i>
DNA synthesis and recombination				
KQ76_03040		-3.24	DNA integration/recombination/inversion protein; recombinase	<i>NA</i>
KQ76_08780	2.00		DNA polymerase I	<i>polA,exo</i>
DNA repair				
KQ76_02680		3.27	Protein/nucleic acid deglycase HchA	<i>hchA</i>
KQ76_02825		-3.41	Uracil-DNA glycosylase, family 1	<i>ung</i>
KQ76_03415		2.59	Deoxyribodipyrimidine photolyase	<i>phrB</i>
KQ76_03790		3.31	Excinuclease ABC subunit B	<i>uvrB</i>
KQ76_03795		3.38	Excinuclease ABC subunit A	<i>uvrA</i>
KQ76_05470		2.60	Excinuclease ABC subunit C	<i>uvrC</i>
KQ76_06205		2.24	DNA recombination/repair protein	<i>recA</i>
KQ76_06600		2.31	SOS-response repressor and protease LexA	<i>lexA</i>
KQ76_06735		5.69	DNA repair protein	<i>umuC</i>
KQ76_07195		-2.14	RecU Holliday junction resolvase	<i>recU</i>
KQ76_07210		2.17	Endonuclease III	<i>nth</i>
KQ76_08530		-2.96	Single-stranded-DNA-specific exonuclease RecJ	<i>recJ</i>
KQ76_10090	2.81	3.35	DNA polymerase IV	<i>dinP / dinB</i>
DNA mismatch repair				
KQ76_06255		-2.32	DNA mismatch repair protein MutS	<i>mutS</i>
KQ76_06260		-2.95	DNA mismatch repair protein MutL	<i>mutL</i>
KQ76_10585		-3.30	DNA mismatch repair protein MutS	<i>mutS</i>
KQ76_13035		-3.00	DNA mismatch repair protein MutT	<i>mutT</i>
Restriction enzyme				
KQ76_00720		-2.06	Type I restriction-modification system, restriction subunit R	<i>COG0610</i>
Energy				
Aerobic respiration				

KQ76_05150		-2.49	Cytochrome d ubiquinol oxidase subunit I	<i>appC</i>
<i>Fermentation of pyruvate</i>				
KQ76_00375	3.80	5.52	acetoin reductase	<i>fabG</i>
KQ76_00580	3.17		Aldehyde dehydrogenase B	<i>adhE</i>
KQ76_13195	-2.66	-4.08	D-lactate dehydrogenase	<i>ldhA</i>
<i>Glycolysis</i>				
KQ76_00925	-2.83	-6.59	L-lactate dehydrogenase	<i>mdh</i>
KQ76_01070		2.83	aryl-phospho-beta-D-glucosidase	<i>bglB</i>
<i>TCA cycle</i>				
KQ76_00625		2.23	NAD-dependent formate dehydrogenase	<i>ldhA?</i>
KQ76_02890	2.23	7.49	Dihydrolipoamide dehydrogenase	<i>lpd</i>
<i>Pentose Phosphate pathway</i>				
KQ76_09260	3.52	2.94	Transaldolase	<i>talA</i>
Epimerase				
KQ76_03865		2.71	epimerase	<i>yfcH</i>
Fatty acid metabolism				
<i>Fatty acid degradation</i>				
KQ76_02795		-2.02	Long-chain-fatty-acid--CoA ligase	<i>caiC</i>
<i>Fatty acid synthesis</i>				
KQ76_06085		-4.34	Phosphatidate cytidyltransferase	<i>cdsA</i>
KQ76_01460		-2.97	Glycerol-3-phosphate transporter	<i>uhpC</i>
KQ76_04480		-2.19	3-oxoacyl-[acyl-carrier-protein] synthase III (KASIII)	<i>fabH</i>
KQ76_06265		-2.42	Glycerol-3-phosphate responsive antiterminator (mRNA-binding)	<i>glpP</i>
KQ76_07980		-2.93	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	<i>accB</i>
KQ76_08975		-4.39	Acyl-ACP:1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>plsC</i>
<i>Glycerolipid</i>				
KQ76_03190	2.67	2.61	Dihydroxyacetone kinase subunit K	<i>dhaK</i>
KQ76_03195	2.63	2.73	Dihydroxyacetone kinase subunit L	<i>dhaL</i>

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KQ76_03200	2.50	2.31	Dihydroxyacetone kinase subunit M	<i>dhaM</i>
KQ76_13780		2.02	Tributylin esterase	<i>frmB</i>
KQ76_13995		2.36	Triacylglycerol lipase	<i>estA</i>
<i>Fermentation of short-chain fatty acids</i>				
KQ76_00885		2.78	propionate CoA-transferase	<i>ydiF</i>
KQ76_00825		-4.04	Pyruvate formate-lyase / formate acetyltransferase	<i>pflD</i>
KQ76_00865		2.51	acetyl-CoA acetyltransferase	<i>paaJ</i>
KQ76_11895	2.14	3.25	Acyl-CoA dehydrogenase	<i>caiA</i>
Hydrolase				
KQ76_01560		3.00	Metal-dependent hydrolase/cyclase	<i>COG1878</i>
KQ76_01770	-2.04	-2.51	histidine phosphatase family protein	<i>phoE</i>
KQ76_02785		-4.54	Haloacid dehalogenase-like hydrolase	<i>gph</i>
KQ76_03975		-5.69	thermonuclease	<i>yncB?</i>
KQ76_04590		-3.71	Oligoendopeptidase F	<i>pepF</i>
KQ76_08775	2.08	2.26	Formamidopyrimidine-DNA glycosylase (EC 3.2.2.23)	<i>nei?</i>
KQ76_08865		3.04	metal-dependent hydrolase	<i>ulaG</i>
KQ76_09095		2.40	MBL fold metallo-hydrolase	<i>gloB?</i>
KQ76_09930	2.05	2.35	metal-dependent hydrolase	<i>ybcI</i>
KQ76_11405		2.13	cell surface alpha/beta hydrolase (putative)	<i>COG4814</i>
KQ76_12745		2.25	phosphohydrolase	<i>cpdA</i>
KQ76_12840		2.69	Carboxylesterase, type B	<i>pnbA?</i>
KQ76_13165		3.81	Phospholipase/carboxylesterase	<i>ypfH</i>
KQ76_13260	2.69	3.82	esterase	<i>aes</i>
KQ76_13490	3.01	5.47	alpha/beta hydrolase	<i>mhpC</i>
KQ76_13485		3.82	amidohydrolase	<i>COG2159</i>
KQ76_13560		2.85	acyl esterase	<i>COG2936</i>
Iron uptake and metabolism				
<i>Cytochrome assembly</i>				
KQ76_08675		-2.30	Heme exporter protein C for the biogenesis of c-type cytochromes	<i>ccmC</i>
<i>Siderophore synthesis</i>				

KQ76_00360	2.34	2.87	Siderophore staphylobactin biosynthesis protein SbnI	<i>sbnI</i>
KQ76_11360		2.71	Siderophore biosynthesis protein near heme transporter HtsABC	<i>htsABC</i>
<i>Staphylobillin</i>				
KQ76_00570	2.34	2.20	Heme-degrading monooxygenase, staphylobillin-producing	<i>hmoA</i>
<i>Iron-regulated surface determinant</i>				
KQ76_05380	-2.31	-4.11	Iron-regulated surface determinant protein A	<i>isdA</i>
KQ76_05385		-3.26	Iron-regulated surface determinant protein C	<i>isdC</i>
KQ76_05405		-3.83	Transpeptidase, SrtB family sortase	<i>srtB</i>
KQ76_05410		-3.38	heme-degrading monooxygenase IsdI	<i>isdI</i>
KQ76_09000		3.54	haptoglobin-binding heme uptake protein/ Iron-regulated surface determinant protein H	<i>harA = isdH</i>
<i>Iron storage</i>				
KQ76_10075	3.20	3.61	Bacterial non-heme ferritin	<i>ftnA</i>
<i>Iron transport</i>				
KQ76_05400		-3.38	heme ABC transporter permease	<i>fepD</i>
KQ76_13340	2.52		ferrous iron transporter A	<i>feoA</i>
Nitrogen metabolism				
<i>Nitrate reductase</i>				
KQ76_12475	-2.79		DNA-binding transcriptional response regulator NreC	<i>nreC</i>
KQ76_12480	-2.63		Sensor histidine kinase NreB	<i>nreB</i>
KQ76_12485	-2.50		Dissimilatory nitrate and nitrite reduction regulatory protein NreA	<i>nreA</i>
KQ76_12490	-3.54	-2.73	Respiratory nitrate reductase gamma chain	<i>narI</i>
KQ76_12495	-5.88	-3.74	Respiratory nitrate reductase delta chain	<i>narJ</i>
KQ76_12500	-5.91	-3.56	Respiratory nitrate reductase beta chain	<i>narH / narY</i>
KQ76_12505	-6.34	-4.17	Respiratory nitrate reductase alpha chain	<i>narG</i>
KQ76_12510	-5.10		Uroporphyrinogen-III C-methyltransferase	<i>cysG</i>
KQ76_12515	-5.61	-4.15	Nitrite reductase [NAD(P)H] small subunit	<i>nirD</i>
KQ76_12520	-5.03	-4.41	Nitrite reductase [NAD(P)H] large subunit	<i>nirB</i>

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Nitrogen compound metabolism				
KQ76_06320	-2.77		Glutamine synthetase type I	<i>glnA</i>
Nitric oxide from arginine				
KQ76_10195		-2.99	Nitric oxide synthase	COG4362
Nucleotide metabolism				
General nucleotide				
KQ76_00125		-3.73	multifunctional 2',3'-cyclic-nucleotide 2'-phosphodiesterase /5'-nucleotidase/3'-nucleotidase	<i>ushA</i>
KQ76_05630	2.46		Pyrimidine 5'-nucleotidase YjjG	<i>yigB</i>
KQ76_02705		-2.31	Deoxyadenosine kinase @ Deoxycytidine kinase	<i>dck</i>
KQ76_02710		-4.09	Deoxyguanosine kinase	<i>dck</i>
KQ76_07285		-3.37	Nucleoside diphosphate kinase	<i>ndk</i>
Pyrimidine				
KQ76_05750	-5.75	-5.86	Uracil permease	<i>uraA</i>
KQ76_05755	-3.45	-3.44	Aspartate carbamoyltransferase	<i>pyrB</i>
KQ76_05760	-4.13	-4.31	Dihydroorotase	<i>pyrC</i>
KQ76_05765	-3.50	-3.86	Carbamoyl-phosphate synthase small chain	<i>carA</i>
KQ76_05770	-3.08	-3.67	Carbamoyl-phosphate synthase large chain	<i>carB</i>
KQ76_05775	-2.23	-2.58	Orotidine 5'-phosphate decarboxylase	<i>pyrF</i>
KQ76_11005		-3.14	Thymidine kinase	<i>tdk</i>
KQ76_11050	-2.60	-4.04	CTP synthase	<i>pyrG</i>
KQ76_13530	-2.19	-2.79	Dihydroorotate dehydrogenase	<i>pyrD</i>
Purine				
KQ76_00425		2.61	Purine nucleoside phosphorylase	<i>deoD</i>
KQ76_01835		-2.45	Xanthine phosphoribosyltransferase	<i>apt</i>
KQ76_02375		-2.17	Hypoxanthine-guanine phosphoribosyltransferase	<i>hptA</i>
KQ76_06580		-3.10	GMP reductase	<i>guaC</i>
KQ76_10160		-4.32	Adenylosuccinate lyase @ SAICAR lyase	<i>purB</i>
KQ76_10920	-2.15	-2.06	ATP synthase CF1 sector epsilon chain	<i>atpC</i>
KQ76_10925	-2.06		ATP synthase CF1 sector subunit beta	<i>atpD</i>
KQ76_10930	-2.14	-2.49	ATP synthase CF1 sector subunit gamma	<i>atpG</i>

KQ76_10935	-2.13	-2.66	ATP synthase CF1 sector subunit alpha	<i>atpA</i>
KQ76_10940	-2.17	-3.28	ATP synthase CF1 sector subunit delta	<i>atpH</i>
KQ76_10945		-2.36	ATP synthase CF0 sector subunit b	<i>atpF</i>
KQ76_10950		-2.07	ATP synthase CF0 sector subunit c	<i>atpE</i>
KQ76_10955		-2.52	ATP synthase CF0 sector subunit a	<i>atpB</i>
KQ76_10960		-2.87	ATP synthase protein I	<i>NA</i>
KQ76_11120		-2.93	Purine nucleoside phosphorylase	<i>deoD</i>
KQ76_11715		-2.00	Hypoxanthine/guanine permease PbuG	<i>pbuG</i>
Other metabolism				
<i>2,3-dihydroxybenzoate biosynthesis</i>				
KQ76_00675	2.55	2.75	isochorismatase	<i>pncA</i>
<i>AAA ATPases (ATPases Associated with diverse cellular Activities)</i>				
KQ76_00165		-3.61	ATPase AAA	<i>NA</i>
<i>acyl-CoA metabolism</i>				
KQ76_08770		2.05	Dephospho-CoA kinase	<i>coaE</i>
KQ76_13270	3.67	3.44	acyl-CoA thioester hydrolase	<i>fadM?</i>
<i>Alcohol degradation</i>				
KQ76_00840		-4.29	membrane-anchoring domain / Glycerophosphoryl diester phosphodiesterase	<i>ugpQ1,ugpQ</i>
<i>Generation of free radical in anaerobic</i>				
KQ76_00830	-2.29	-5.48	Pyruvate formate-lyase activating enzyme	<i>pflA</i>
KQ76_13680		-2.51	anaerobic ribonucleoside-triphosphate reductase activating protein	<i>nrdG</i>
<i>Flavin</i>				
KQ76_09190	-2.45	-3.52	6,7-dimethyl-8-ribityllumazine synthase	<i>ribE</i>
KQ76_09195	-2.31	-3.49	3,4-dihydroxy-2-butanone 4-phosphate synthase / GTP cyclohydrolase II	<i>ribBA</i>
KQ76_09200	-2.18	-3.65	Riboflavin synthase	<i>RibC</i>
KQ76_09205		-3.69	Diaminohydroxyphosphoribosylaminopyrimidine deaminase / 5-amino-6-(5-phosphoribosylamino)uracil reductase	<i>ribD</i>

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<i>Cell division</i>				
KQ76_05290		-2.14	cell division protein FtsW	<i>ftsW</i>
KQ76_05655		-2.72	Cell division protein FtsL	<i>ftsL</i>
KQ76_07810		2.07	Site-specific tyrosine recombinase XerD	<i>xerD</i>
KQ76_11290	3.00	2.46	Chromosome partitioning protein ParA?	<i>parA</i>
<i>Cobalamin</i>				
KQ76_02025		3.00	cobalamin biosynthesis protein CobW	<i>cobW</i>
<i>Mo-molybdopterin cofactor biosynthetic process</i>				
KQ76_11810		2.09	GTP 3',8-cyclase / cyclic pyranopterin monophosphate synthase	<i>moaA</i>
KQ76_11835		2.07	Molybdopterin molybdenumtransferase	<i>moaA</i>
KQ76_11870	2.15		Sulfur carrier protein FdhD	<i>fdhD</i>
<i>Formaldehyde assimilation</i>				
KQ76_09260	3.52	2.94	Transaldolase	<i>talA</i>
<i>Folate biosynthesis</i>				
KQ76_01570		3.92	bifunctional 5,10-methylenetetrahydrofolate reductase / homocysteine S-methyltransferase	<i>metH1,metF</i>
<i>Holin-antiholin</i>				
KQ76_01040		-2.61	Autolysis response regulator LytR	<i>lytR</i>
KQ76_01045		-2.72	Antiholin-like protein LrgA	<i>lrgA</i>
KQ76_01050		-2.09	Antiholin-like protein LrgB	<i>lrgB</i>
KQ76_13290		3.29	Holin-like protein CidA	<i>cidA</i>
<i>Phosphopantothenate Biosynthesis</i>				
KQ76_13570		2.43	Pantoate--beta-alanine ligase	<i>panC</i>
<i>Polyprenyl biosynthesis</i>				
KQ76_06080		-4.34	Phosphatidate cytidyltransferase	<i>cdsA</i>
KQ76_05780		-3.06	Undecaprenyl diphosphate synthase / isoprenyl transferase	<i>uppS</i>
<i>Porphyrin Compound Biosynthesis</i>				
KQ76_05300		-3.67	Heme A synthase, cytochrome oxidase biogenesis protein Cox15-CtaA	<i>ctaA</i>
KQ76_05305		-3.11	Heme O synthase, protoheme IX farnesyltransferase, COX10-CtaB	<i>cyoE</i>
KQ76_08670		-2.82	Porphobilinogen deaminase / hydroxymethylbilane synthase	<i>hemC</i>
KQ76_13700		2.30	Precorrin-2 oxidase	<i>cysG2</i>

<i>preQ0 biosynthesis</i>				
KQ76_03515		2.58	7-carboxy-7-deazaguanine synthase	<i>queE</i>
KQ76_03520		2.12	6-carboxy-5,6,7,8-tetrahydropterin synthase	<i>queD</i>
<i>Pyridoxine (vitamin B6)</i>				
KQ76_02510		-3.05	Pyridoxal 5'-phosphate lyase subunit	<i>pdxS</i>
KQ76_02515		-2.95	Pyridoxal 5'-phosphate synthase glutaminase subunit	<i>pdxT</i>
<i>Quinol and Quinone synthesis</i>				
KQ76_04905		-2.08	1,4-dihydroxy-2-naphthoate polyprenyltransferase	<i>menA</i>
<i>Thiamine biosynthesis (vitamin B1)</i>				
KQ76_10875		-3.33	Thiaminase II involved in salvage of thiamin pyrimidine moiety	<i>tenA</i>
<i>Urea cycle</i>				
KQ76_04365		5.50	Argininosuccinate lyase	<i>argH</i>
KQ76_04370		5.71	Argininosuccinate synthase	<i>argG</i>
Oxidative stress				
<i>Thioredoxin</i>				
KQ76_03830	2.75	2.36	Thioredoxin reductase	<i>trxB</i>
KQ76_04050		2.63	thiol reductase thioredoxin	<i>trxA?</i>
KQ76_04080		2.14	thiol reductase thioredoxin	<i>?</i>
KQ76_05460	3.01	2.08	thiol reductase thioredoxin	<i>NA</i>
KQ76_06300		2.78	Glutathione peroxidase @ Thioredoxin peroxidase	<i>btuE</i>
KQ76_07055		2.07	Peptide-methionine (S)-S-oxide reductase MsrA	<i>msrA</i>
KQ76_10275		2.29	thioredoxin	<i>trxA</i>
KQ76_12375	3.60	4.13	Thioredoxin reductase (EC 1.8.1.9) / ferredoxin--NADP(+) reductase	<i>trxB?</i>
KQ76_13715		2.73	Glutathione peroxidase @ Thioredoxin peroxidase	<i>btuE</i>
<i>ROS</i>				
KQ76_06565	3.69	4.85	Catalase	<i>katA</i>
KQ76_08110	3.52	3.38	Superoxide dismutase	<i>sodA</i>
<i>Iron-sulfur cluster biosynthesis</i>				

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KQ76_08250	2.60		Molecular chaperone protein	<i>dnaK</i>
KQ76_04125		2.15	Iron-sulfur cluster assembly protein SufD	<i>sufD</i>
KQ76_04130		3.01	Cysteine desulfurase => SufS / aminotransferase	<i>sufS</i>
KQ76_04135		2.93	Iron-sulfur cluster assembly scaffold protein for SUF system, SufE2	<i>sufE2</i>
KQ76_04140		2.49	Iron-sulfur cluster assembly protein SufB	<i>sufB</i>
KQ76_04260	2.27	2.12	Iron-sulfur cluster assembly accessory protein	<i>sufA</i>
Glutathione metabolism				
KQ76_06300	2.54		Glutathione peroxidase (EC 1.11.1.9)	<i>btuE</i>
KQ76_08900	2.97		2-Cys peroxiredoxin (EC 1.11.1.15)	<i>tpx</i>
KQ76_09895	2.99		Thiol peroxidase, Bcp-type (EC 1.11.1.15); peroxiredoxin	<i>bcp</i>
Oxidoreductase				
KQ76_00755		2.07	FMN-dependent NADH-azoreductase	<i>azoR</i>
KQ76_00785		-3.43	NADH-dependent dehydrogenase	<i>mviM</i>
KQ76_00790		-3.40	NADH-dependent dehydrogenase	<i>mviM</i>
KQ76_02720		-3.18	haloacid dehalogenase	<i>cof??</i>
KQ76_02725		-2.07	FMN-dependent NADH-azoreductase	<i>ssuE</i>
KQ76_02955	-2.49	-4.95	Zn-dependent alcohol dehydrogenase	<i>adhP</i>
KQ76_03090		-3.21	haloacid dehalogenase	<i>gph</i>
KQ76_04035		3.25	organic hydroperoxide reductase	<i>osmC/ohrA</i>
KQ76_04200		-2.08	haloacid dehalogenase	<i>nagD?</i>
KQ76_07145	-4.65	-5.90	sulfite reductase subunit alpha	<i>piuB?</i>
KQ76_07825	2.30		aldo/keto reductase	<i>yqkF</i>
KQ76_09890	3.02	2.62	2-hydroxyacid dehydrogenase	<i>serA</i>
KQ76_10505		2.20	Nitroreductase family protein	<i>FMR2</i>
KQ76_10560	2.27	4.06	oxidoreductase	<i>tusA</i>
KQ76_11025		3.62	Aldehyde dehydrogenase family protein	<i>adhE</i>
KQ76_11150		4.44	Oxidoreductase	<i>ybjT</i>
KQ76_11395	2.55	3.33	Zinc-type alcohol dehydrogenase-like protein	<i>qor</i>
KQ76_11400	3.46	3.94	NADP-dependent oxidoreductase YfmJ	<i>curA</i>
KQ76_12125	2.33	4.57	Oxidoreductase, short-chain dehydrogenase/reductase family	<i>fabG</i>

KQ76_12945		3.08	short chain dehydrogenase/reductase	<i>fabG</i>
KQ76_12990	2.38		oxidoreductase	<i>ydfG</i>
KQ76_13190	2.69	2.68	Oxygen-insensitive NAD(P)H nitroreductase / Dihydropteridine reductase	<i>nfnB</i>
Peptidase				
KQ76_00760	3.30	3.81	peptidase M23	<i>nlpD</i>
KQ76_01750		2.05	peptidase	<i>ykoI</i>
KQ76_04385		-2.48	inactive Signal peptidase IA	<i>lepB</i>
KQ76_06170		-2.28	peptidase M16	<i>pqqL</i>
KQ76_07990		-2.72	peptidase M24	<i>pepP</i>
KQ76_12860		2.21	peptidase M28	<i>frvX</i>
KQ76_13305		-2.05	CHAP domain-containing protein / peptidase M23B	COG3942
KQ76_14170	-3.18	-3.78	peptidase	<i>aprE</i>
Post-translational process				
<i>Protein metabolism</i>				
KQ76_04270		-2.60	Cytosol aminopeptidase PepA	<i>pepA</i>
KQ76_13525		3.96	fructosamine 3-kinase	FN3K?
<i>Protein modification</i>				
KQ76_01410	2.73	2.21	NAD-dependent protein deacetylase of SIR2	<i>SIR2</i>
KQ76_01415	2.33		Lipoate-protein ligase A	<i>lplA</i>
KQ76_05735	-2.64	-3.60	Lipoprotein signal peptidase	<i>ispA</i>
KQ76_11450		3.10	NAD-dependent protein deacetylase of SIR2 family	<i>SIR2</i>
<i>Protein degradation</i>				
KQ76_02545	4.03	6.52	ATP-dependent Clp protease, ATP-binding subunit ClpC	<i>clpA</i>
<i>Protein folding</i>				
KQ76_08255	2.49	5.29	heat shock protein GrpE	<i>grpE</i>
KQ76_10485	2.18	4.96	Heat shock protein 60 kDa family chaperone GroEL	<i>groEL</i>
KQ76_10490		4.41	Heat shock protein 10 kDa family chaperone GroES	<i>groES</i>
Protease				

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KQ76_01715		-2.67	CAAX amino protease	NA
KQ76_01735		-2.78	CAAX amino protease	NA
KQ76_04705		-4.06	Serine protease, DegP/HtrA, do-like	<i>degQ</i>
KQ76_08090		-2.20	rhomboid family intramembrane serine protease	<i>glpG</i>
KQ76_09455		2.37	Serine protease	<i>eMpr</i>
KQ76_09460		2.53	Serine protease	<i>eMpr</i>
KQ76_09465		2.15	serine protease SplC	<i>spIC</i>
KQ76_10385		-2.69	CAAX amino protease	NA
KQ76_10495		-2.23	CPBP family intermembrane metalloprotease	<i>ydiL</i>
KQ76_12050		-4.73	CPBP family intermembrane metalloprotease	<i>ydiL</i>
RNA metabolism				
<i>mRNA degradation</i>				
KQ76_06150		-2.03	Polyribonucleotide nucleotidyltransferase	<i>pnp</i>
<i>RNA helicase</i>				
KQ76_08130		-2.20	Endonuclease IV	<i>nfo</i>
KQ76_08135		-2.83	DEAD-box ATP-dependent RNA helicase CshB	<i>srnB/cshB</i>
KQ76_10800	-2.39	-3.59	DEAD-box ATP-dependent RNA helicase DeaD (= CshA)	<i>srnB</i>
KQ76_13030		-2.12	DNA/RNA helicase of DEAD/DEAH box family	<i>SSL2, COG3886</i>
<i>RNA maturation</i>				
KQ76_05435		-3.42	Ribonuclease HIII	<i>rnhC</i>
<i>RNA synthesis</i>				
KQ76_11055		-2.24	DNA-directed RNA polymerase delta subunit	NA
KQ76_11570	-2.18		DNA-directed RNA polymerase alpha subunit	<i>rpoA</i>
KQ76_14075		3.36	DNA-directed RNA polymerase subunit delta	<i>COG1912</i>
Stress response				
<i>General stress</i>				
KQ76_01760		2.64	GlsB/YeaQ/YmgE family stress response membrane protein	<i>yeaQ</i>
KQ76_02540	4.40	6.77	Protein-arginine kinase McsB	<i>mcsB</i>
KQ76_05270	-2.77	-3.68	translational GTPase TypA	<i>typA</i>
KQ76_08885		3.03	universal stress protein UspA	<i>uspA</i>

KQ76_11375		2.19	Asp23/Gls24 family envelope stress response protein	<i>yloU</i>
KQ76_12425		2.26	General stress protein 26	<i>NA</i>
<i>Stress-induced multi chaperone system</i>				
KQ76_03855	2.67	3.80	ATP-dependent Clp protease proteolytic subunit ClpP (EC 3.4.21.92)	<i>clpP</i>
KQ76_04440	3.49	7.55	Chaperone protein ClpB (ATP-dependent unfoldase)	<i>clpB</i>
KQ76_08245		3.84	Chaperone protein DnaJ	<i>dnaJ</i>
<i>Sugar metabolism</i>				
<i>Galactose</i>				
KQ76_00385	-3.05		UDP-glucose 4-epimerase, NAD-dependent	<i>wcaG</i>
<i>Gluconeogenesis</i>				
KQ76_00680	2.78	2.42	Pyruvate decarboxylase; Alpha-keto-acid decarboxylase	<i>ipdC</i>
KQ76_03860	2.34	2.85	Malolactic enzyme	<i>sfcA</i>
KQ76_08765		-3.50	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	<i>gapA</i>
KQ76_09295		-2.54	Phosphoenolpyruvate carboxykinase [ATP]	<i>pckA</i>
KQ76_13150	3.39	3.82	Fructose-1,6-bisphosphatase	<i>fbp2</i>
<i>Glycolysis</i>				
KQ76_03885		2.05	Phosphoglycerate kinase	<i>pgk</i>
KQ76_03890		2.26	Triosephosphate isomerase	<i>tpiA</i>
KQ76_03895		2.28	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	<i>gpmI</i>
<i>D-mannose</i>				
KQ76_11145		-3.42	Mannose-6-phosphate isomerase, class I	<i>manA</i>
<i>Fructose</i>				
KQ76_03455	-2.29		1-phosphofructokinase	<i>fruK</i>
<i>Carboxylate degradation</i>				
KQ76_00870		2.51	3-hydroxyacyl-CoA dehydrogenase,3-hydroxyacyl-CoA dehydrogenase	
KQ76_00875		3.14	glutaryl-CoA dehydrogenase	
KQ76_13630	2.30	2.11	Acyl-coenzyme A synthetases	<i>acs</i>
<i>Secondary metabolite</i>				

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KQ76_00700		-2.07	N-acetylmuramic acid 6-phosphate etherase (EC 4.2.1.126)	<i>murQ</i>
KQ76_00970		2.12	sorbitol dehydrogenase	<i>tdh</i>
KQ76_11210		2.33	Mannitol-1-phosphate 5-dehydrogenase (EC 1.1.1.17)	<i>mtlD</i>
<i>Other</i>				
KQ76_00980		2.18	galactitol-1-phosphate 5-dehydrogenase	<i>tdh</i>
KQ76_13615	2.46		Fructose-bisphosphate aldolase class I	<i>NA</i>
Transcription				
KQ76_03980		-3.07	Cold shock protein of CSP family	<i>cspC</i>
KQ76_06920		-2.40	Cold shock protein CspA	<i>cspA</i>
KQ76_10715		-2.37	anti-sigma B protein RsbW	<i>rsbW</i>
KQ76_10720		-2.13	Anti-sigma B factor antagonist RsbV	<i>rsbV</i>
KQ76_11020		-2.49	Transcription termination factor Rho	<i>rho</i>
KQ76_14175		-3.97	Cold shock protein of CSP family	<i>cspC</i>
Transcriptional regulators				
KQ76_00810		-2.39	DNA-binding response regulator, AraC family	<i>yesN</i>
KQ76_00815		-2.31	Two-component system sensor histidine kinase	<i>yesM</i>
KQ76_01105		2.58	LacI family transcriptional regulator	<i>purR?</i>
KQ76_01635		-2.50	transcriptional regulator	<i>hipB</i>
KQ76_02530	4.29		Transcriptional regulator CtsR	<i>ctsR</i>
KQ76_02895	2.12		transcriptional regulator	<i>iscR</i>
KQ76_03285		-2.36	MarR family transcriptional regulator	<i>NA</i>
KQ76_03390		-3.04	MarR family transcriptional regulator	<i>marR</i>
KQ76_03450	-2.32		DeoR family transcriptional regulator	<i>glpR</i>
KQ76_05215	-2.81		Cro/CI family transcriptional regulator	<i>hipB</i>
KQ76_06315	-3.03		MerR family transcriptional regulator	<i>soxR</i>
KQ76_06720		-3.40	regulatory protein MsrR	<i>msrR</i>
KQ76_07865	2.16		LacI family transcriptional regulator	<i>purR</i>
KQ76_08260	2.54		HrcA family transcriptional regulator	<i>hrcA</i>
KQ76_09660	-2.62		Cro/CI family transcriptional regulator	<i>XRE</i>
KQ76_10475		-4.16	transcriptional regulator	<i>hipB</i>

KQ76_10550		-2.58	LacI family transcriptional regulator	<i>purR?</i>
KQ76_10790		2.18	Osmosensitive K ⁺ channel histidine kinase KdpD	<i>kdpD</i>
KQ76_10795		2.54	DNA-binding response regulator KdpE	<i>kdpE</i>
KQ76_11155		2.94	Zn(II) or Co(II)-specific transcriptional repressor protein	<i>arsR?</i>
KQ76_11780	-2.35		Transcriptional regulator, MarR family	<i>marR</i>
KQ76_11940	-2.88		MarR transcriptional regulator	<i>marR</i>
KQ76_11955	-2.50		transcriptional regulator	<i>marR</i>
KQ76_11960	-2.66		AraC family transcriptional regulator	<i>araC</i>
KQ76_12105		-4.91	Transcriptional regulator GlvR, RpiR family	<i>glvR</i>
KQ76_12255		-2.44	TetR family transcriptional regulator	<i>acrR</i>
KQ76_12320	2.08		TetR family transcriptional regulator	<i>acrR</i>
KQ76_12400		-2.29	TetR family transcriptional regulator	<i>acrR</i>
KQ76_13175		3.67	MarR family transcriptional regulator	<i>marR</i>
KQ76_13245		-2.34	Transcriptional regulator pfoR	<i>frwC</i>
KQ76_14265	4.01		Transcriptional regulator	<i>arsR</i>
Transferase				
KQ76_09965		3.52	glutamine amidotransferase	<i>thiJ</i>
KQ76_03950		2.99	N-acetyltransferase	<i>yhbS</i>
KQ76_07045		2.48	glucose-specific phosphotransferase enzyme IIA component	<i>nagE</i>
KQ76_13180		2.08	N-acetyltransferase,	<i>yidJ</i>
KQ76_13425		-4.97	O-acetyltransferase OatA	<i>oatA</i>
KQ76_13605		2.72	aspartate Aminotransferase	<i>gabT</i>
KQ76_12370	3.28		N-acetyltransferase	<i>rimI</i>
KQ76_14130	3.06		N-hydroxyarylamine O-acetyltransferase (EC 2.3.1.118)	<i>nhoA</i>
Translation				
<i>Ribosomal protein</i>				
KQ76_01610	-4.00	-4.99	30S ribosomal protein S6	<i>rpsF</i>
KQ76_01615	-4.01	-4.91	Single-stranded DNA-binding protein	<i>ssb</i>

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KQ76_01620	-3.91	-4.51	30S ribosomal protein S18	<i>rpsR</i>
KQ76_02330	-2.46		50S ribosomal protein L25	<i>rplY</i>
KQ76_02615	-2.33	-2.74	50S ribosomal protein L1	<i>rplA</i>
KQ76_02620	-3.47	-4.28	50S ribosomal protein L10	<i>rplJ</i>
KQ76_02625	-3.33	-3.92	50S ribosomal protein L7/L12	<i>rplL</i>
KQ76_02645	-3.47	-5.47	50S ribosomal protein L7ae-like protein	<i>rpl7Ae</i>
KQ76_02650	-3.45	-4.61	30S ribosomal protein S12	<i>rpsL</i>
KQ76_02655	-3.56	-4.41	30S ribosomal protein S7	<i>rpsG</i>
KQ76_02660	-3.52		elongation factor G	<i>fusA</i>
KQ76_05955	-2.69	-2.84	30S ribosomal protein S16	<i>rpsP</i>
KQ76_05960	-3.21		16S rRNA processing protein	<i>rimM</i>
KQ76_05965	-2.82		tRNA (guanine(37)-N(1))-methyltransferase	<i>trmD</i>
KQ76_06055	-2.89	-2.36	30S ribosomal protein S2	<i>rpsB</i>
KQ76_06570	2.28	3.22	50 ribosomal protein L33p, zinc-dependent	<i>rpmG</i>
KQ76_07165		-3.20	23S rRNA (guanine(2445)-N(2))-methyltransferase	<i>rlmL</i>
KQ76_08100	-2.21	-2.90	50S ribosomal protein L33p, zinc-dependent	<i>rpmG</i>
KQ76_08240		3.67	Ribosomal protein L11 methyltransferase	<i>prmA</i>
KQ76_08275		-2.33	30S ribosomal protein S20	<i>rpsT</i>
KQ76_08585	-2.83		50S ribosomal protein L21p	<i>rplU</i>
KQ76_08720	-2.91	-4.74	50S ribosomal protein L20p	<i>rplT</i>
KQ76_08725	-3.03	-4.90	50S ribosomal protein L35p	<i>rpmI</i>
KQ76_08935	-2.66	-3.04	30S ribosomal protein S4, zinc-independent	<i>rpsD</i>
KQ76_10600		-2.55	Ribosomal-protein-S18p-alanine acetyltransferase	<i>rimI</i>
KQ76_11015	-2.56	-2.82	50S ribosomal protein L31 type B, zinc-independent	<i>rpmE</i>
KQ76_11540		-2.08	50S ribosomal protein L13	<i>rplM</i>
KQ76_11565	-2.31		50S ribosomal protein L17	<i>rplQ</i>
KQ76_11575	-2.04		30S ribosomal protein S11	<i>rpsK</i>
KQ76_11585	-2.36	-2.24	50S ribosomal protein L36, zinc-dependent	<i>rpmJ</i>
KQ76_11600	-2.11		Protein translocase subunit SecY	<i>secY</i>
KQ76_11605	-2.65	-2.46	50S ribosomal protein L15	<i>rplO</i>

KQ76_11610	-3.26	-3.46	50S ribosomal protein L30	<i>rpmD</i>
KQ76_11615	-3.35	-3.38	30S ribosomal protein S5	<i>rpsE</i>
KQ76_11620	-3.50	-3.72	50S ribosomal protein L18	<i>rplR</i>
KQ76_11625	-3.48	-3.97	50S ribosomal protein L6	<i>rplF</i>
KQ76_11630	-3.83	-4.63	30S ribosomal protein S8	<i>rpsH</i>
KQ76_11635	-3.85	-5.26	30S ribosomal protein S14	<i>rpsN</i>
KQ76_11640	-3.97	-5.54	50S ribosomal protein L5	<i>rplE</i>
KQ76_11645	-4.03	-5.73	50S ribosomal protein L24	<i>rplX</i>
KQ76_11650	-3.83	-5.34	50S ribosomal protein L14	<i>rplN</i>
KQ76_11655	-3.85	-6.27	30S ribosomal protein S17	<i>rpsQ</i>
KQ76_11660	-3.74	-5.54	50S ribosomal protein L29	<i>rpmC</i>
KQ76_11665	-3.67	-5.37	50S ribosomal protein L16	<i>rplP</i>
KQ76_11670	-3.69	-5.21	30S ribosomal protein S3	<i>rpsC</i>
KQ76_11675	-3.71	-5.52	50S ribosomal protein L22	<i>rplV</i>
KQ76_11680	-3.56	-5.18	30S ribosomal protein S19	<i>rpsS</i>
KQ76_11685	-3.40	-5.05	50S ribosomal protein L2	<i>rplB</i>
KQ76_11690	-3.54	-5.81	50S ribosomal protein L23	<i>rplW</i>
KQ76_11695	-3.47	-5.46	50S ribosomal protein L4	<i>rplD</i>
KQ76_11700	-3.50	-6.10	50S ribosomal protein L3	<i>rplC</i>
KQ76_11705	-3.52	-6.40	30S ribosomal protein S10	<i>rpsJ</i>
KQ76_14225	-2.30	-3.38	50S ribosomal protein L34	<i>rpmH</i>
<i>Ribosomal RNA</i>				
KQ76_02580		2.80	Mini-ribonuclease III	<i>mrnC</i>
KQ76_02585		2.98	23S rRNA (guanosine(2251)-2'-O)-methyltransferase	<i>rlmB</i>
KQ76_02630		-2.70	16S rRNA (guanine(1207)-N(2))-methyltransferase	<i>rsmC</i>
<i>tRNA-related</i>				
KQ76_05720		-2.02	Isoleucyl-tRNA synthetase	<i>ileS</i>
KQ76_02575		2.65	CysteinyI-tRNA synthetase	<i>cysS</i>

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KQ76_02715		-2.21	tRNA-specific adenosine-34 deaminase	<i>tadA</i>
KQ76_02970		-2.56	Arginyl-tRNA synthetase	<i>argS</i>
KQ76_04570		-2.33	Tryptophanyl-tRNA synthetase	<i>trpS</i>
KQ76_07845		-3.41	Ribonuclease Z	<i>elaC</i>
KQ76_10115	-2.19	-2.90	aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit B	<i>gatB</i>
KQ76_10120	-2.03	-4.09	glutamyl-tRNA(Gln) amidotransferase subunit A	<i>gatA</i>
KQ76_10125		-5.70	Aspartyl-tRNA(Asn) amidotransferase subunit C	<i>gatC</i>
KQ76_10610		-2.31	tRNA threonylcarbamoyladenosine biosynthesis protein TsaE	<i>tsaE</i>
General translation				
KQ76_03745	2.04	3.10	Ribosome hibernation promoting factor Hpf	<i>hpf</i>
KQ76_08270		-2.03	Translation elongation factor 4 LepA	<i>lepA</i>
KQ76_08730	-2.76	-4.97	translation initiation factor IF-3	<i>infC</i>
KQ76_10515		-5.06	RNAIII (delta-hemolysin)	<i>NA</i>
KQ76_10995	-2.25	-2.63	Peptide chain release factor N(5)-glutamine methyltransferase	<i>prmC</i>
KQ76_11000	-2.36	-3.26	Peptide chain release factor 1	<i>prfA</i>
KQ76_11590	-2.10		translation initiation factor IF-1	<i>infA</i>
Transporters				
Amino acid				
KQ76_00670		-3.70	branched-chain amino acid transporter II carrier protein	<i>brnQ</i>
KQ76_01370	-2.11	-3.59	histidine uptake transporter	<i>yjiH</i>
KQ76_01810		3.33	L-cystine uptake protein TcyP, sodium:anion symporter family	<i>tcyP</i>
KQ76_02085		3.54	Methionine import ATP-binding protein	<i>metN</i>
KQ76_02090		4.25	Methionine ABC transporter permease	<i>metP</i>
KQ76_02095		5.11	Methionine ABC transporter substrate-binding protein	<i>nlpA</i>
KQ76_02840		-2.32	amino acid permease	<i>potE</i>
KQ76_04095		2.45	Methionine ABC transporter substrate-binding protein	<i>nlpA</i>
KQ76_06950		-3.14	branched-chain amino acid ABC transport system II carrier protein	<i>brnQ</i>
KQ76_07120	-2.47	-3.31	amino acid permease	<i>potE</i>
KQ76_08815		-3.23	D-serine/D-alanine/glycine permease	<i>ansP</i>
KQ76_09720		3.92	Glutamine ABC transporter, ATP-binding protein GlnQ	<i>hisJ</i>

KQ76_09725		3.68	glutamate ABC transporter permease	<i>hisM, hisJ</i>
KQ76_12210	-2.59	-5.27	Sodium/glutamate symporter	<i>gltS</i>
KQ76_12585	2.28	2.65	L-cystine ABC transporter, ATP-binding protein TcyC	<i>tcyC</i>
KQ76_12590	2.25	2.43	L-cystine ABC transporter, permease protein TcyB	<i>tcyB</i>
KQ76_12595	2.11		L-cystine ABC transporter, substrate-binding protein TcyA	<i>tcyA</i>
KQ76_12750		-2.12	D-serine/D-alanine/glycine permease	<i>ansP</i>
<i>ABC transporters</i>				
KQ76_01135	-2.10	-2.22	multidrug ABC transporter ATP-binding protein	<i>ccmA</i>
KQ76_01140	-2.19	-3.44	ABC transporter	<i>NA</i>
KQ76_01320		2.25	peptide ABC transporter ATP-binding protein	<i>lolD</i>
KQ76_02985		-3.42	ABC transporter, substrate-binding protein	<i>fepB</i>
KQ76_04780		2.33	bacteriocin ABC transporter ATP-binding protein	<i>lolD</i>
KQ76_06870		-2.96	ABC transporter ATP-binding protein	<i>uup</i>
KQ76_09600		-2.30	multidrug ABC transporter, permease protein EscB	<i>ecsB</i>
KQ76_13145		-2.16	abc transporter	<i>nosY</i>
KQ76_14140	3.49	3.05	bactracin ABC transporter, ATP-binding protein	<i>lolD</i>
KQ76_14145	2.91	2.22	bacitracin ABC transporter permease	<i>salY</i>
<i>Carbohydrate</i>				
KQ76_00765		-3.25	Maltose/maltodextrin transport ATP-binding protein MalK (EC 3.6.3.19); Maltodextrin ABC transporter, ATP-binding protein MsmX	<i>malK</i>
KQ76_00770		-2.93	maltose ABC transporter substrate-binding protein	<i>mdxE</i>
KQ76_00775		-3.69	arabinogalactan ABC transporter permease	<i>ugpA</i>
KQ76_00780		-3.96	maltose ABC transporter permease	<i>mdxG</i>
KQ76_01435		-2.27	PTS ascorbate transporter subunit IIA	<i>ptsN</i>
KQ76_06270		-3.21	Glycerol uptake facilitator protein	<i>glpF</i>
KQ76_11200		2.52	PTS lactose transporter subunit IIB, PTS lactose transporter subunit IIB	<i>bglG, ptsN</i>
KQ76_11205		3.00	PTS system, mannitol-specific IIA component	<i>mtlA2</i>
KQ76_13080		2.00	Gluconate permease, Bsu4004 homolog	<i>gntT</i>

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<i>Membrane protein</i>				
KQ76_00800	-4.00	-3.68	membrane protein	<i>ypbQ</i>
KQ76_01285		-2.17	membrane protein	<i>NA</i>
KQ76_01145	-2.05	-6.15	membrane protein	<i>NA</i>
KQ76_01480		-3.22	membrane protein	<i>yeiH</i>
KQ76_01880	3.96	3.70	membrane protein	<i>COG3759</i>
KQ76_03025	-2.30	-3.33	Integral membrane protein	<i>ycdZ, ydcZ</i>
KQ76_03930		-2.53	membrane protein	<i>NA</i>
KQ76_04490		-5.29	membrane protein	<i>NA</i>
KQ76_05310		-3.49	membrane protein	<i>yozeB</i>
KQ76_06630		-2.29	membrane protein	<i>ccdC</i>
KQ76_07095	-2.72	-6.81	membrane protein	<i>yhhQ</i>
KQ76_09310	2.12		Membrane protein insertion efficiency factor YidD	<i>yidD</i>
KQ76_10050		-2.01	Integral membrane protein	<i>ygaE</i>
KQ76_10270		-2.74	membrane protein	<i>NA</i>
KQ76_10855	-2.06		Inner membrane protein translocase and chaperone	<i>yidC</i>
KQ76_14135		2.13	membrane protein	<i>hdeD</i>
<i>PTS transporters</i>				
KQ76_03460	-2.61		PTS fructose transporter subunit IIC	<i>frwC, frwB, ptsN</i>
KQ76_12100	2.91	3.47	PTS alpha-glucoside transporter subunit IIBC	<i>PtsG1, PtsG2</i>
KQ76_12410	2.50	3.15	PTS sucrose transporter subunit IIBC	<i>ptsG1, ptsG2</i>
KQ76_13275		4.59	PTS system, glucose-specific IICBA	<i>ptsG1, ptsG2, nagE</i>
<i>Ions</i>				
KQ76_00275		-2.84	Na/Pi cotransporter	<i>nptA</i>
KQ76_03105		-4.69	phosphonate ABC transporter ATP-binding protein	<i>znuC</i>
KQ76_11855		-2.64	Molybdenum ABC transporter ATP-binding protein ModC	<i>modC</i>
KQ76_11860		-3.67	Molybdenum ABC transporter permease protein ModB	<i>modB</i>
KQ76_11865		-5.10	Molybdenum ABC transporter, substrate-binding protein ModA	<i>modA</i>
KQ76_12190		-2.49	ABC-type Na ⁺ efflux pump, permease component	<i>natB</i>
KQ76_12195		-3.36	sodium efflux ABC transporter, ATP-binding protein	<i>yhaQ</i>

KQ76_12440	-2.01	-4.73	proton/sodium-glutamate symport protein	<i>gltT</i>
KQ76_12460	-6.45	-7.19	Nitrate/nitrite transporter	<i>narT</i>
KQ76_12535		-2.15	formate/nitrite transporter	<i>focA</i>
<i>Metal</i>				
KQ76_03095		-2.44	Manganese/Zinc ABC transporter, periplasmic-binding protein SitA	<i>sitA</i>
KQ76_03100		-3.17	Manganese/Zinc ABC transporter, inner membrane permease protein SitD	<i>sitD</i>
KQ76_12915	2.14	3.19	Nickel ABC transporter permease	<i>dppB / nikB</i>
KQ76_12920	2.23	3.74	Nickel ABC transporter, nickel/metallophore periplasmic binding prot	<i>ddpA / nika</i>
<i>Secondary metabolite</i>				
KQ76_03175		2.44	iron-dicitrate ABC transporter ATP-binding protein	
KQ76_03180		2.26	Ferrichrome ABC transporter permease protein	
<i>Other transporter</i>				
KQ76_02525		-3.76	pyrimidine Nucleoside permease NupC	<i>nupC</i>
KQ76_03045	-2.10	-2.38	Na(+) H(+) antiporter subunit A	<i>nuoL, mnhB</i>
KQ76_03050	-2.28		Na(+) H(+) antiporter subunit B	<i>mnhB</i>
KQ76_03160		-2.14	nucleoside permease	<i>nupC</i>
KQ76_04275	-2.79	-5.30	sodium:proton antiporter / histidine transporter YuiF	<i>YuiF</i>
KQ76_04495		-2.85	(Oligo)peptide ABC transporter, permease protein OppB	<i>oppB</i>
KQ76_04645		-2.07	AI-2E family transporter	<i>perM</i>
KQ76_05220	-2.55	-2.75	Spermidine/putrescine import ATP-binding protein	<i>potA</i>
KQ76_05225	-2.32	-2.66	Spermidine/putrescine import ABC transporter permease	<i>potB</i>
KQ76_05230	-2.62	-3.04	Spermidine/putrescine import ABC transporter permease	<i>potC</i>
KQ76_05610		-2.25	Permease	<i>tehA</i>
KQ76_06650	-2.65	-6.42	Glycine betaine transporter OpuD/choline transporter	<i>betT</i>
KQ76_07885		-3.35	MFS-type transporter	<i>araJ</i>
KQ76_10375		-2.74	permease	<i>tehA</i>
KQ76_11365	2.51	2.32	Uncharacterized MFS-type transporter	<i>NA</i>
KQ76_11760		2.07	RND multidrug efflux transporter; Acriflavin resistance protein	<i>acrB</i>

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KQ76_12340	-2.80	-5.37	L-lactate permease	<i>lldP</i>
KQ76_13140		-2.75	lantibiotic ABC transporter ATP-binding protein	<i>ccmA</i>
KQ76_13690		-4.20	citrate transporter	<i>citM</i>
KQ76_13365	3.80	3.64	Maltose O-acetyltransferase	<i>wbbJ?</i>
KQ76_13665	-3.11	-5.00	choline transporter	<i>betT</i>
KQ76_14115	2.03		chloramphenicol-sensitive protein RarD, metabolite transporter	<i>rarD</i>
Transposase				
KQ76_01200	-2.10	-2.13	Transposase	<i>NA</i>
Virulence factors				
Camphor resistance protein				
KQ76_09270		-4.34	camphor resistance protein CrcB	<i>crcB</i>
KQ76_09275		-2.39	camphor resistance protein CrcB	<i>crcB</i>
Type VII secretion protein				
KQ76_01165	-2.38	-4.96	Putative type VII secretion accessory protein EsaA	<i>esaA</i>
KQ76_01170	-2.94	-4.42	Protein EssA	<i>essaA</i>
KQ76_01175	-2.53	-5.04	Putative type VII secretion accessory protein EsaB	<i>esaB</i>
KQ76_01180	-2.29	-3.91	Putative type VII secretion system component EssB	<i>essB</i>
KQ76_01185	-2.63	-2.90	putative protein EssC	<i>essC</i>
KQ76_01190	-3.06	-3.25	type VII secretion effector	<i>NA</i>
KQ76_01195	-2.91		DUF5082 domain-containing protein	<i>NA</i>
Secretion system				
KQ76_06590		-2.00	secretion protein	<i>ykwD</i>
KQ76_13420		-3.40	CHAP domain-containing protein / Secretory antigen precursor SsaA	<i>COG3942</i>
KQ76_13875		2.42	accessory Sec system glycosylation chaperone GtfB	<i>gtfB</i>
KQ76_13880		2.58	Poly(glycerol-phosphate) alpha-glucosyltransferase GftA (EC 2.4.1.52)	<i>gtfA</i>
KQ76_13885		2.55	Protein translocase subunit SecA clustered with accessory secretion system	<i>secA2</i>
KQ76_13905		-3.07	Protein translocase subunit SecY clustered with accessory secretion system	<i>secY2</i>
Cell surface-associated - adhesion and pathogenesis				
KQ76_02730	-2.98	-7.08	Serine-aspartate repeat-containing protein C	<i>sdrC</i>
KQ76_02740		-3.52	Serine-aspartate repeat-containing protein E	<i>sdrE</i>

KQ76_02735	-2.54	-6.58	serine-aspartate repeat-containing protein D	<i>sdrD</i>
KQ76_02770	2.17		Glucosamine-6-phosphate deaminase	<i>nagB</i>
KQ76_13785	-3.11	-4.90	Clumping factor B ClfB, fibrinogen binding protein	<i>clfB</i>
KQ76_13975		2.77	poly-beta-1,6 N-acetyl-D-glucosamine synthase	<i>plc</i>
<i>Quinolone resistance</i>				
KQ76_00270		4.08	MFS-type transporter quinolone resistance protein NorB/NorC	<i>norB/norC</i>
KQ76_03435		2.10	multidrug MFS transporter NorA	<i>norA</i>
KQ76_07115	-2.10	-2.82	quinolone resistance protein	<i>norB</i>
<i>Resistance to other antibiotics</i>				
KQ76_04990		-3.54	methicillin resistance protein FmtA	<i>fmtA</i>
KQ76_12150		-3.17	metallothiol transferase fosB	<i>gloA</i>
<i>Exotoxin</i>				
KQ76_09505	-2.36	-4.30	Exotoxin, phage associated	NA
KQ76_09510	-2.90	-5.07	Exotoxin, phage associated @ Superantigen enterotoxin SEI	NA
KQ76_09515	-3.40	-5.10	Exotoxin, phage associated	NA
KQ76_09520	-3.02	-7.89	Exotoxin, phage associated	NA
<i>Regulator of virulence factors</i>				
KQ76_10520	-2.01	-3.79	Accessory gene regulator B	
KQ76_10525		-3.96	Accessory gene regulator D (pheromone precursor, type III)	NA
KQ76_10530		-3.87	Accessory gene regulator C (sensor histidine kinase)	<i>agrC</i>
KQ76_10535		-3.71	Accessory gene regulator A (response regulator)	<i>lytT</i>
<i>Toxin</i>				
KQ76_01625		-3.04	mRNA interferase PemK	<i>pemK</i>
KQ76_11505	2.12	4.61	Truncated cell surface protein map-w / toxin	NA
KQ76_12640	3.55	6.60	Cytolytic pore-forming protein S component => Gamma-hemolysin HlgA	<i>hlgA</i>
KQ76_12645	3.26	6.46	Cytolytic pore-forming protein S component => Gamma-hemolysin HlgC	<i>hlgC</i>
KQ76_12650	2.97	5.82	Cytolytic pore-forming protein F component => Gamma-hemolysin HlgB	<i>hlgB</i>
<i>Pathogenesis</i>				

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KQ76_12630		2.42	IgG-binding protein SBI	<i>sbi</i>
<i>Phage integrase</i>				
KQ76_07765		2.30	Phage/site-specific integrase	
KQ76_10480		-4.21	site-specific integrase, superantigen-encoding pathogenicity islands SaPI	
<i>Pathogenicity island</i>				
KQ76_01700		3.93	Pathogenicity islands protein	NA
KQ76_10400		3.93	Pathogenicity islands protein	NA
<i>Sporulation</i>				
KQ76_03845		2.76	Sporulation transcription regulator WhiA	<i>whiA</i>
<i>Biofilm formation</i>				
KQ76_00395		-2.95	Exopolysaccharide biosynthesis glycosyltransferase	<i>epsF</i>
<i>General virulence factor</i>				
KQ76_00250		-4.04	1-phosphatidylinositol phosphodiesterase	<i>plc</i>
KQ76_05625		-2.70	hemolytic protein	NA
KQ76_06865		-2.07	Virulence factor B	<i>cvfB</i>
Unknown				
KQ76_01470		3.11	Luciferase-like monooxygenase YhbW	<i>yhbW</i>
KQ76_03725	2.53	2.06	YigZ family protein	<i>YIH1</i>
KQ76_03840		2.88	YvcK family protein	<i>cofD</i>
KQ76_04160		2.95	nitronate monooxygenase	<i>yrpB?</i>
KQ76_04380		-2.84	TVP38/TMEM64 family protein	<i>TVP38</i>
KQ76_05010		-2.70	quinol oxidase subunit 2	<i>cyoA?</i>
KQ76_05520	-2.14	-2.44	formyl peptide receptor	NA
KQ76_05570		2.16	Transposase, IS4 family	COG3666, COG3666
KQ76_06220		-2.50	metallophosphoesterase	<i>ymdB</i>
KQ76_06725		2.06	IS1182 family transposase ISSau3	COG3666, COG3666
KQ76_06860		-2.18	Phosphate-binding protein PstS	<i>pstS</i>
KQ76_07880		-2.54	carbohydrate kinase	<i>rbsK</i>
KQ76_11965	-2.20		CHAP domain-containing protein	COG3942

KQ76_12130		2.69	N-acetyl-L,L-diaminopimelate deacetylase homolog	<i>abgB</i>
KQ76_13635		2.85	antibiotic biosynthesis monooxygenase	<i>hmoA</i>
KQ76_14080		2.46	polyisoprenoid-binding protein	<i>yceI</i>
KQ76_14280	-2.83	-3.55	lactococcin 972 family bacteriocin	NA

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Table S6.6. List and the functional groups of the differentially expressed genes of Ag⁺-resistant (Ag-r) *S. aureus* strain at high Ag⁺ concentration (high-Ag, 4.5 µg Ag/mL) relative to the untreated Ag-r strain. Highlighted tables are the genes or pathways discussed in the main text.

Locus tag	Log2 fold change	Annotations	Gene
	Ag-r high-Ag vs Ag-r NT		
Amino acid metabolism			
L-asparagine			
KQ76_07350	-2.31	L-asparaginase	ansA
KQ76_10115	-2.36	Aspartyl-tRNA(Asn) amidotransferase subunit B	gatB
KQ76_10120	-3.08	Aspartyl-tRNA(Asn) amidotransferase subunit A	gatA
KQ76_10125	-4.03	Aspartyl-tRNA(Asn) amidotransferase subunit C	gatC
L-glutamate			
KQ76_13360	2.04	L-glutamate gamma-semialdehyde dehydrogenase	adhE
L-isoleucine			
KQ76_06230	2.21	2-oxoacid ferredoxin oxidoreductase subunit beta	porB
KQ76_07125	-2.48	serine/threonine dehydratase	ilvA
KQ76_10665	2.19	Threonine dehydratase biosynthetic	ilvA
KQ76_11515	5.05	Acetolactate synthase	alsS
L-leucine			
KQ76_04450	3.83	2-isopropylmalate synthase	leuA
KQ76_10650	2.15	3-isopropylmalate dehydrogenase	leuB
KQ76_10655	2.14	3-isopropylmalate dehydratase large subunit	leuC
L-lysine			
KQ76_00355	2.59	diaminopimelate decarboxylase	lysA = sbnH
KQ76_10340	-2.61	succinyl-diaminopimelate desuccinylase	NA
KQ76_10345	3.15	succinyl-diaminopimelate desuccinylase	argE

<i>L-methionine</i>			
KQ76_09290	-2.79	S-adenosylmethionine synthetase	<i>metK</i>
KQ76_11515	5.05	Acetolactate synthase	<i>alsS</i>
<i>L-alanine</i>			
KQ76_07130	-3.04	Alanine dehydrogenase	<i>ald</i>
<i>L-arginine</i>			
KQ76_05590	-4.44	Ornithine carbamoyltransferase	<i>argF</i>
KQ76_13360	2.04	L-glutamate gamma-semialdehyde dehydrogenase	<i>adhE</i>
KQ76_13810	-2.46	Arginine deiminase	<i>arcA</i>
<i>L-proline</i>			
KQ76_13360	2.04	L-glutamate gamma-semialdehyde dehydrogenase	<i>adhE</i>
<i>L-glutamine</i>			
KQ76_00890	-2.10	GMP synthase	<i>guaA1</i>
KQ76_05765	-2.54	carbamoyl phosphate synthase small subunit	<i>carA</i>
KQ76_05770	-2.18	carbamoyl phosphate synthase large subunit	<i>carB</i>
KQ76_11050	-2.17	CTP synthetase	<i>pyrG</i>
<i>L-serine</i>			
KQ76_07125	-2.48	serine/threonine dehydratase	<i>ilvA</i>
<i>L-tryptophan</i>			
KQ76_10200	-2.39	Prephenate dehydratase	<i>pheA2</i>
<i>L-glycine</i>			
KQ76_13645	-3.01	Choline dehydrogenase	<i>betA</i>
KQ76_13650	-3.43	Betaine aldehyde dehydrogenase	<i>adhE</i>

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Cell wall metabolism			
<i>Teichoic acid</i>			
KQ76_06085	-3.45	phosphatidate cytidyltransferase	<i>cdsA</i>
<i>General cell-wall</i>			
KQ76_01130	3.45	glycyl-glycine endopeptidase	<i>lytM</i>
KQ76_04795	-4.25	Poly(glycerol-phosphate) alpha-glucosyltransferase	<i>rfaB</i>
KQ76_13430	-2.10	transglycosylase IsaA	<i>isaA</i>
KQ76_14050	-2.49	Polysaccharide Deacetylase	NA
Cysteine metabolism			
<i>L-cysteine biosynthesis</i>			
KQ76_09290	-2.79	S-adenosylmethionine synthetase	<i>metK</i>
<i>Inhibitor of cysteine protease</i>			
KQ76_10165	-4.39	Staphopain A precursor	NA
KQ76_10170	-3.85	staphostatin A	NA
Detoxification			
<i>Methylglyoxal</i>			
KQ76_01465	4.69	glyoxalase	<i>gloA</i>
KQ76_09280	2.84	glyoxal reductase	<i>ARA1</i>
KQ76_13170	2.99	glyoxalase	<i>gloA</i>
KQ76_13185	3.96	glyoxalase	<i>catE</i>
KQ76_13460	2.01	glyoxalase	NA
DNA metabolism			
<i>DNA mismatch repair</i>			
KQ76_10585	-5.90	DNA mismatch repair protein MutS	<i>mutS</i>
KQ76_13035	-2.14	DNA mismatch repair protein MutT	<i>mutT</i>

<i>DNA repair</i>			
KQ76_02535	5.79	excinuclease ABC subunit B	<i>uvrB</i>
KQ76_02825	-2.17	Uracil-DNA glycosylase	<i>Ung</i>
KQ76_03790	2.19	Excinuclease ABC subunit B	<i>uvrB</i>
KQ76_05470	2.68	Excinuclease ABC subunit C	<i>uvrC</i>
KQ76_06735	4.28	DNA repair protein	<i>umuC</i>
KQ76_08530	-2.20	Single-stranded-DNA-specific exonuclease RecJ	<i>recJ</i>
KQ76_10090	2.95	DNA polymerase IV	<i>dinP/dinB</i>
<i>Restriction modification</i>			
KQ76_01940	-2.18	type I restriction-modification system subunit M	<i>hsdM</i>
KQ76_09430	-2.06	Type I restriction endonuclease subunit S	<i>hsdS</i>
<i>Foreign DNA uptake</i>			
KQ76_06200	2.18	damage-inducible protein ClnA	<i>cinA</i>
Energy			
<i>Glycolysis</i>			
KQ76_00925	-3.75	L-lactate dehydrogenase	<i>mdh</i>
KQ76_08765	-2.13	aldehyde dehydrogenase	<i>gapA</i>
KQ76_13150	3.24	Fructose-1,6-bisphosphatase	<i>fbp2</i>
<i>TCA cycle</i>			
KQ76_02890	6.02	dihydrolipoamide dehydrogenase	<i>lpd</i>
KQ76_06000	2.06	Succinyl-CoA ligase [ADP-forming] alpha chain	<i>sucD</i>
KQ76_08805	2.34	Isocitrate dehydrogenase [NADP]	<i>lcd</i>
KQ76_08810	2.51	Citrate synthase (si)	<i>gltA</i>

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<i>Pentose phosphate pathway</i>			
KQ76_09260	3.02	Transaldolase	<i>talA</i>
<i>Fermentation of pyruvate</i>			
KQ76_00375	3.46	acetoin reductase	<i>fabG</i>
KQ76_00825	-2.32	formate acetyltransferase	<i>pflD</i>
KQ76_11510	4.98	Alpha-acetolactate decarboxylase	<i>alsD</i>
KQ76_11515	5.05	Acetolactate synthase	<i>alsS</i>
<i>Fermentation of short-fatty acids</i>			
KQ76_00825	-2.32	formate acetyltransferase	<i>pflD</i>
KQ76_00865	3.27	acetyl-CoA acetyltransferase	<i>paaJ</i>
<i>Other energy</i>			
KQ76_02890	6.02	dihydrolipoamide dehydrogenase	<i>lpd</i>
Epimerase			
KQ76_12780	4.26	epimerase	<i>wcaG</i>
Fatty acid metabolism			
<i>CDP-diacylglycerol</i>			
KQ76_06085	-3.45	phosphatidate cytidyltransferase	<i>cdsA</i>
KQ76_08975	-3.80	1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>plsC</i>
<i>Phosphatidylglycerol biosynthesis</i>			
KQ76_06085	-3.45	phosphatidate cytidyltransferase	<i>cdsA</i>
KQ76_08975	-3.80	1-acyl-sn-glycerol-3-phosphate acyltransferase	<i>plsC</i>
<i>Glycerol degradation</i>			
KQ76_00840	-2.43	glycerophosphodiester phosphodiesterase	<i>ugpQ1,ugpQ</i>
<i>Glycerolipid</i>			
KQ76_03190	3.32	dihydroxyacetone kinase subunit K	<i>dhaK</i>

KQ76_03195	3.43	dihydroxyacetone kinase subunit L	<i>dhaL</i>
KQ76_03200	3.56	PTS-dependent dihydroxyacetone kinase phosphotransferase subunit M	<i>dhaM</i>
Hydrolase			
KQ76_00265	2.77	amidohydrolase	<i>abgB</i>
KQ76_02785	-2.66	Haloacid dehalogenase-like hydrolase	<i>gph</i>
KQ76_03975	-7.00	thermonuclease	<i>yncB</i>
KQ76_11405	2.25	alpha/beta hydrolase	<i>COG4814</i>
KQ76_12745	2.90	phosphohydrolase	<i>cpdA</i>
KQ76_13165	2.39	carboxylesterase	<i>ypfH</i>
KQ76_13260	2.78	Esterase/lipase	<i>aes</i>
KQ76_13485	2.34	amidohydrolase	<i>COG2159</i>
KQ76_13490	3.61	hydrolase	<i>mhpC</i>
Iron uptake and metabolism			
<i>Siderophore synthesis</i>			
KQ76_00325	5.62	Siderophore staphylobactin biosynthesis protein SbnB	<i>sbnB</i>
KQ76_00345	2.66	Siderophore staphylobactin biosynthesis protein SbnF	<i>sbnF</i>
KQ76_00350	3.27	Siderophore staphylobactin biosynthesis protein SbnG	<i>sbnG</i>
KQ76_00360	2.59	Siderophore staphylobactin biosynthesis protein SbnI	<i>sbnI</i>
KQ76_11360	2.03	Siderophore biosynthesis protein near heme transporter	<i>htsABC</i>
<i>Iron-regulated surface determinant</i>			
KQ76_05380	-4.66	Iron-regulated surface determinant protein A	<i>isdA</i>
KQ76_05385	-3.75	Iron-regulated surface determinant protein C	<i>isdC</i>
<i>Iron storage</i>			

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KQ76_10075	4.46	Bacterial non-heme ferritin	<i>ftn</i>
Nitrogen metabolism			
KQ76_12505	-3.13	Respiratory nitrate reductase alpha chain	<i>narG</i>
KQ76_12520	-2.29	Nitrite reductase [NAD(P)H] large subunit	<i>nirB</i>
Nucleotide metabolism			
<i>Purine</i>			
KQ76_00085	-3.80	Adenylosuccinate synthetase	<i>purA</i>
KQ76_00425	2.04	Purine nucleoside phosphorylase	<i>deoD</i>
KQ76_00890	-2.10	GMP synthase	<i>guaA1</i>
KQ76_01835	-2.58	Xanthine phosphoribosyltransferase	<i>apt</i>
KQ76_02710	-2.50	Deoxyguanosine kinase	<i>dck</i>
KQ76_05035	-2.15	N5-carboxyaminoimidazole ribonucleotide mutase	<i>purE</i>
KQ76_05040	-3.41	N5-carboxyaminoimidazole ribonucleotide synthase	<i>purK</i>
KQ76_05045	-3.82	Phosphoribosylaminoimidazole-succinocarboxamide synthase	<i>purC</i>
KQ76_05050	-4.09	Phosphoribosylformylglycinamide synthase, PurS subunit	<i>purS</i>
KQ76_05055	-4.08	phosphoribosylformylglycinamide synthase, PurQ subunit	<i>purQ</i>
KQ76_05060	-3.97	phosphoribosylformylglycinamide synthase, PurL subunit	<i>purL</i>
KQ76_05065	-4.04	Amidophosphoribosyltransferase	<i>purF</i>
KQ76_05070	-4.10	Phosphoribosylformylglycinamide cyclo-ligase	<i>purM</i>
KQ76_05075	-3.99	Phosphoribosylglycinamide formyltransferase	<i>purN</i>
KQ76_05080	-3.95	IMP cyclohydrolase	<i>purH</i>
KQ76_05085	-3.75	Phosphoribosylamine--glycine ligase	<i>purD</i>
KQ76_06580	-2.20	GMP reductase	<i>guaC</i>
KQ76_10160	-3.86	Adenylosuccinate lyase	<i>purB</i>
KQ76_11595	-2.01	Adenylate kinase	<i>adk</i>

KQ76_13495	2.23	GTPase	<i>yejR</i>
<i>Pyrimidine</i>			
KQ76_05745	-2.99	phosphoribosyl transferase	<i>pyrR</i>
KQ76_05760	-2.42	dihydroorotase	<i>allB</i>
KQ76_05765	-2.54	carbamoyl phosphate synthase small subunit	<i>carA</i>
KQ76_05770	-2.18	carbamoyl phosphate synthase large subunit	<i>carB</i>
KQ76_11005	-2.70	thymidine kinase	<i>tdk</i>
KQ76_11050	-2.17	CTP synthetase	<i>pyrG</i>
<i>General nucleotide/nucleoside</i>			
KQ76_00125	-3.73	multifunctional 2',3'-cyclic-nucleotide 2'-phosphodiesterase/5'-nucleotidase/3'-nucleotidase	<i>ushA</i>
KQ76_05630	2.63	5'-nucleotidase	<i>yigB</i>
KQ76_07285	-3.59	Nucleoside diphosphate kinase	<i>ndk</i>
Other metabolism			
<i>Lipoate biosynthesis</i>			
KQ76_01415	2.36	Lipoate-protein ligase A	<i>lplA</i>
<i>Phosphopantothenate biosynthesis</i>			
KQ76_13570	2.21	Pantoate--beta-alanine ligase	<i>panC</i>
<i>Polyprenyl biosynthesis</i>			
KQ76_06080	-2.33	Undecaprenyl diphosphate synthase	<i>uppS</i>
<i>Porphyrin biosynthesis</i>			
KQ76_05300	-2.23	heme A synthase	<i>ctaA</i>
KQ76_08670	-2.52	hydroxymethylbilane synthase	<i>hemC</i>
<i>S-adenosylmethionine synthase</i>			

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KQ76_09290	-2.79	S-adenosylmethionine synthetase	<i>metK</i>
<i>ppGpp biosynthesis</i>			
KQ76_07285	-3.59	Nucleoside diphosphate kinase	<i>ndk</i>
<i>2,3-dihydroxybenzoate biosynthesis</i>			
KQ76_00675	3.72	isochorismatase	<i>pncA</i>
<i>Glutaminyl-tRNA^{Gln} biosynthesis</i>			
KQ76_10115	-2.36	Aspartyl-tRNA(Asn) amidotransferase subunit B	<i>gatB</i>
KQ76_10120	-3.08	Aspartyl-tRNA(Asn) amidotransferase subunit A	<i>gatA</i>
KQ76_10125	-4.03	Aspartyl-tRNA(Asn) amidotransferase subunit C	<i>gatC</i>
<i>N-acetylglucosamine degradation</i>			
KQ76_02770	2.18	Glucosamine-6-phosphate deaminase	<i>nagB</i>
<i>Urea cycle</i>			
KQ76_05765	-2.54	carbamoyl phosphate synthase small subunit	<i>carA</i>
KQ76_05770	-2.18	carbamoyl phosphate synthase large subunit	<i>carB</i>
KQ76_13805	-2.65	Ornithine carbamoyltransferase	<i>argF</i>
<i>Generation of free radical in anaerobic</i>			
KQ76_00830	-3.39	pyruvate formate lyase-activating protein	<i>pflA</i>
<i>Bile acid biosynthesis</i>			
KQ76_01125	5.07	Choloylglycine hydrolase	<i>yxeI</i>
<i>Isoprenoid biosynthesis</i>			
KQ76_02290	-2.70	4-diphosphocytidyl-2C-methyl-D-erythritol kinase	<i>ispE</i>
<i>Acyl-CoA metabolism</i>			
KQ76_13270	3.37	acyl-CoA thioester hydrolase	<i>fadM</i>
<i>ATPase AAA</i>			
KQ76_00165	-2.05	ATPase AAA	<i>NA</i>

<i>Holin-antiholin</i>			
KQ76_01045	-4.88	Antiholin-like protein LrgA	<i>irgA</i>
KQ76_01050	-3.51	Antiholin-like protein LrgB	<i>irgB</i>
KQ76_13290	3.59	Holin-like protein CidA	<i>yohJ</i>
<i>Cobalamin biosynthesis</i>			
KQ76_02025	4.09	Zinc-binding GTPase YciC	<i>yejR</i>
<i>Cell division</i>			
KQ76_05655	-2.79	Cell division protein FtsL	<i>ftsL2</i>
KQ76_07810	3.21	Site-specific tyrosine recombinase XerD	<i>xerD</i>
KQ76_11290	2.74	Chromosome partitioning protein ParA?	<i>parA</i>
<i>nitric oxide synthesis from L-arginine</i>			
KQ76_10195	-2.29	Nitric oxide synthase oxygenase	COG4362
Oxidative stress			
<i>Iron-sulphur cluster</i>			
KQ76_04260	2.32	iron-sulphur cluster assembly accessory protein	<i>iscA</i>
KQ76_08250	3.36	Chaperone protein DnaK	<i>dnaK</i>
KQ76_01030	-2.09	iron-sulphur cluster repair protein ScdA	<i>scdA</i>
<i>Thioredoxin</i>			
KQ76_03830	2.58	Thioredoxin reductase	<i>trxB</i>
KQ76_04050	3.46	thiol reductase thioredoxin	<i>trxA</i>
KQ76_05460	2.81	thiol reductase thioredoxin	NA
KQ76_12375	3.70	ferredoxin--NADP(+) reductase	<i>trxB</i>
<i>ROS degradation</i>			

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KQ76_06565	4.20	Catalase	<i>katE</i>
KQ76_08110	3.43	Superoxide dismutase	<i>sodA</i>
<i>Cell redox homeostasis</i>			
KQ76_01795	4.31	Alkyl hydroperoxide reductase protein F	<i>ahpF</i>
KQ76_01800	4.22	Alkyl hydroperoxide reductase protein C	<i>ahpC</i>
<i>Peroxides</i>			
KQ76_08900	2.53	2-Cys peroxiredoxin	<i>tpx</i>
KQ76_08945	2.08	peroxiredoxin	<i>osmC</i>
KQ76_09895	3.03	peroxiredoxin	<i>bcp</i>
<i>Glutathione</i>			
KQ76_06300	2.58	glutathione peroxidase	<i>btuE</i>
KQ76_13715	2.23	glutathione peroxidase	<i>btuE</i>
Oxidoreductase			
KQ76_00580	3.46	Aldehyde dehydrogenase B	<i>adhE</i>
KQ76_00755	3.80	FMN-dependent NADH-azoreductase	<i>azoR</i>
KQ76_01395	3.14	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
KQ76_01420	2.12	Aldehyde reductase	<i>wcaG</i>
KQ76_01470	4.23	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
KQ76_01805	4.29	NADPH-dependent oxidoreductase	<i>nfnB</i>
KQ76_01885	3.63	NAD(P)-dependent oxidoreductase	<i>ybjT</i>
KQ76_02720	-2.08	haloacid dehalogenase	<i>cof</i>
KQ76_02930	2.09	oxidoreductase	<i>tas</i>
KQ76_02935	2.04	NAD(P)H-dependent oxidoreductase	<i>wrbA</i>
KQ76_02955	-3.26	zinc-dependent alcohol dehydrogenase	<i>adhP</i>
KQ76_04285	6.10	pyridine nucleotide-disulfide oxidoreductase	<i>NA</i>

KQ76_04340	4.15	NADH-dependent flavin oxidoreductase	<i>fadH</i>
KQ76_06225	2.23	2-oxoglutarate ferredoxin oxidoreductase subunit alpha	<i>porA,porG</i>
KQ76_07145	-5.28	sulfite reductase subunit alpha	<i>piuB</i>
KQ76_07825	2.70	oxidoreductase	<i>tas</i>
KQ76_08465	5.20	LLM class flavin-dependent oxidoreductase	<i>ssuD</i>
KQ76_09890	3.01	2-hydroxyacid dehydrogenase	<i>serA</i>
KQ76_10505	3.38	Nitroreductase family protein	<i>FMR2</i>
KQ76_11025	2.54	Aldehyde dehydrogenase family protein	<i>adhE</i>
KQ76_11150	2.98	oxidoreductase	<i>ybjT</i>
KQ76_11395	2.66	Zinc-type alcohol dehydrogenase-like protein	<i>qor</i>
KQ76_11400	3.67	NADP-dependent oxidoreductase	<i>curA</i>
KQ76_12125	2.62	oxidoreductase	<i>fabG</i>
KQ76_12360	2.42	quinone oxidoreductase	<i>qor</i>
KQ76_12945	2.02	short-chain dehydrogenase	<i>fabG</i>
KQ76_12990	2.68	oxidoreductase	<i>ydfG</i>
KQ76_13190	3.54	NAD(P)H nitroreductase	<i>nfnB</i>
KQ76_13195	-2.33	lactate dehydrogenase	<i>ldhA</i>
Peptidase			
KQ76_00760	4.40	peptidase M23	<i>nlpD</i>
KQ76_14170	-4.77	peptidase	<i>aprE</i>
Protease			
KQ76_10495	-2.04	CPBP family intramembrane metalloprotease	<i>ydiL</i>
KQ76_12050	-3.35	CPBP family intramembrane metalloprotease	<i>ydiL</i>

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Post-translational process			
Protein modification			
KQ76_01400	3.25	glycine cleavage system protein H	<i>gcvH</i>
KQ76_01410	2.64	deacetylase SIR2	<i>SIR2</i>
Protein degradation			
KQ76_02545	5.22	ATP-dependent Clp protease, ATP-binding subunit ClpC	<i>clpC</i>
Protein binding			
KQ76_04580	2.30	adapter protein MecA	<i>mecA</i>
KQ76_08245	2.57	Chaperone protein DnaJ	<i>dnaJ</i>
Protein folding			
KQ76_10485	2.89	Heat shock protein 60 kDa family chaperone GroEL	<i>groEL</i>
KQ76_10490	2.58	Heat shock protein 10 kDa family chaperone GroES	<i>groES</i>
RNA metabolism			
RNA maturation			
KQ76_05435	-2.37	ribonuclease HIII	<i>rnhC</i>
Stress response			
Stress-induced multi chaperone system			
KQ76_02540	5.67	Protein-arginine kinase	<i>mcsB</i>
KQ76_03855	3.60	ATP-dependent Clp protease proteolytic subunit	<i>clpP</i>
KQ76_04440	5.80	Chaperone protein ClpB (ATP-dependent unfoldase)	<i>clpB</i>
KQ76_05270	-2.22	transational GTPase TypA	<i>typA</i>
KQ76_08245	2.57	Chaperone protein DnaJ	<i>dnaJ</i>
Sugar metabolism			
Gluconeogenesis			
KQ76_00680	3.96	Pyruvate decarboxylase	<i>PDC1</i>

KQ76_03860	3.61	Malolactic enzyme	<i>sfcA</i>
KQ76_08765	-2.13	aldehyde dehydrogenase	<i>gapA</i>
KQ76_13150	3.24	Fructose-1,6-bisphosphatase	<i>fbp2</i>
<i>D-mannose degradation</i>			
KQ76_11145	-3.00	Mannose-6-phosphate isomerase	<i>manA</i>
<i>N-Acetylneuraminate and N-Acetylmannosamine Degradation</i>			
KQ76_01350	-2.98	N-acetylneuraminate lyase	<i>dapA</i>
<i>D-gluconate degradation</i>			
KQ76_13085	-2.32	Gluconate kinase	<i>xyfB</i>
<i>Fermentation to acetate</i>			
KQ76_00825	-2.32	formate acetyltransferase	<i>pflD</i>
<i>Glutaryl-CoA degradation</i>			
KQ76_00865	3.27	acetyl-CoA acetyltransferase	<i>paaJ</i>
KQ76_00870	2.79	3-hydroxyacyl-CoA dehydrogenase	<i>fadB, caiD</i>
KQ76_00875	2.51	glutaryl-CoA dehydrogenase	<i>caiA</i>
<i>Other</i>			
KQ76_13365	4.52	Maltose O-acetyltransferase	<i>wbbJ</i>
Transcription			
<i>Cold shock protein</i>			
KQ76_03980	-2.48	Cold shock protein of CSP family	<i>cspC</i>
KQ76_14175	-2.92	Cold shock protein of CSP family	<i>cspC</i>
<i>Anti sigma B protein</i>			
KQ76_10715	-2.03	anti-sigma B protein RsbW	<i>rsbW</i>

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Transcriptional regulators			
KQ76_01040	-3.55	DNA-binding response regulator	<i>lytT</i>
KQ76_01060	-2.52	GntR family transcriptional regulator	<i>mngR</i>
KQ76_02505	3.12	GntR family transcriptional regulator	<i>ARO8</i>
KQ76_02530	5.70	CtsR family transcriptional regulator	<i>ctsR</i>
KQ76_02895	6.35	transcriptional regulator	<i>iscR</i>
KQ76_03285	-2.48	MarR family transcriptional regulator	<i>NA</i>
KQ76_03390	-2.11	MarR family transcriptional regulator	<i>marR</i>
KQ76_05215	-2.05	Cro/CI family transcriptional regulator	<i>hipB</i>
KQ76_07815	2.51	Fur family transcriptional regulator	<i>fur</i>
KQ76_08115	2.32	Fur family transcriptional regulator	<i>fur</i>
KQ76_08260	3.23	HrcA family transcriptional regulator	<i>hrcA</i>
KQ76_09660	-3.75	Cro/CI family transcriptional regulator	<i>XRE</i>
KQ76_10475	-2.53	transcriptional regulator	<i>hipB</i>
KQ76_11940	-3.39	MarR family transcriptional regulator	<i>marR</i>
KQ76_11955	-2.66	transcriptional regulator	<i>marR</i>
KQ76_11960	-2.89	AraC family transcriptional regulator	<i>araC</i>
KQ76_12105	-4.01	RpiR family transcriptional regulator	<i>rpiR</i>
KQ76_12320	3.07	TetR family transcriptional regulator	<i>acrR</i>
KQ76_12450	-2.18	MarR family transcriptional regulator	<i>marR</i>
KQ76_13090	-2.69	GntR family transcriptional regulator	<i>gntR</i>
Transferase			
KQ76_02245	-2.60	O-methyltransferase	<i>trmN6</i>
KQ76_01080	-2.01	SAM-dependent methyltransferase	<i>smtA</i>
KQ76_03950	2.11	N-acetyltransferase	<i>yhbS</i>

KQ76_11870	2.16	sulfurtransferase FdhD	<i>fdhD</i>
KQ76_12370	2.93	N-acetyltransferase	<i>rimI</i>
KQ76_12530	2.02	N-acetyltransferase	<i>rimI</i>
KQ76_13425	-2.91	O-acyltransferase	<i>oafA</i>
KQ76_14130	2.91	arylamine N-acetyltransferase	<i>nhoA</i>
Translations			
<i>rRNA</i>			
KQ76_00130	-2.22	23S rRNA (pseudouridine(1915)-N(3))-methyltransferase	<i>rlmH</i>
KQ76_02630	-2.05	16S rRNA (guanine(1207)-N(2))-methyltransferase	<i>rsmC</i>
KQ76_05960	-2.34	16S rRNA processing protein	<i>rimM</i>
KQ76_05650	-2.02	16S rRNA methyltransferase H	<i>rmsH</i>
KQ76_07165	-2.13	23S rRNA (guanine(2445)-N(2))-methyltransferase	<i>rlmL</i>
<i>Ribosomal protein</i>			
KQ76_01610	-4.22	30S ribosomal protein S6	<i>rpsF</i>
KQ76_01615	-4.24	single-stranded DNA-binding protein	<i>Ssb</i>
KQ76_01620	-4.14	30S ribosomal protein S18	<i>rpsR</i>
KQ76_02330	-2.37	50S ribosomal protein L25	<i>rplY</i>
KQ76_02615	-2.00	50S ribosomal protein L1	<i>rplA</i>
KQ76_02620	-2.55	50S ribosomal protein L10	<i>rplJ</i>
KQ76_02625	-2.49	50S ribosomal protein L7/L12	<i>rplL</i>
KQ76_02645	-2.99	50S ribosomal protein L7Ae-like protein	<i>rpl7Ae</i>
KQ76_02650	-3.09	30S ribosomal protein S12	<i>rpsL</i>
KQ76_02655	-2.97	30S ribosomal protein S7	<i>rpsG</i>

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KQ76_05955	-2.98	30S ribosomal protein S16	<i>rpsP</i>
KQ76_06055	-2.04	30S ribosomal protein S2	<i>rpsB</i>
KQ76_06570	2.53	50S ribosomal protein L33	<i>rpmG</i>
KQ76_06575	5.83	30S ribosomal protein S14	<i>rpsN</i>
KQ76_08100	-2.51	50S ribosomal protein L33	<i>rpmG</i>
KQ76_08240	2.46	ribosomal protein L11 methyltransferase	<i>prmA</i>
KQ76_08585	-2.11	50S ribosomal protein L21	<i>rplU</i>
KQ76_08720	-2.57	50S ribosomal protein L20	<i>rplT</i>
KQ76_08725	-2.50	50S ribosomal protein L35	<i>rpmI</i>
KQ76_08935	-2.73	30S ribosomal protein S4	<i>rpsD</i>
KQ76_11605	-2.36	50S ribosomal protein L15	<i>rplO</i>
KQ76_11610	-2.45	50S ribosomal protein L30	<i>rpmD</i>
KQ76_11615	-2.69	30S ribosomal protein S5	<i>rpsE</i>
KQ76_11620	-2.62	50S ribosomal protein L18	<i>rplR</i>
KQ76_11625	-2.91	50S ribosomal protein L6	<i>rplF</i>
KQ76_11630	-3.05	30S ribosomal protein S8	<i>rpsH</i>
KQ76_11635	-3.07	30S ribosomal protein S14	<i>rpsN</i>
KQ76_11640	-3.04	50S ribosomal protein L5	<i>rplE</i>
KQ76_11645	-2.99	50S ribosomal protein L24	<i>rplX</i>
KQ76_11650	-3.04	50S ribosomal protein L14	<i>rplN</i>
KQ76_11655	-3.00	30S ribosomal protein S17	<i>rpsQ</i>
KQ76_11660	-3.00	50S ribosomal protein L29	<i>rpmC</i>
KQ76_11665	-2.93	50S ribosomal protein L16	<i>rplP</i>
KQ76_11670	-2.82	30S ribosomal protein S3	<i>rpsC</i>
KQ76_11675	-2.74	50S ribosomal protein L22	<i>rplV</i>

KQ76_11680	-2.89	30S ribosomal protein S19	<i>rpsS</i>
KQ76_11685	-2.82	50S ribosomal protein L2	<i>rplB</i>
KQ76_11690	-2.73	50S ribosomal protein L23	<i>rplW</i>
KQ76_11695	-2.73	50S ribosomal protein L4	<i>rplD</i>
KQ76_11700	-2.80	50S ribosomal protein L3	<i>rplC</i>
KQ76_11705	-2.90	30S ribosomal protein S10	<i>rpsJ</i>
<i>tRNA-related</i>			
KQ76_02715	-5.22	tRNA-specific adenosine-34 deaminase	<i>tadA</i>
KQ76_05965	-2.26	tRNA (guanine(37)-N(1))-methyltransferase	<i>trmD</i>
KQ76_07845	-2.54	ribonuclease Z	<i>elaC</i>
<i>General translation</i>			
KQ76_02660	-2.93	Translation elongation factor G	<i>fusA</i>
KQ76_08730	-2.31	Translation initiation factor 3	<i>infC</i>
KQ76_10515	-2.43	RNAIII (delta-hemolysin)	<i>NA</i>
Transporters			
<i>Amino acid</i>			
KQ76_00670	-2.62	branched-chain amino acid transporter II carrier protein	<i>brnQ</i>
KQ76_02085	-2.33	methionine import ATP-binding protein	<i>metN</i>
KQ76_06950	-2.82	branched-chain amino acid transporter II carrier protein	<i>brnQ</i>
KQ76_07120	-2.14	amino acid permease	<i>potE</i>
KQ76_08815	-2.28	D-alanine/glycine transporter	<i>ansP</i>
KQ76_12210	-2.97	Sodium/glutamate symporter	<i>gltS</i>
<i>Carbohydrate</i>			

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KQ76_00690	-2.89	PTS glucose EIICBA component	<i>ptsG1,nagE,ptsG2</i>
KQ76_00765	-5.33	sugar ABC transporter ATP-binding protein	<i>malK</i>
KQ76_00770	-4.52	maltose ABC transporter substrate-binding protein	<i>malE</i>
KQ76_00775	-3.17	arabinogalactan ABC transporter permease	<i>ugpA</i>
KQ76_00780	-2.71	maltose ABC transporter permease	<i>malG</i>
KQ76_01435	-2.52	PTS ascorbate transporter subunit IIA	<i>ptsN</i>
KQ76_06270	-2.92	Glycerol uptake facilitator protein	<i>glpF</i>
<i>lons</i>			
KQ76_00275	-2.89	Na/Pi cotransporter	<i>nptA</i>
KQ76_00460	-2.41	phosphonate ABC transporter substrate-binding protein	<i>phnD</i>
KQ76_00585	2.63	cation efflux protein	<i>czcD</i>
KQ76_11855	-2.94	Molybdenum ABC transporter ATP-binding protein	<i>modC</i>
KQ76_11860	-3.23	Molybdenum ABC transporter permease protein	<i>moB</i>
KQ76_11865	-4.83	molybdate ABC transporter substrate-binding protein	<i>modA</i>
KQ76_12190	-2.05	sodium ABC transporter permease	<i>natB</i>
KQ76_12195	-3.06	sodium ABC transporter ATP-binding protein	<i>yhaQ</i>
KQ76_12460	-3.73	nitrate transporter	<i>narT</i>
KQ76_12440	-3.00	proton/sodium-glutamate symport protein	<i>gltT</i>
<i>Membrane protein</i>			
KQ76_00800	-4.39	membrane protein	<i>ypbQ</i>
KQ76_01145	-4.60	membrane protein	NA
KQ76_01285	-2.84	membrane protein	NA
KQ76_01480	-2.82	membrane protein	<i>yeiH</i>
KQ76_01880	3.38	membrane protein	COG3759
KQ76_04490	-2.29	membrane protein	NA

KQ76_05245	2.03	membrane protein	NA
KQ76_05310	-2.02	membrane protein	yozeB
KQ76_07095	-4.97	membrane protein	yhhQ
KQ76_12385	-2.06	membrane protein	NA
<i>Metal</i>			
KQ76_00820	-2.55	iron ABC transporter substrate-binding protein	afuA
KQ76_01495	2.32	Ferrous iron transport periplasmic protein EfeO	NA
KQ76_03095	-2.43	Manganese ABC transporter, periplasmic-binding protein SitA	sitA
KQ76_03100	-2.95	Manganese ABC transporter, inner membrane permease protein SitD	siD
KQ76_03105	-3.60	Manganese ABC transporter, ATP-binding protein SitB	sitB
KQ76_08120	2.44	iron ABC transporter permease	znuB
KQ76_08125	2.19	Zinc ABC transporter, ATP-binding protein	znuC
KQ76_12550	3.82	Zinc ABC transporter substrate-binding protein	zinT,znuA
KQ76_12915	3.62	Nickel ABC transporter permease	nikB
KQ76_12920	3.45	Nickel ABC transporter	nikA
<i>Secondary metabolite</i>			
KQ76_03175	2.23	iron-dicitrate ABC transporter ATP-binding protein	fepC
<i>Other transporters</i>			
KQ76_00740	-3.36	peptide ABC transporter permease	dppC
KQ76_00745	-2.19	peptide ABC transporter substrate-binding protein	ddpA
KQ76_00430	2.02	multidrug MFS transporter	araJ
KQ76_01120	2.63	MFS transporters	proP
KQ76_02525	-3.75	nucleoside permease	nupC

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KQ76_03160	-2.10	nucleoside permease	<i>nupC</i>
KQ76_05750	-3.20	Uracil permease	<i>uraA</i>
KQ76_06650	-3.32	choline transporter	<i>betT</i>
KQ76_07885	-2.10	MFS-type transporter	<i>araJ</i>
KQ76_08255	3.04	nucleotide exchange factor	<i>grpE</i>
KQ76_10210	-2.04	sodium-dependent dicarboxylate transporter	<i>sdcS</i>
KQ76_10375	-2.03	permease	<i>tehA</i>
KQ76_11365	2.36	MFS-type transporter	NA
KQ76_12340	-3.80	L-lactate permease	<i>lldP</i>
KQ76_13500	3.85	transporter	<i>feoB</i>
KQ76_13665	-2.73	choline transporter	<i>betT</i>
KQ76_13690	-2.31	citrate transporter	<i>citM</i>
KQ76_14145	3.57	bacitracin ABC transporter permease	<i>salY</i>
ABC transporters			
KQ76_01135	-2.59	multidrug ABC transporter	<i>ccmA</i>
KQ76_01140	-2.18	ABC transporters	NA
KQ76_02985	-3.03	ABC transporters	<i>fepB</i>
KQ76_03585	2.20	ABC transporters permease	<i>osmF, opuBB</i>
KQ76_09915	2.38	multidrug ABC transporter	<i>mdlB</i>
KQ76_13140	-2.30	lantibiotic ABC transporter ATP-binding protein	<i>ccmA</i>
KQ76_14140	4.02	bacitracin ABC transporter ATP-binding protein	<i>lolD</i>
Virulence factor			
General virulence factor			
KQ76_00250	-4.45	1-phosphatidylinositol phosphodiesterase	NA
Toxin			

KQ76_01625	-3.34	mRNA interferase PemK	NA
KQ76_10335	-2.07	gamma-hemolysin subunit B	NA
KQ76_11505	3.68	Truncated cell surface protein map-w	NA
<i>Biofilm formation</i>			
KQ76_00395	-3.43	Exopolysaccharide biosynthesis glycosyltransferase	<i>epsF</i>
<i>Quinolone resistance</i>			
KQ76_00270	2.36	MFS-type transporter quinolone resistance protein NorB/NorC	NA
<i>Secretion protein</i>			
KQ76_01165	-4.19	type VII secretion protein EsaA	<i>esaA</i>
KQ76_01170	-4.60	type VII secretion protein EssA	<i>essA</i>
KQ76_01175	-4.72	type VII secretion protein EsaB	<i>esaB</i>
KQ76_01180	-3.41	type VII secretion protein EssB	<i>essB</i>
KQ76_01185	-2.64	type VII secretion protein EssC	<i>essC</i>
KQ76_01190	-2.51	type VII secretion factor	NA
KQ76_11600	-2.04	Protein translocase subunit SecY	<i>secY</i>
KQ76_13905	-2.75	accessory Sec system protein translocase subunit	<i>secY2</i>
<i>Cell surface adhesion</i>			
KQ76_02730	-3.04	Serine-aspartate repeat-containing protein C	<i>sdrC</i>
KQ76_02735	-4.77	serine-aspartate repeat-containing protein D	<i>sdrD</i>
KQ76_13060	2.42	Fibronectin binding protein A	<i>fnbA</i>
KQ76_13785	-3.34	Clumping factor B	<i>clfB</i>
<i>Resistance to antibiotic</i>			
KQ76_04990	-3.51	methicillin resistance protein FmtA	<i>fmtA</i>

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KQ76_12150	-3.01	Fosfomycin resistance protein FosB	<i>gloA</i>
<i>Exotoxin</i>			
KQ76_09505	-2.72	Exotoxin, phage associated	NA
KQ76_09510	-3.50	Exotoxin, phage associated @ Superantigen enterotoxin SEI	NA
KQ76_09515	-4.86	Exotoxin, phage associated	NA
KQ76_09520	-4.56	Exotoxin, phage associated	NA
<i>Regulator of virulence factor</i>			
KQ76_10520	-2.56	Accessory gene regulator B	<i>agrB</i>
KQ76_10525	-2.55	Accessory gene regulator D (pheromone precursor, type III)	NA
KQ76_10530	-2.74	Accessory gene regulator C (sensor histidine kinase)	<i>agrC</i>
KQ76_10535	-2.66	Accessory gene regulator A (response regulator)	<i>lytT</i>
<i>Phage integrase</i>			
KQ76_10480	-3.87	site-specific Integrase, superantigen-encoding pathogenicity islands SaPI	<i>xerC</i>
Unknown			
KQ76_01195	-2.03	DUF5082 domain-containing protein	NA
KQ76_01155	-3.58	CHAP domain-containing protein	COG3942
KQ76_01965	-2.70	Tandem lipoprotein within Pathogenicity island	NA
KQ76_01970	-4.26	Tandem lipoprotein within Pathogenicity island	NA
KQ76_03935	-2.99	DUF5067 domain-containing protein	NA
KQ76_04160	2.20	nitronate monooxygenase	<i>yrpB</i>
KQ76_04465	-2.34	phosphatidylethanolamine-binding protein	<i>PEBP</i>
KQ76_05010	-2.04	quinol oxidase subunit 2	<i>cyoA</i>
KQ76_06220	-2.19	metallophosphoesterase	<i>ymdB</i>
KQ76_12200	-2.53	DUF805 domain-containing protein	<i>yhaH</i>
KQ76_13420	-2.27	CHAP domain-containing protein	COG3942

KQ76_14080	3.50	polyisoprenoid-binding protein	<i>yceI</i>
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Chapter 7

General Discussion and Future Directions

7.1. General discussion

Previous studies have identified the emergence of silver nanoparticle (NAg) resistance in an environmental Gram-positive bacterium, *B. subtilis*¹², and in Gram-negative pathogenic bacteria, *E. coli* and *P. aeruginosa*^{14–16}. Although resistance mechanisms of Gram-negative bacteria to NAg have been suggested^{14,15}, the molecular basis of these remain unclear. Additionally, the emergence and defence mechanisms of NAg resistance in Gram-positive pathogenic bacteria are currently still unknown.

To address this, this thesis study sought to investigate the unknown potential spread of NAg resistance and defence mechanisms and hence, selected *Staphylococcus aureus* as the target bacteria. *S. aureus* is a Gram-positive clinically-relevant pathogenic bacterium and known as the leading cause of nosocomial (hospital-related) infections. In addition, *S. aureus* do not possess the already-known silver resistance traits such as *omp*, *cus*, and *sil* system reported as ionic silver (Ag^+) resistance mechanisms identified in *E. coli* and *S. typhimurium*^{84,88,92}, as well as presence of the protein flagellin reported as NAg resistance mechanisms¹⁴. An increased production of the latter has been observed in *E. coli*, which was thought to function as the defence mechanisms of the bacterium to aggregate the nanoparticle and therefore, passivating the toxicity.

The investigation to reveal the emergence of NAg and Ag^+ resistance in *S. aureus* was the first aim of this study. This was addressed by conducting a prolonged exposure experiment to NAg and Ag^+ and subsequently, examining the dose-dependent growth responses when compared to the wild-type (WT) strain (Chapter 3). Next, to understand the NAg antibacterial mechanism, particularly in terms of the individual role of the main biologically active components of NAg, the solid particulate and soluble silver, the second aim was to elucidate the toxicological responses of *S. aureus* to NAg and Ag^+ by performing a whole transcriptomic study (Chapter 5). Ultimately, the study aimed to elucidate the molecular basis of the NAg and Ag^+ defence mechanisms in NAg- and Ag^+ -resistant *S. aureus*. This was addressed by the analysis and interpretation of the whole genomic data (Chapter 4) and whole transcriptomic data (Chapter 6).

7.1.1. The emergence of NAg resistance is likely widespread across different species.

This study discovered that *S. aureus* has the ability to develop phenotypic resistance to NAg and Ag⁺ (Chapter 3). Upon 50 days exposure to increasing NAg or Ag⁺ concentrations, the resistant *S. aureus* displayed significantly higher growth at the concentration used to inhibit the wild-type (WT) strain. NAg-resistant (NAg-r) *S. aureus* was associated with a 5-7 fold minimum inhibitory concentration (MIC) increase (of NAg) while the Ag⁺-resistant (Ag-r) *S. aureus* strain was associated with ~3 fold MIC increase (of Ag⁺) when compared to the WT strain.

Prior to this work, studies have shown the NAg and Ag⁺ resistant characteristic in *E. coli* and *P. aeruginosa*^{14–16,84,88}, Gram-negative pathogenic bacteria, as well as *B. subtilis*¹², a Gram-positive environmental bacterium. With the discovery of NAg- and Ag⁺-resistant *S. aureus*, this study suggests that the emergence of resistance could be widespread across phylogenetically-different species.

In real-life settings, for example in wound dressings, NAg is incorporated at milligram dosages³⁵, which is 1000 times higher than the generally reported bacterial NAg (microgram range). At such relatively high concentration, there should be a rather little chance for the bacteria to develop resistance spontaneously²⁷⁰. Interestingly, studies have reported that NAg can be systematically absorbed into the bloodstream and has been detected in organs and tissues, including in urinary tract at the microgram (per L, ppb, parts-per-billion) levels, following prolonged use of NAg dressings³⁵. The widespread use of NAg in consumer products has also led to its release into the environment, through disposal of effluent from waste water treatment or silver leaching into ground water from landfills or even from the use of biosolids in agriculture²⁷¹. It is estimated that the silver concentration in water and soil are in the range of ng/L and mg/kg, respectively¹²².

Such release of the biologically active silver species into the human and environmental microbial habitats can facilitate for resistance development. Wounds and urinary tract, for example, as well as water and soil, are densely populated by microorganisms. The potential prolonged exposure of these microorganisms to low, sub-inhibitory concentrations of silver can gradually select for resistant microorganisms²⁷⁰ and indeed, this work have shown that resistance development is possible across phylogenetically-different bacteria.

The findings here imply that NAg can still be a great alternative to antibiotics as the development of resistance was slow and lower than that of ciprofloxacin (the antibiotic

MIC increased by 32-fold for the ciprofloxacin-passaged strain when compared to the WT strain, see Chapter 3). Nevertheless, with the now indiscriminate use of NAg, and therefore, the increasing release and build-up of toxic silver species in human body and environment, the potential for resistance development is real. In support of public health, the knowledge generated from this study is anticipated to inform strategies for better management and regulation of the NAg use and disposal, as well as, for the much needed evidence-based regulation to balance the obligatory risk versus benefits assessment of NAg applications.

7.1.2. Potential defence mechanisms in NAg and Ag⁺-resistant *S. aureus*

7.1.2.1. Changes in bacterial metabolism

This study herein found changes in specific metabolic pathways in *S. aureus*. There are changes in pathways that give rise to the physiological adaptations of the bacterium, that is, the defence traits that resulted from the prolonged silver exposure, which will manifest even in the absence of silver (Chapter 6). These include increased expression of nucleotide synthesis genes, nitrogen metabolism genes, as well as downregulation of cell envelope metabolism genes. There are also changes that have roles in the resistance characteristics. These, like the upregulation of ROS scavenger genes and iron-sulphur (Fe-S) cluster assembly, although were also due to the prolonged exposure, yet, will only manifest in the presence of silver (Chapter 6).

Genetically-encoded resistance, either due to genomic mutations or acquisition of external resistance determinants, can lead to changes in bacterial metabolism²⁷². Indeed, this study has identified a single nucleotide mutation in the nucleotide synthesis regulator gene *purR* that encodes the purine synthesis regulator in the genome of the NAg-r strain (Chapter 4), which was hypothesised to result in the increased expression of the purine synthesis genes. Further RNA analysis confirmed this hypothesis, whereby upregulation of the whole purine operon genes was observed (Chapter 6) relative to the WT strain, when in the absence of NAg. In the case of Ag⁺ resistance, mutation was identified in *cymR* encodes a regulator for cysteine metabolism (Chapter 4), possibly intended for the later observed increased expression of cysteine synthesis and uptake genes (Chapter 6). Intrinsic resistance, on the other hand, is a natural metabolic responses that mediates resistance to a certain antibacterial agent, examples of these are the multi-efflux pumps²⁷². It is currently unclear as whether some of the herein identified metabolic changes were to a direct consequence of the mutation or were natural responses to the silver exposure.

An earlier study in *P. aeruginosa* with a mutation in *lasR*, encodes a quorum sensing regulator, reported a metabolic shift in the bacterium when compared to the parental strain²⁷³. The mutation not only caused the bacterium to grow in otherwise stressing conditions e.g. at low oxygen and high nitrate level, but also facilitated the resistance to antibiotics (tobramycin and ciprofloxacin), even with no prior exposure to these antibiotics. This study, along with others^{274,275}, have shown correlation between changes in metabolic activity to resistance. A follow-up metabolomics work is therefore particularly important in this silver study to more thoroughly understand the roles of the observed metabolic changes in the developed resistance, as further described in Section 7.2.2.

7.1.2.2. Responses to oxidative stress

This study detected unique responses in the NAg-r strain upon exposure to NAg, not seen when in the absence of the nanoparticle, which implies that these responses were not part of the physiological adaptation of the bacterium, but rather a potentially important defence features against NAg toxicity.

The transcriptomic data showed the upregulation of genes that are ‘directly’ associated with oxidative stress coping mechanism (Chapter 6), these include genes that encode cellular ROS scavengers, such as catalase and thioredoxin. This study also detected increased expression on genes that ‘indirectly’ associated with oxidative stress, for example, those that encode the Fe-S cluster assembly, cysteine metabolism and uptake, iron uptake, as well as SOS response/DNA repair (Chapter 6). It was thought that the upregulation of Fe-S clusters synthesis genes along with the cysteine metabolism and uptake as well as the iron uptake genes are intended to repair the damages on the clusters, potentially inflicted by the NAg-induced generation of oxygen radicals (the superoxide radicals). At the same time, the cell may also attempt to repair oxidative damages on DNA *via* the SOS/DNA repair responses. Oxidative stress has been known as one of the NAg’s major toxicity paradigm, ultimately leading to cell death^{9,31,99}. It is, therefore, reasonable to deduce that these ‘direct’ and ‘indirect’ responses to oxidative stress are the bacterium’s defence mechanisms to NAg toxicity, most likely one of the major resistance determinants in *S. aureus*.

It is anticipated that the herein generated knowledge of NAg resistance mechanisms could contribute to the development of the much needed strategies to overcome the emergence of NAg resistance and to monitor its potential to spread. For example, the knowledge can be used to identify novel antibacterial targets to eradicate resistant bacterial population and guide the engineering of new generation antibacterial

nanoparticles with less tendency for resistance development. The knowledge of resistance determinants, following further follow-up studies, could also benefit the tracking the resistance spread. The study also hopes to raise awareness for a more appropriate use of NAg. With no discovery of new effective antibiotics over the past three decades, it is necessary to ensure the long term success of the valuable alternative antimicrobial nanoparticle to fight untreatable infections. Future study is discussed in more detail in Section 7.2.2.

7.1.3. The defence mechanisms to NAg is unlikely a species-specific mechanism.

This study suggests that the defence mechanisms to NAg toxicity is unlikely a species-specific mechanism, instead the mechanisms are likely to be conserved functional responses across different species. One of the examples is the copper transporter genes. *Cus*, a copper transporter system, has been indicated to mediate silver resistance in Gram-negative bacteria, with the studies reporting upregulation of the genes in this system in *E. coli* upon exposure to NAg⁷². *S. aureus*, on the other hand, has no *cus* system in their genome, however, the transcriptomic data herein showed upregulation of the equivalent copper transporter system (Cop system) in the NAg-r strain, indicating that the defence response in *S. aureus* and the Gram-negative bacteria share commonalities, although the specific pathways and genes responsible differ between different species.

The ‘shared’ mechanism also applies to genes that associated with oxidative stress, that is, the upregulation of the ROS scavenger genes as well as the Fe-S assembly, cysteine metabolism and uptake, and iron uptake genes, herein observed in *S. aureus*. Indeed, these genes were also found to be upregulated in *E. coli* and *P. aeruginosa* upon exposure to NAg^{72,252}.

The effects of NAg to cell envelope metabolism, particularly the D-alanylation of teichoic acid and capsular polysaccharide biosynthesis, were observed in both *S. aureus* WT and NAg-r strain (Chapter 5 and Chapter 6). Although there has been no previous work on the NAg defence mechanisms in Gram-positive bacteria, the latter findings are consistent with the reported NAg targeting on Gram-positive cell envelope observed in *B. subtilis*⁹⁸ and *S. aureus*⁵⁴.

Taken together, while there may be unique mechanisms of NAg resistance in specific bacterial species (e.g. the reported phenotypic changes such as increased production of flagellin protein in *E. coli*¹⁴), this study highlights a likely core sets of resistance mechanisms that are conserved among bacterial species. This means that the

fundamental knowledge of the NAg defence mechanism herein generated for the Gram-positive *S. aureus* will enable the development of technologies to overcome the resistance phenomena, not only in *S. aureus*, but also, potentially in other species. This study has also shown that the silver-resistant *S. aureus*, both the NAg-r and Ag-r strains, are susceptible to the model antibiotic tested (ciprofloxacin, methicillin, vancomycin, and cefotaxime, Chapter 3), indicating no cross-resistances to these antibiotics, which implies the potential efficacy of commercial antibiotics on silver-resistant bacteria in clinical settings.

7.1.4. NAg source of toxicity potentially originates from soluble silver and solid particulate.

The transcriptomic data presented in Chapter 5 showed that the WT strain upregulated similar genes when treated with sub-inhibitory concentration of NAg or Ag⁺. These genes mapped to the siderophore biosynthesis (iron metabolism), D-alanylation of teichoic acid (cell envelope metabolism), and metal transporter functional pathways. Regardless, the WT strain was found to also significantly upregulate a substantial number of additional genes in the presence of NAg that mapped to different pathways, e.g. oxidative stress.

The increased expressions of the common NAg/Ag⁺ set of genes support the already developed notion of NAg toxicity originating from the soluble silver ions that leached from the nanoparticle. As extensively described in Chapter 2, the nanoparticle has been known to undergo oxidative dissolution when in contact with aqueous environment, releasing soluble silver ions^{18,21}. Further, the unique responses found in the NAg-treated, not seen with the Ag⁺ counterpart, recognizes the additional toxicity contribution, most likely originating from the solid particulate (that remain after leaching) of the nanoparticle.

This study underlines the potential contribution of both the leached soluble silver and the solid particulate in NAg toxicity, in agreement with similar findings by others^{22,99,112}. The knowledge herein addresses the ‘knowledge gap’ raised by regulating bodies on the toxicity characteristics of NAg, as opposed to other forms of silver. The knowledge can contribute towards the development of ‘unique’ regulation for NAg for its ‘unique’ toxicological characteristics, to move away from the current ‘silver (bulk)’ classification and toxicity reporting requirements imposed on the nanoparticle. The latter has created commercialization loopholes for NAg-incorporated products, as well as on the assessment of their human health and environmental impacts³⁵.

7.2. Future directions

This study highlighted the emergence of resistance, and for the first time, the molecular basis of physiological adaptation and resistance responses in the Gram-positive pathogenic bacterium, *S. aureus*, to the otherwise toxic NAg. It is implicated from the results that phylogenetically-diverse bacteria can develop resistance to the nanoparticle with a potentially conserved defence mechanisms to the toxicity.

The knowledge generated from this study has advanced the understanding in development of bacterial resistance to the most developed and engineered products of nanotechnology, including the molecular basis for the resistances mechanisms. Nevertheless, it is important to keep in mind that the approaches and methods used here can be considered exploratory as no prior work, particularly on the defence mechanisms of resistant *S. aureus* to NAg has ever been reported. To move forward, it will be interesting to investigate the following phenomena in future studies.

7.2.1. The potential spread and development of NAg resistance.

An important implication from the studies is that as whether the discovery of the heritable NAg resistance characteristic in *S. aureus* is relevant to other bacterial species. As earlier described in Chapter 2, the findings herein could be relevant to other species of Gram-positive bacteria, of which many, like *S. aureus*, are lack of the known silver resistance determinants (for silver ions). Future studies can be dedicated to explore this, as well as, how the different conditions of the real-world environmental and clinical settings may affect the resistance phenomena.

First, it is suggested to investigate the emergence of NAg resistance in a species with a distinct-phylogenetic profile from what has been reported previously. For instance, *Mycobacterium tuberculosis* is a pathogenic bacterium that causes tuberculosis particularly in developing countries and has evolved many mechanisms to tolerate antibiotic treatment.

The potential spread of NAg resistance could also be explored further by the investigation of NAg resistance in a real-life setting where NAg is accessible to. For instance, NAg has been incorporated in wound dressings, therefore, future studies could investigate the emergence of NAg resistance to a multi-species bacterial culture that is often found in wounds e.g. the combination of *S. aureus* and *P. aeruginosa* via a prolonged exposure. Both planktonic and biofilm forms of this multi-species culture could be considered as to emulate the real condition.

By using multi-species bacterial culture, future studies could elucidate the evolution of this bacterial culture to NAg resistance, as well as understand the pathogen-pathogen interaction, including the possibility of bacterial competition and horizontal gene transfer that allows for exchange of resistance traits, in facilitating NAg resistance development.

7.2.2. Identification of novel targets to fight off resistance

7.2.2.1. Role of the mutations in the defence mechanisms.

The genomic changes in the NAg- and Ag⁺-resistant *S. aureus* have revealed potential genetic-based defence mechanisms to NAg and Ag⁺, respectively. To confirm and to understand the effect of the mutation to resistance mechanisms, future studies could perform gene recombinant or gene knock-out of the targeted genes. As explained in Chapter 4, this study has attempted to apply CRISPRi method to knock out *purR*, *tcyA*, and *cymR* individually in the *S. aureus* wild-type (WT) strain. However, this study has not been completed and further optimization has to be conducted.

Following CRISPRi, future studies could determine the MIC of the *S. aureus* WT mutant to NAg or Ag⁺ to confirm if the repression to the targeted genes alters the susceptibility of the strain when compared to *S. aureus* WT. Other than that, the *S. aureus* generated mutant could be subjected to whole transcriptomic study as to investigate the resistance responses in the presence of NAg.

7.2.2.2. Impact of NAg and Ag⁺ to the metabolic pathways of the silver resistant strains.

It has been shown from the findings herein that changes in metabolic pathways may be one of the bacterial defence mechanisms to NAg and Ag⁺ toxicity, however, it is currently unclear if the metabolic changes detected here were a direct consequence of the mutation or responses to the silver exposure.

Intracellular metabolic pathways are a complex network consisting of inter correlated series of reactions. This can be seen from purine metabolic pathway that, in addition to nucleotide synthesis, is closely associated with amino acid metabolism, e.g. thiamine, histidine, glycine, and alanine, and ATP synthesis. The purine changes within the cell may result a chain reaction that influences important cellular processes such as translation process and DNA replication. In addition, purine metabolism has been reported to influence virulence factors or metal homeostasis in bacteria^{267,268}. Therefore, to understand the relationship between metabolic pathways and the silver exposure, future work could consider the approach of global metabolomics study in the

NAg-r, Ag-r and WT strains. The results could point out the metabolic pathway that NAg or Ag⁺ affect directly and unravel the intrinsic bacterial defence mechanisms to NAg or Ag⁺.

Modulating the metabolism responses might be one of the follow up studies after metabolomics analysis. Indeed, studies have shown that modulating the metabolism responses could be one of the solutions to acquire susceptibility of a resistant bacteria to a particular antibacterial agent^{276,277}. Vestergaard *et al.*, for example, reported the possibility of turning intrinsically-resistant *S. aureus* to polymyxin B (MIC = 128 µg/mL) to susceptible (MIC = 16 µg/mL) by inhibiting ATP synthesis, one of the basic, yet essential, cellular processes, with olygomycin A²⁷⁶. Therefore, it was thought that, the identification of directly-affected metabolic pathway by NAg or Ag⁺ may become the future target for metabolic intervention in NAg-r or Ag-r strains, to assess the possibility for the resistant strain to lose their resistance characteristics.

Additionally, the findings here also imply that the NAg-r strain, in the presence of NAg, was not only showing expression of genes associated with direct oxidative stress scavengers, *e.g.* thioredoxin and antioxidant, but also genes associated with metabolism, *e.g.* iron and cysteine, to cope with the oxidative stress impact to Fe-S cluster. Therefore, it was thought that there is possibly a link between ROS and bacterial metabolism that could be further used as a novel target to fight NAg toxicity off or to perhaps control the level of cellular ROS *via* metabolic shifts.

Indeed, a recent study reported the effect of extracellular iron addition to *E. coli* in the presence of toxic manganese (Mn) ion²⁷⁸. *Via* a biochemical assay and proteomic study, iron addition was observed to restore not only growth of *E. coli*, but also the level of ATP and aconitase, one of the enzymes in TCA cycle, to a comparable level of the untreated *E. coli*. These results were not observed when extracellular ROS quencher, *e.g.* tiron, thiourea or sodium pyruvate, was added. Interestingly, the ROS level was seen to be repressed by the addition of iron, altogether indicating a link between ROS and bacterial metabolism.

Collectively, although still currently unclear here, it was evident from earlier studies that metabolism may contribute to the resistance mechanisms. It is therefore essential to validate the findings in this study, to confirm the indicated changes in *S. aureus* cellular pathways and their roles in NAg resistance development using metabolomic study, for detection of small molecule metabolites, and bacterial phenotypic characterization, to identify changes in cell wall characteristics and defence against oxidative stress.

7.2.2.3. Identification of conserved resistance responses.

It was implied from the transcriptomic results that the defence mechanisms to NAg are unlikely to be specific to a species (Chapter 6), instead this study suggests that the mechanisms of defence have conserved functional responses across diverse bacteria. For example, responses to oxidative stress, e.g. upregulation of antioxidant, glutathione, and SOS responses genes, have been reported by earlier studies that exposed *E. coli* or *P. aeruginosa* to NAg^{72,184,252}. Similar cases were also identified in upregulation of iron uptake and copper transporters, albeit in different molecular systems. However, these earlier studies have only been conducted in NAg-susceptible bacteria, thus, there are still possibilities that the resistant *E. coli* or *P. aeruginosa* may respond differently.

To investigate the deduction made in this study, it will be interesting for a future study to perform multi-omics studies (genomic, transcriptomic, and/or metabolomics studies) to these NAg-resistant strain from different species, e.g. in *E. coli*, *P. aeruginosa*, and *S. aureus*, in the absence or presence of NAg. From there, scholars could identified signature gene expression or conserved pathways, hence a deeper understanding to NAg resistance mechanisms.

In addition to that, future studies could identify common resistance responses from the NAg-resistant species in the presence or absence of NAg to the responses from an antibiotic-resistant species in the presence or absence of the respective antibiotic. This was suggested as some of the responses identified here was also seen when the bacteria was exposed to a certain antibiotic, for instance, upregulation of purine biosynthesis genes in vancomycin-resistant *S. aureus*²³². Investigating the conserved resistance responses between NAg and antibiotics may aid the discovery of the cross-resistance phenomenon between the two antibacterial agents, and subsequently, may prevent the emergence of cross-resistance bacteria.

Altogether, these future studies (Section 7.2.2.1 - 7.2.2.2) may serve one purpose, to identify novel targets to overcome resistance, for example, the impact of purine, iron, and cysteine metabolisms, the role of metal transporters, as well as the cellular responses to oxidative stress. By having a prior knowledge on the conserved gene or pathway identified as the resistance determinants, regulatory bodies could distinguish silver resistance from a diverse bacterial species and at the same time, facilitate tracking down silver resistance spread or development. Healthcare providers may also administer suitable treatment to treat patients infected by silver-resistant bacteria, possibly using different antibacterial agents with different toxicity targets or antibacterial

agents that do not confer cross-resistance. The identified novel targets may also be used to engineer a new antimicrobial nanoparticle. Having the knowledge of the resistance mechanisms, the new antibacterial nanoparticle can be designed to prevent the occurrence of resistance development or to have a greater impact on eradicating bacterial population, *e.g.* a combination therapy of nanoparticle and antibiotic or antioxidant. Subsequently, the new antimicrobial nanoparticle could be further used to treat silver-resistant bacteria in a clinical setting.

In summary, the generated knowledge has advanced our understanding of NAg resistance phenomena, both on the emergence and molecular basis. The knowledge will enable development of technologies to overcome the phenomena through design of new-generation antimicrobial NAg. Regulatory bodies can also use the knowledge to inform strategies for the better management of NAg use and disposal. With the use of NAg as an alternative antimicrobial to fight stubborn infections, the issue is even more urgent in this era of increasing antibiotic resistance, both in the context of public health management and prevention of future outbreaks

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