Antibiotic resistance and dissemination in MRSA: novel antimicrobial coatings to combat a clinical MRSA strain

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Title of the publication	My contribution to the publication in %
Problematic groups of multi-drug resistant bacteria and their resistance mechanisms In: Ahmad I., Ahmad S., Rumbaugh K. (eds) Antibacterial Drug Discovery to Combat MDR. Springer, Singapore. (2019). Kohler V., **Vaishampayan A**., & Grohmann E.	40%
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A novel antimicrobial coating represses biofilm and virulence-related genes in methicillin-resistant *Staphylococcus aureus.* Frontiers in Microbiology. (2018). **9**, 1-14. **Vaishampayan A**., de Jong A., Wight DJ., Kok J., & Grohmann E.	70%
Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain (submitted to Materials Science and Engineering: C) **Vaishampayan A**., Ahmed R., Wagner O., de Jong A., Haag R., Kok J., & Grohmann E.	70%

List of publications and author's contribution

This thesis is based on the following publications and a manuscript

1. Problematic groups of multi-drug resistant bacteria and their resistance mechanisms

Kohler V[1]., **Vaishampayan A**[2]., & Grohmann E[2*].

[1]Department Molecular Biosciences, The Werner-Gren Institute, Stockholm University, Stockholm, Sweden

[2]Life Sciences and Technology, Beuth University of Applied Sciences Berlin, D-13347 Berlin, Germany

In: Ahmad I., Ahmad S., Rumbaugh K. (eds) Antibacterial Drug Discovery to Combat MDR. Springer, Singapore. (2019). https://doi.org/10.1007/978-981-13-9871-1_2

Ankita Vaishampayan has written the following parts of this publication: Vancomycin-Resistant *Enterococcus faecium,* Methicillin- and Vancomycin-Resistant *Staphylococcus aureus,* Clarithromycin-Resistant *Helicobacter pylori,* Fluoroquinolone-Resistant *Campylobacter* spp., Fluoroquinolone-Resistant *Salmonella* spp., Cephalosporin- and Fluoroquinolone-Resistant *Neisseria gonorrhoeae,* and Penicillin-Non-susceptible *Streptococcus pneumoniae*.

2. Broad-host-range Inc18 plasmids: occurrence, spread and transfer mechanisms

Kohler V[1]., **Vaishampayan A**[2]., & Grohmann E[2*].

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Ankita Vaishampayan has written the following parts of this publication: Occurrence of Inc18-type plasmids in the clinical setting, Occurrence of Inc18-type plasmids in environmental settings, Dissemination of Inc18-type plasmids, and Glycopeptide resistance reservoirs shared by humans and animals

3. Multi-resistant biofilm-forming pathogens on the International Space Station

Vaishampayan A[1]., & Grohmann E[1*].

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Journal of Biosciences. (2019). 44:125. https://doi.org/10.1007/s12038-019-9929-8

Ankita Vaishampayan has written all parts of this mini-review and has prepared the figure.

4. A novel antimicrobial coating represses biofilm and virulence-related genes in methicillin-resistant *Staphylococcus aureus*

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Frontiers in Microbiology. (2018). **9**, 1-14. https://doi.org/10.3389/fmicb.2018.00221

Ankita Vaishampayan has performed all the microbiological and molecular experiments, analyzed RNA-sequencing data, drafted the manuscript, and designed the figures used in this publication.

5. Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain (submitted to Materials Science and Engineering: C)

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Prof. Dr. Elisabeth Grohmann, the corresponding author of all the four publications and one manuscript mentioned in this document approves of Ankita Vaishampayan's contribution as stated above.

List of Manuscripts used in this thesis

#1. Problematic groups of multi-drug resistant bacteria and their resistance mechanisms

Kohler V., **Vaishampayan A**., & Grohmann E. In: Ahmad I., Ahmad S., Rumbaugh K. (eds) Antibacterial Drug Discovery to Combat MDR. Springer, Singapore. (2019). https://doi.org/10.1007/978-981-13-9871-1_2

#2. Broad-host-range Inc18 plasmids: occurrence, spread and transfer mechanisms

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#3. Multi-resistant biofilm-forming pathogens on the International Space Station

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Journal of Biosciences. (2019). 44:125. https://doi.org/10.1007/s12038-019-9929-8

#4. A novel antimicrobial coating represses biofilm and virulence-related genes in methicillin-resistant *Staphylococcus aureus*

Vaishampayan A., de Jong A., Wight DJ., Kok J., & Grohmann E.

Frontiers in Microbiology. (2018). **9**, 1-14. https://doi.org/10.3389/fmicb.2018.00221

#5. Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain (submitted to Materials Science and Engineering: C)

Vaishampayan A., Ahmed R., Wagner O., de Jong A., Haag R., Kok J., & Grohmann E.

The work presented in this thesis was conducted and prepared at the Department of Microbiology, Faculty of Life Sciences and Technology, Beuth Hochschule für Technik Berlin under the supervision of Prof. Dr. Elisabeth Grohmann.

Abstract

Bacterial resistance to antibiotics and spread of antibiotic resistance, especially among pathogens is one of the biggest problems today. It is not only a serious concern in the clinical and environmental settings but also in space, on manned space crafts. Bacteria use horizontal gene transfer, a highly effective mechanism for dissemination of antibiotic resistance. Some bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) are multi-drug resistant. MRSA is also capable of forming biofilms. It is a major human pathogen causing infections of skin and soft tissue that can culminate in fatal sepsis. It is a leading cause of nosocomial infections and has been declared as a high priority pathogen by the World Health Organization. Innovative approaches and antimicrobials are needed to combat infections caused by this multi-drug resistant, biofilm forming pathogen. We tested novel antimicrobial surface coatings (AGXX® and its combination with functionalized graphene oxide GOX referred to as GOX-AGXX®) against a clinical MRSA strain *S. aureus* 04-02981. The antimicrobials successfully inhibited the growth of our strain. In order to examine the effect of these antimicrobials on the transcriptome of the strain, we exposed cultures of *S. aureus* 04-02981 to the antimicrobials for different time-periods and subjected the cultures to RNA-sequencing. All the antimicrobials had a huge impact on the transcriptome of *S. aureus* 04-02981; more than 2500 genes were differentially expressed. The antimicrobials primarily down-regulated the expression of genes associated with biofilm formation and virulence in *S. aureus*; genes critical for survival in biofilms were also down-regulated. On the contrary, the expression of genes involved in heat shock, metal stress, and oxidative stress response was induced. Our data suggest that these antimicrobials impose significant stress on *S. aureus* 04-02981 as evident from the strong stress response elicited by the strain. These antimicrobials aid in alleviating biofilm formation and virulence in *S. aureus* 04-02981. Based on our data, AGXX® and GOX-AGXX® are efficient antimicrobials and can be considered suitable for various applications including antimicrobial surface coating applications.

Abstract (German)

Eines der größten Probleme der Gegenwart sind antibiotikaresistente Bakterien und ihre Ausbreitung, insbesondere die von Krankheitserregern. Sie stellen ein ernstes Problem für die Umwelt dar und sind nicht nur im klinischen Umfeld problematisch, sondern auch auf bemannten Raumfahrzeugen im Weltraum. Bakterien nutzen horizontalen Gentransfer, einen hochwirksamen Mechanismus zur Verbreitung von Antibiotikaresistenzen. Der Methicillin-resistente *Staphylococcus aureus* (MRSA) ist multiresistent und in der Lage, Biofilme zu bilden. Als bedeutender humanpathogener Erreger ruft er Haut- und Gewebsinfektionen hervor, die sich zu einer lebensbedrohlichen Sepsis entwickeln können. Zusätzlich wurde er von der Weltgesundheitsorganisation in die Gruppe von „Erregern mit hoher Priorität" aufgenommen, da er ein Auslöser nosokomialer Infektionen ist. Innovative Ansätze und antimikrobielle Mittel sind erforderlich, um Infektionen zu bekämpfen, die durch diesen multiresistenten und biofilmbildenden Erreger verursacht werden. Wir haben neuartige antimikrobielle Oberflächenbeschichtungen (AGXX® und ihre Kombination mit funktionalisiertem Graphenoxid GOX, GOX-AGXX®) gegen einen klinischen MRSA-Stamm, *S. aureus* 04-02981, getestet und festgestellt, dass sie das Wachstum unseres Stammes erfolgreich hemmen. Um die Wirkung dieser antimikrobiellen Mittel auf das Transkriptom des Stammes zu untersuchen, haben wir *S. aureus* 04-02981-Kulturen den antimikrobiellen Mitteln ausgesetzt und sie nach unterschiedlichen Expositionszeiträumen mittels RNA-Sequenzierung untersucht. Alle antimikrobiellen Substanzen hatten einen großen Einfluss auf das Transkriptom von *S. aureus* 04-02981; mehr als 2500 Gene wurden unterschiedlich exprimiert. Die antimikrobiellen Substanzen haben in erster Linie die Expression von Genen herunterreguliert, die mit der Biofilmbildung und der Virulenz von *S. aureus* assoziiert sind, sowie von Genen, die für das Überleben in Biofilmen essentiell sind. Im Gegensatz dazu wurde die Expression von Genen induziert, die an Hitzeschock-, Metall- und oxidativen Stressreaktionen beteiligt sind. Unsere Daten deuten darauf hin, dass diese antimikrobiellen Substanzen signifikant Stress auf *S. aureus* 04-02981 ausüben, wie die starke Stressreaktion des Stamms zeigt. Diese antimikrobiellen Mittel tragen zu einer verminderten Biofilmbildung und Virulenz in *S. aureus* 04-02981 bei. Unsere Daten zeigen, dass AGXX® und GOX-AGXX® antimikrobiell wirken und geeignet für verschiedene Anwendungen einschließlich antimikrobieller Oberflächenbeschichtungen sind.

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Abbreviations

MRSA	Methicillin-resistant *Staphylococcus aureus*
GO	graphene oxide
GOX	functionalized graphene oxide
VRSA	Vancomycin-resistant *Staphylococcus aureus*
VISA	Vancomycin intermediate *Staphylococcus aureus*
ROS	Reactive oxygen species
ISS	International Space Station
Fur	Ferric uptake regulator
AIP	Autoinducer peptide
OD	Optical density
CFU	Colony forming units
mL	Milliliter
L	Liter
µg	Microgram
min	Minute(s)
mg	Milligram
Ag	Silver
AGXX®	Silver and Ruthenium coating
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
RT-qPCR	Reverse transcription quantitative polymerase chain reaction

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1. Introduction

1.1. Antibiotic resistance in bacteria

Antibiotics are used in large quantities in the human health care, as well as in animal farms to treat and sometimes even to prevent bacterial infections (Kohler et al., 2019). However, excessive and improper use of antibiotics has resulted in antibiotic resistant bacteria. Some bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA) have become resistant to multiple antibiotics and such bacteria are termed as multi-drug resistant bacteria. Antibiotic resistance is a serious problem and needs immediate attention. According to Center for Disease Control and Prevention's *Antibiotic Resistant Threats in the United States*, 2019 report, more than 2.8 million people get antibiotic resistant infections in the U.S alone. These infections result in more than 35,000 deaths, annually (CDC, 2019). Moreover, antibiotic resistance related issues if unchecked, are predicted to result in about 10 million deaths every year by 2050 (O'Neill, 2016). The problem, prevalence and spread of crucial multi-drug resistant pathogens have been discussed in **Manuscript #1** (Kohler et al., 2019). **Manuscript #1** also introduces a pathogen priority list described by the World Health Organization focusing on antibiotic resistant pathogens that need urgent attention and research.

1.2. General characteristics of *Staphylococcus aureus*

Staphylococcus aureus is a Gram-positive, facultative anaerobic bacterium and is a part of normal human microflora. It predominantly occupies the respiratory tract, nasal passages, gut and the skin of healthy individuals (Lakhundi and Zhang, 2018). *S. aureus* is a commensal as well as a human pathogen and it typically colonizes the anterior nares of approximately 30% of the healthy population (Tong et al., 2015). *S. aureus* resistant to methicillin, also called as MRSA cause various infections like skin and soft tissue infections, medical-device related, surgical site and osteoarticular infections, infective endocarditis, bacteraemia, toxic shock syndrome, up to fatal sepsis (Kallen et al., 2012; Marathe et al., 2015; Tong et al., 2015). MRSA is a key human pathogen and it greatly impacts the patients in health care settings as it causes lethal nosocomial infections (Kohler et al., 2019); it is also responsible for causing about 80% of prosthetic infections (Kawada-Matsuo and Komatsuzawa, 2012).

1.3. History of antibiotic resistance, occurrence and spread of MRSA

Alexander Fleming accidentally contaminated *Staphylococcus* plates while examining them, which led to the growth of *Penicillium* on these plates. Fleming discovered that this fungus produced a substance which could lyse the *Staphylococcus* colonies on the agar plates. He examined this antibacterial substance and thus penicillin was discovered (Fleming, 1929). Penicillin was introduced as an antibiotic in the early 1940s, however, already after a few years of use (in 1945), 80% of the clinical isolates developed resistance against penicillin (Rammelkamp et al., 1992; Lowy et al., 2003; Lakhundi and Zhang, 2018). These bacteria produced plasmid-encoded β-lactamase which hydrolyzes the β-lactam ring rendering bacteria resistant to these antibiotics (Lakhundi and Zhang, 2018). Such plasmid-encoded resistances are easily disseminated by horizontal gene transfer, either by transformation, transduction, or by conjugation as thoroughly explained in **Manuscript #2** (Kohler et al., 2018).

Methicillin, which was specifically designed against β-lactamase producing staphylococci was the first semi-synthetic penicillin and was insensitive to β-lactamase. Methicillin was clinically introduced in 1959 (Lakhundi and Zhang, 2018). However, within two years of its introduction, MRSA strains had already emerged (Jevons et al., 1961; Lakhundi and Zhang, 2018). MRSA strains harbour an extra penicillin-binding protein PBP2' encoded by *mecA*; this protein has low affinity to β-lactam antibiotics. The gene *mecA* is carried on a mobile genetic element called staphylococcal chromosome cassette SCCmec, and confers resistance to β-lactam antibiotics (Chambers and DeLeo, 2009; Lakhundi and Zhang, 2018; Kohler et al., 2019).

The emergence of MRSA was reported for the first time in 1961 in the U.K. (Jevons, 1961; Katayama et al., 2000). Following the emergence in the U.K., first cases of MRSA outbreaks were reported in Australia in 1965 (Rountree et al., 1968), in the USA in 1968 (Barrett et al., 1968), and in Japan in 1989 (Kayaba et al., 1997). MRSA is now spread globally and is a leading cause of nosocomial infections (Lakhundi and Zhang, 2018).

1.4. Problems with current antibiotics in use for the treatment of MRSA infections

Development of antimicrobials has been beneficial for health-care, but the abuse of antimicrobials has led to the problem of drug-resistance making MRSA multi-drug resistant. Vancomycin is often used as a last resort antibiotic to treat MRSA infections. However,

overuse of vancomycin has led to the development of vancomycin resistant *Staphylococcus aureus* (VRSA) inhibited only at concentrations of 16 µg/ mL, and vancomycin intermediate *Staphylococcus aureus* (VISA) inhibited only at concentrations of 4-8 µg/ mL (Hiramatsu et al., 1997; Tenover et al., 2001; CDC, 2002). The emergence and prevalence of VISA and VRSA are described in detail in **Manuscript #1** (Kohler et al., 2019). If this resistance trend continues, MRSA and other bacteria will soon be pan-resistant posing a tremendous threat to the health of humans as well as animals. Hence, it is crucial to develop new classes of antimicrobials.

In addition to being multi-drug resistant, MRSA also forms firm biofilms on medical devices such as catheters and causes medical device related infections; hence biofilms are considered as a major determinant of prevalence of MRSA (Tan et al., 2015). Biofilms comprise of polysaccharides, proteins, and extracellular DNA. Biofilms protect MRSA from damage caused by physical forces as well as by antimicrobial agents rendering the bacteria recalcitrant. Biofilm formation further enhances development of antibiotic resistance in MRSA. Extenuating biofilms is a useful approach to deal with the problem of increasing spread of antibiotic resistance (Selvaraj et al., 2019). Thus, novel alternative antimicrobials are required to prevent bacterial contamination of surfaces and their transmission to patients via the surfaces.

1.5. Alternative antimicrobials

As the bacterial resistance to antibiotics increases, several alternative strategies to prevent and combat bacterial infections are being developed and tested. Bacteriophages, antimicrobial peptides, antibodies, and graphene oxide (GO) are among the relatively newer alternative approaches. Metals such as silver, gold, copper and titanium are more commonly used as antimicrobials, especially in the form of nanoparticles. Silver (17^{th} century) and copper (5^{th} and 6^{th} millennia B.C.) have been in use as antimicrobials since ancient times (Chopra et al., 2007; Grass et al., 2011). Several transition metals and metal complexes have been studied for their role as antimicrobials (Li et al., 2015). Among transition metal complexes, ruthenium-based complexes have been extensively studied for their antimicrobial nature (Gopinath et al., 2014; Li et al., 2015; Abebe and Hailemariam et al., 2016; Vaidya et al., 2019; Yadav et al., 2019).

1.5.1. Silver as an antimicrobial

Silver has been in use as an antimicrobial since the 17th century (Edward-Jones, 2009; Maillard and Hartemann, 2013) and its use was approved by the U.S. Food and Drug Administration in the 1920s (Maillard and Hartemann, 2013). Silver is still used in a wide variety of applications such as in home consumer products, clothing and fabrics, in wall paints, air conditioning, healthcare, food industry, and agricultural and industrial disinfectants (Maillard and Hartemann, 2013). Silver and silver nanoparticles are used as antimicrobials due to the increasing antibiotic resistance in bacteria (Sim et al., 2018). At least three mechanisms of the antimicrobial action of silver are known, i) silver cations react with the peptidoglycan creating pores and puncturing the cell wall (Jung et al., 2008; Sim et al., 2018), ii) silver ions inhibit cellular respiration and interfere with metabolic pathways and generate reactive oxygen species (ROS) which damage the cell leading to cell death (Morones-Ramirez et al., 2013; Sim et al., 2018), and iii) silver ions enter the cell and disrupt the replication cycle (Yakabe et al., 1980; Sim et al., 2018). However, one of the main mechanisms of action of silver is by interaction of silver with sulfhydryl groups on the surface of bacteria; silver replaces the hydrogen atoms of the thiol groups forming an 'S-Ag' bond which ultimately completely blocks respiration and electron transfer chain. This leads to cell death due to plummeting proton motive force (Mijnendockx et al., 2013). However, bacteria also demonstrate resistance towards silver (Gupta et al., 1999) which prompts us to develop new antimicrobials.

1.5.2. AGXX®

AGXX® is a surface coating antimicrobial produced by Largentec GmbH, Berlin. It is a catalyst system of transition metals that permanently generates ROS on contact with water. AGXX® can be electroplated on several carriers such as steel meshes, glass, cellulose, polydimethylsoloxane, fleece, and polypropylene and can also be processed in the form of powder. The antimicrobial coating has been tested and has proven effective against bacteria, fungi, as well as viruses (Clauss-Lendzian et al., 2018; Landau et al., 2017a; Landau et al., 2017b). AGXX® has previously shown strong antibacterial activity against *Legionella* spp. (Guridi et al., 2015), and *E. faecalis* (Clauss-Lendzian et al., 2018). AGXX® has also been successfully used in decontamination of water in industrial setting (Landau et al., 2013) and as a surface coating on the International Space Station (ISS) (Sobisch et al., 2019). The application of AGXX® to mitigate microbial contamination on the ISS is discussed in

Manuscript #3 (Vaishampayan and Grohmann, 2019). The effect of AGXX® on the growth and transcriptome of a clinical strain of MRSA is presented in **Manuscript #4** (Vaishampayan et al., 2018). AGXX® displays very low cytotoxicity as analyzed by Bouchard and co-workers on a human lung fibroblast cell line MRC-5, making it suitable in a wide range of applications including in health care settings (Bouchard et al., 2011). The antimicrobial action of AGXX® is based on the production of ROS and can be explained by the proposed mechanism presented below in figure 1.

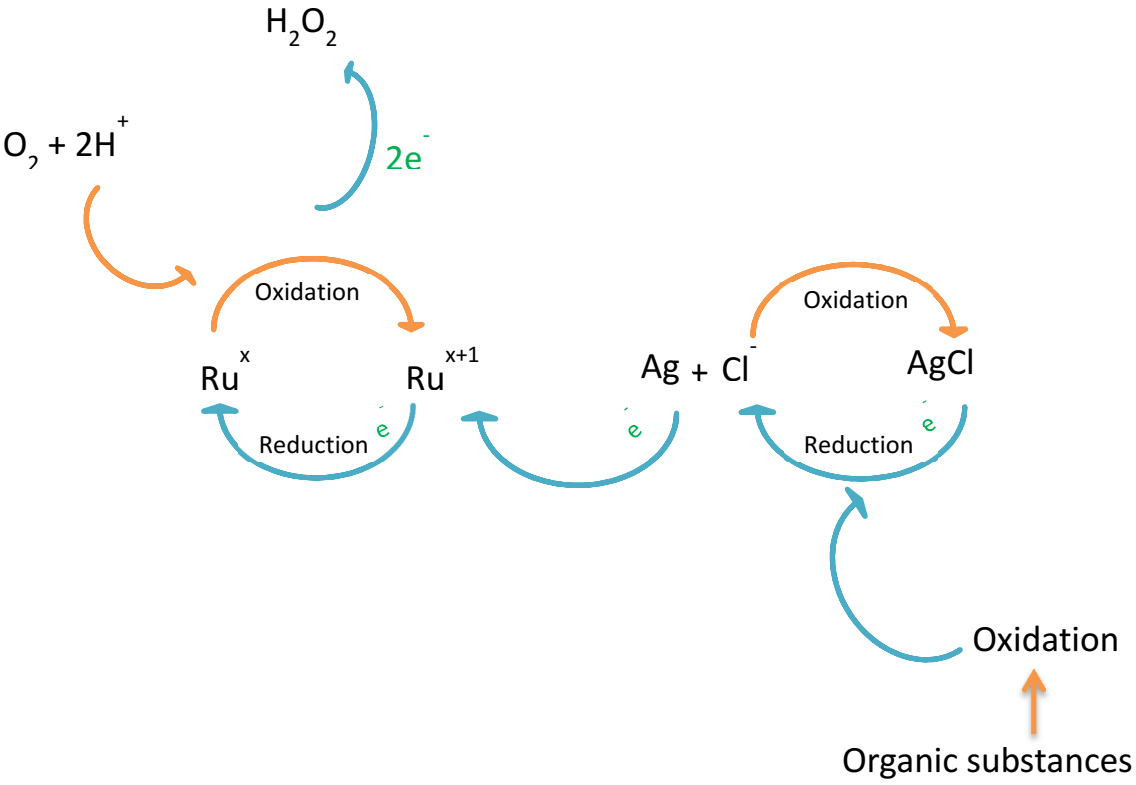

Figure 1. Putative mode of action of AGXX®. The mechanism of action of AGXX® is based on two inter-connected redox cycles resulting in its antimicrobial effect and self-renewal. The chloride (Cl^-) from the electrolyte oxidizes elementary silver (Ag^0) to form silver chloride (AgCl). Subsequently, oxidation of organic matter like sugars in bacteria leads to the reduction of AgCl to form Ag and Cl^-. The electron (e^-) from this step is taken up by Ru^{x+1} (x is used because the precise oxidation state of ruthenium is not known) forming Ru^x. All these reactions occur simultaneously. Finally, the reduction of oxygen results in the formation of H_2O and H_2O_2 and at the same time the lower valent Ru^x is oxidized to its initial state by oxygen, thus completing the cycle (the blue arrows show the pathway of electrons). Figure adapted from Clauss-Lendzian et al., 2018.

1.5.3. Graphene oxide

Graphene oxide is a derivative of graphene with hydroxyl and epoxy groups at the base and carboxyl groups at the edges. The oxygen-containing functional groups make GO a suitable candidate for biomedical applications due to its hydrophilicity, and dispersibility (Liu et al., 2017). GO is a good candidate to adsorb metal or inorganic precursors, due to its abundant functional groups on its surface (Liu et al., 2017). Since antimicrobials can be more efficient when used in combination instead of when used individually (Bauer et al., 2019) we decided to use functionalized GO (GOX) alone as well as in combination with AGXX® in **Manuscript #5** (Vaishampayan et al., personal communication). The combination may also help reduce the problem of antimicrobial resistance (Bauer et al., 2019). These two antimicrobials likely have a synergistic effect on bacteria. The proposed mode of action of the combination of GOX and AGXX® as two component system is demonstrated in figure 2.

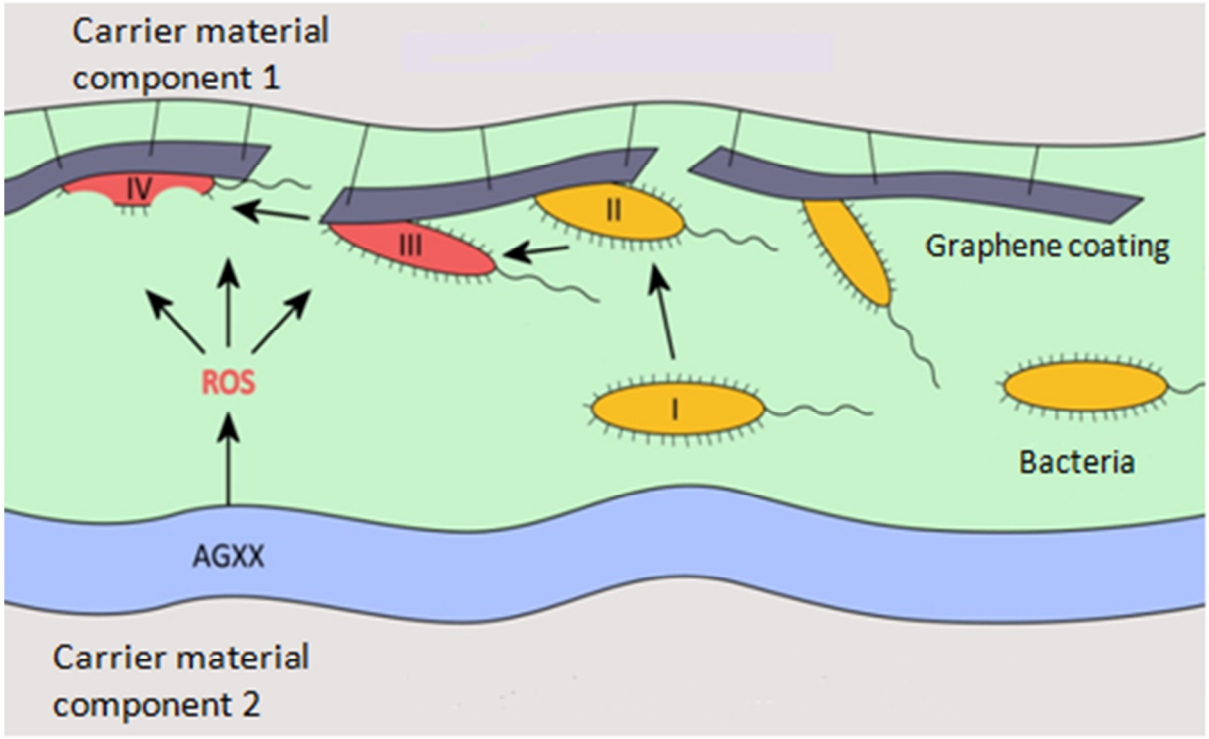

Figure 2. Mechanism of action of the two-component antimicrobial GOX-AGXX®. Component one consists of GOX that binds free bacteria (I) to be trapped on the surface of component one (II). The adsorption of bacteria is ensured by modification of the graphene layers with ionic groups. Component two consists of a carrier material which is coated with the antimicrobial surface AGXX® that forms ROS. ROS completely kill the bacteria present

in the environment (III), and subsequently, the cell structure of the bacteria is destroyed (IV). This figure has been taken from Largentec GmbH.

1.6. Bacterial stress response

Bacteria are exposed to various stresses such as changes in temperature, pH, nutrient limitation, change in metal ion concentration, etc. Bacteria respond to such stresses by modulating gene expression through regulatory networks (Ron et al., 2013). Coping up with the stresses is essential for bacterial survival. Metal stress, oxidative stress, and heat shock stress response are among the well-studied and conserved stress response pathways in bacteria.

1.7. Metal stress response

Transition metals such as iron, manganese, copper, nickel, zinc, etc., serve as crucial micronutrients for the growth and survival of *S. aureus*. These metal ions are also involved in several biochemical processes such as metabolism, regulation of virulence factors, defense against oxidative stress, and DNA synthesis (Cassat and Skaar, 2012). As essential as these metals are for *S. aureus*, they can have a detrimental effect when present in excess or scarce amounts. *S. aureus* generally responds to metal stress by producing enzymes such as superoxide dismutase (to overcome the oxidative stress imposed by heavy metals), misfolded protein responses that enhance peptide degradation, and by using heavy metal efflux pumps (Clements and Foster 1999; Wozniak et al., 2012). *S. aureus* is known to alter its gene expression mediated by ferric uptake regulator protein (Fur) to overcome the stress caused by iron-deficient conditions. Fur binds a consensus DNA sequence called the Fur box in presence of iron. The Fur box is located upstream of Fur regulated genes. Under iron-deficient conditions, Fur releases from DNA and reduces Fur-regulated transcriptional repression. This leads to repression of Fur regulon when *S. aureus* is iron-replete and de-repression when iron is depleted (Hammer and Skaar, 2011). *S. aureus* induces the transcription of siderophore biosynthesis genes (*sbn*) under iron-starvation conditions which is modulated by Fur. Fur also regulates staphylococcal virulence factors involved in attachment to host cells, and biofilm formation (Hammer and Skaar, 2011). In contrast to deficient conditions, effect of high doses of metals such as copper and silver have previously been studied on *S. aureus* and *E. faecalis* (Baker et al., 2010; Clauss-Lendzian et al., 2018). Baker et al. (2010) exposed *S. aureus* to a high concentration of copper and found that *S. aureus* responds to copper stress in mainly four ways, it induces direct copper homeostasis

mechanism, oxidative stress response, expression of misfolded protein response, and repression of global staphylococcal regulators such as Agr and Sae (Baker et al., 2010). In the study on *E. faecalis*, Clauss-Lendzian and collaborators exposed the pathogen to a metal composite of silver and ruthenium, AGXX®, and found that *E. faecalis* responded to the metal stress by up-regulating genes involved in copper homeostasis, heat shock, DNA damage, and oxidative stress response genes (Clauss-Lendzian et al., 2018).

1.8. Heat shock response

Molecular chaperones crucial for refolding of degraded and damaged proteins (heat shock proteins) are key players in bacterial stress response (Visick et al., 1995; Gottesman et al., 1997; Fleury et al., 2009). Two chaperone-involving stress response pathways have been identified in *S. aureus* (Gottesman et al., 1997; Fleury et al., 2009), one which encodes classical chaperones DnaK, GroES, and GroEL (the HrCA regulon), and the second including ClpC, ClpB, and ClpP which are regulated by the CtsR regulon. DnaK, GroES, and GroEL ensure proper assembly or preventing misfolding or correcting and refolding of proteins (Gottesman et al., 1997; Fleury et al., 2009). In *S. aureus*, the HrCA regulon is embedded within the CtsR regulon and these regulons are induced upon heat shock, oxidative or chemical stress (Chastanet et al., 2003; Anderson et al., 2006; Fleury et al., 2009). The induction of heat shock response is crucial for the bacterial cells to survive the damage caused by heat shock stress and is a conserved response in bacteria (Fleury et al., 2009).

1.9. Oxidative stress response

Oxidative stress can be endogenous or exogenous. Endogenous oxidative stress can be caused by aerobic respiration, intracellular redox reactions, and antibiotics, whereas, exogenous oxidative stress is caused by host-pathogen interaction (when the pathogen interacts with the immune system of the host) (Imlay, 2003; Gaupp et al., 2012). Oxygen is the terminal electron acceptor in the electron transport chain under aerobic conditions and its complete reduction results in generation of H_2O. However, sometimes, oxygen undergoes incomplete reduction when it comes in contact with flavoproteins such as oxidases and monooxygenases or metals like iron, or copper which leads to production of ROS such as H_2O_2 instead of H_2O in a Fenton-type reaction (Gaupp et al., 2012). Metals like silver can also enhance ROS production (Gordon et al., 2010; Mijnendockx et al., 2013). ROS oxidize nucleic acids, lipids and proteins in bacterial cells causing cell damage which ultimately may lead to cell death (Imlay, 2003). Generation of ROS produces oxidative stress in bacteria (Gordon et al., 2010).

S. aureus has several stress responses to combat the oxidative stress such as pigmentation, production of detoxifying enzymes, metal homeostasis, DNA repair, general stress response, SOS response, etc. *S. aureus* produces pigments like carotenoids which protect the bacterium from desiccation as well as quenches toxic singlet oxygen. Superoxide dismutase catalyzes the dismutation of superoxide anion to H_2O_2. Catalase and alkyl hydroperoxide reductase further reduce H_2O_2 to water and oxygen (Gaupp et al., 2012). Catalase is also known to detoxify H_2O_2 by degrading H_2O_2 to oxygen and water. Another mechanism employed by *S. aureus* to repair protein damage caused by oxidative stress is using thioredoxins. The thioredoxin system consists of two components, thioredoxin (*trxA*) and thioredoxin reductase (*trxB*). Thioredoxins catalyze the reduction of H_2O_2 to H_2O and scavenging of hydroxyl radical to peroxiredoxins and peroxidase (Gaupp et al., 2012).

Stress response, like biofilm formation and virulence is tightly regulated by extensive gene networks in *S. aureus*. MRSA contains several virulence regulators and two-component regulatory systems, some crucial regulatory systems are discussed below.

1.10. Global virulence regulators in MRSA

The major virulence regulators in *S. aureus* include DNA binding proteins such as SarA and its homologs, and two-component systems such as AgrCA, and SaeRS (Liu et al., 2016). Community-associated MRSA is highly virulent because of its quorum-sensing systems. The quorum-sensing systems use the autoinducing peptide to regulate the expression of several virulence factors such as toxins and enzymes (Quave and Horswill, 2014). Accessory gene regulator (Agr) is a quorum-sensing system in *S. aureus* (Baker et al., 2010). The *agr* locus consists of two transcripts RNA II and RNA III. The transcript RNA II contains the *agrBDCA* genes; these genes encode factors which are key to synthesize autoinducing peptide. AgrD accumulates outside the cell and serves as the peptide inducer. AgrD teams up with the membrane protease AgrB. AgrBD produce and secrete autoinducer peptide (AIP). AgrC, a sensor histidine kinase gets activated on binding with AIP (Quave and Horswill, 2014). Phosphorylation of AgrC activates AgrA. AgrA is a response regulator and promotes the production of phenol soluble modulins. (Quave and Horswill, 2014). Agr positively regulates expression of the extracellular proteins Eap and Emp which are crucial for biofilm formation in *S. aureus* (Baker et al., 2010).

The staphylococcal accessory regulator protein SarA regulates the expression of several virulence factors in *S. aureus*. *sarA* promotes biofilm formation (Li et al., 2016) and plays a

significant role in the pathogenesis in *S. aureus* (Wang et al., 2019). It allows formation of immature biofilms by repressing proteases and thermostable nuclease (Beenken et al., 2010; Slany et al., 2017).

Another important two-component system in *S. aureus* is SaeRS. It positively regulates more than 20 virulence factors such as hemolysins *hla, hlb,* and *hlgABC*, leukocidins *lukED*, and proteins such as Staphylocoagulase *coa* (Giraudo et al., 1999; Liu et al., 2016; Guo et al., 2017). SaeRS also controls the survival of *S. aureus* in human blood through regulation of coagulase production (Guo et al., 2017). In addition, SaeRS regulates the expression of adhesins and invasins which enable *S. aureus* to adhere to and invade human epithelial and endothelial cells (Giraudo et al., 1999; Guo et al., 2017).

All of these global regulators are crucial in virulence and pathogenesis of MRSA (Liu et al., 2016). Targeting these regulators using antimicrobials could be a useful approach towards alleviating the problem of MRSA infections.

2. Aims of this study

The principal aim of this thesis is to investigate the effect of the metal-based surface coating antimicrobial AGXX®, alone or in combination with GOX on the growth and transcriptome of the clinical MRSA strain *S. aureus* 04-02981 (strain reference Nübel et al., 2010). For this purpose, the experimental part of this thesis was carried out as summarized in **Manuscript #4** (Vaishampayan et al., 2018), and **Manuscript #5** (Vaishampayan et al., submitted). In **Manuscript #4**, the effect of AGXX® on the growth and biofilm forming capacity of *S. aureus* 04-02981 was tested. In **Manuscript #5**, the transcriptomic response of *S. aureus* 04-02981 on exposure to GOX or AGXX® fibers, or their combination (GOX-AGXX®) was analyzed. Basic microbiology techniques followed by total RNA-sequencing and RT-qPCR were performed in both the manuscripts.

3. Discussion

S. aureus has various virulence determinants which facilitate its evasion from the immune response of the host via adhesion, and immune subversion making it a successful pathogen (Burda et al., 2014). The expression of these virulence determinants is modulated by an intricate network of regulatory framework allowing *S. aureus* to adapt to stresses caused by external factors such as temperature, host immune response, and antimicrobials such as disinfectants, antibiotics and metal-based surface coatings (Burda et al., 2014).

We tested the effect of such a metal-based surface coating, AGXX® on MRSA. AGXX® has previously been used in a variety of applications including its application as a coating on the exterior side of the toilet door surfaces on the ISS. Microbial diversity, antibiotic resistance, and biofilm formation on the ISS along with potential solutions such as using AGXX® to moderate the microbial contamination on the ISS are discussed in detail in **Manuscript #3** (Vaishampayan and Grohmann, 2019). A study on the long term effect of AGXX® on the ISS was conducted, recently. The authors isolated and characterized the bacteria from AGXX®, Ag, and uncoated controls exposed for 6, 12, and 19 months on the ISS. Their data show that AGXX® displayed strong antimicrobial activity as the number of bacteria isolated from AGXX® was remarkably low as compared with Ag and uncoated controls (Sobisch et al., 2019). In this thesis, we have studied the gene expression patterns of *S. aureus* 04-02981 in response to stress imposed by AGXX® individually in **Manuscript #4** (Vaishampayan et al., 2018) and AGXX® in combination with GOX in **Manuscript #5** (Vaishampayan et al., submitted).

In **Manuscript #4**, we investigated the effect of AGXX® on the growth of *S. aureus* 04-02981 using basic microbiological methods such as disk diffusion assay, and growth inhibition assay by measuring optical density at 600 nm (OD_{600}) and standard plate count to determine colony forming units (CFU) per mL. A conventional silver coating, Ag, served as control. AGXX® efficiently inhibited the growth of *S. aureus* 04-02981 by 99.99%, while Ag only inhibited it by 26.58% in comparison with the untreated control. In order to test if AGXX® interfered with the biofilm forming capacity of *S. aureus* 04-02981, we performed a biofilm assay via crystal violet staining and confocal microscopy with Live/ Dead staining. In the crystal violet assay, we normalized the values obtained at OD_{570} (after crystal violet staining) to OD_{600} to consider the antimicrobial effect of AGXX®. Upon normalization, AGXX® reduced biofilm formation by 46% while Ag by 41%. In addition to the OD values,

we also determined the CFU/ mL of the biofilm. In the biofilm, AGXX® showed 99.99% reduction in CFU/ mL in comparison with the untreated control, while Ag showed only 9.13% reduction. AGXX® had a profound effect on the biofilm as observed even through confocal microscopy. We observed very few cells in presence of AGXX® and most of them appeared to be dead (stained red) in contrast to the densely packed cells observed in the untreated control. Further, to analyze the effect of AGXX® on the transcriptome of *S. aureus* 04-02981, we exposed the cultures of *S. aureus* 04-02981 to AGXX® or Ag for five different time-periods (6, 12, 24, 80, and 120 min) and subjected them to total RNA-sequencing. High sequence depth was achieved in RNA-seq with an average of 12.4 million reads. In total, 2864 genes were differentially expressed on exposure to Ag and AGXX®. These differentially expressed genes majorly belonged to biofilm and virulence regulators, heat shock response, oxidative stress response, pathogenesis, lipopolysaccharide synthesis, serine protease activity, and transcriptional regulators. Numerous genes associated with biofilm formation and virulence such as *agrABCD*, *saeRS*, *cap* genes, *srrA*, *sdrC*, *lukE*, *isdC*, etc. were downregulated. Representative genes selected from these groups, namely, *agrC, lukE, sdrC, srrA*, and *cap5A* were further tested via RT-qPCR and *gyrB* was used as a house-keeping gene. The data obtained in RNA-seq was confirmed using RT-qPCR.

In the next part of our study, presented in **Manuscript #5**, we used AGXX® in combination with GOX as using combinations of antimicrobials typically has a stronger inhibitory effect than when the antimicrobials are used individually (Cihalova et al., 2015; Slany et al., 2017; Bauer et al., 2019; Jin et al., 2019; Vaidya et al., 2019). Previously, GO and AGXX® have both been shown to have antibacterial activity (Akhavan et al., 2011; Gurunathan et al., 2012; He et al., 2015; Guridi et al., 2015; Clauss-Lendzian et al., 2018; Baptista et al., 2018; Vaishampayan et al., 2018; Jin et al., 2019; Sobisch et al., 2019). The electrostatic interaction between positively charged metal complexes and negatively charged biological structures like DNA, RNA, and phospholipids could help binding the intracellular targets of a biological cell (Li et al., 2015). Hence, we functionalized the negatively charged GO with polycationic groups to make the final product GOX positively charged which would help with binding free bacteria. The proposed mechanism of action of this two-component antimicrobial system is as follows. Component one which is GOX binds free bacteria, and component two AGXX® generates ROS causing damage to bacterial cells which ultimately leads to cell death. Thus, we examined the response of *S. aureus* 04-02981 on exposure to GOX or AGXX® fibers or combination of these two fibers GOX-AGXX® via a simple microbiology technique of

standard plate count as well molecular analyzes using RNA-seq. The microbiological assays displayed the efficiency of the combination of antimicrobials as GOX-AGXX® showed maximum growth inhibition of *S. aureus* 04-02981 (99.98%) compared with the untreated control. RNA-seq demonstrated surprising results and the effect of the GOX-AGXX® compared to GOX or AGXX® used individually was not as prominent as in the microbiology assays. Nevertheless, the data obtained via RNA-seq were intriguing. An average sequence depth of 14 million reads was achieved and 2650 genes were differentially expressed (in total) on exposure to cellulose (negative control), GOX, AGXX®, and GOX-AGXX® fibers for 0, 30, 60, 120, and 180 min. We encountered two odd trends of gene expression in the RNA-seq data, i) cellulose (uncoated) differentially expressed a vast number of genes, and ii) hardly any differential expression was observed in samples at t60. Since we did not expect uncoated cellulose to have such a major impact on the expression of genes, we looked into genes putatively associated with cellulose utilization or with carbohydrate metabolism (6-phospho-β-glucosidase *bglA*, 6-phospho-β-galactosidase *lacG*, α-D-1,4-glucosidase *malA*, catabolite control protein A *ccpA*, and the phosphotransferase system PTS IIC component). *bglA* was induced in presence of cellulose at t30, t120, and t180, in presence of GOX-AGXX® at t0 and in presence of GOX at t0, the highest up-regulation was at t30 in presence of cellulose. *ccpA* was down-regulated in presence of GOX-AGXX® at t120, and cellulose at t120 and at t180. *lacG* and PTS IIC component were induced and highest induction was observed in presence of cellulose at t30. The gene *rpiR* was also induced at t0 (GOX-AGXX®) and t30 in presence of cellulose (highest differential expression), and then repressed at t120 (GOX-AGXX®) and t180 (AGXX® and cellulose). RpiR and CcpA regulate the tricarboxylic acetic acid cycle in *S. aureus* in response to the availability of sugars, amino acids, and environmental stresses (Hartmann et al., 2013). These expression patterns display the extraordinary behaviour of cellulose fibers, particularly the cellulose fibers at t30. The exact reason behind cellulose fibers altering the expression of genes putatively involved in carbohydrate metabolism is not very clear and certainly needs further experiments which might explain the tremendous effect that cellulose fibers at t30 have on the transcriptome of the strain. Apart from some unexpected trends observed in the RNA-seq data, the antimicrobial fibers GOX, AGXX®, and GOX-AGXX® influenced the expression of several interesting genes. The antimicrobial fibers down-regulated genes involved in biofilm formation and virulence such as *sarA, agrC, saeRS*, etc., genes essential for survival in biofilms like *arcABCD*, and antibiotic resistance genes like *mecA*. On the other hand, expression of the *kdp* genes crucial for intracellular survival and pathogenesis, oxidative

stress response genes *trxA, ahpF*, and *katA*, and siderophore biosynthesis (*sbn*) genes was induced in presence of GOX, AGXX®, and GOX-AGXX® fibers.

Previous investigations on MRSA have reported similar trends of gene expression on exposure to antimicrobials as we observed in our **Manuscripts #4** and **#5**. A study was conducted scrutinizing the effect of high concentrations of copper on MRSA, measured via microarray. Their data showed an increase in the expression of *copAZ*, *ahpF*, *sodM*, and *trxAB* genes. On the other hand, the high level of copper repressed the expression of Agr and Sae suggesting the role of copper in preventing biofilms (Baker et al., 2010). In another study, the effect of two natural products, ursolic acid and resveratrol was checked on the biofilms of a clinical MRSA strain. Ursolic acid repressed genes related to metabolism, *arcA, arcB2*, and *arcD* (which are also required to survive in biofilms), and genes encoding adhesins, *isdB, srtB*, and *sdrC*. Resveratrol repressed quorum-sensing associated genes *agrABCD*, and the capsular polysaccharide synthesis genes, *cap5ABCFG* (Qin et al., 2014). Recently, a group of researchers cultivated *S. aureus* biofilms in a flow system and profiled intracellular and extracellular biofilm proteomes as well as the extracellular metabolome in comparison with planktonic cultures. The virulence regulators SigB, SaeRS, and SrrAB were more abundantly found in biofilms than in planktonic cultures (Graf et al., 2019). These genes which were more abundantly found in biofilms in their study were repressed in our data. In contrast, SodA, KatA, and AhpC were less abundant in biofilms than in planktonic cultures, most likely due to reduced oxygen levels in biofilms compared with planktonic cultures (Graf et al., 2019). These oxidative stress response genes found less abundantly in biofilms in the study by Graf and co-workers, were up-regulated in presence of the antimicrobials in our data. The studies by Baker et al. (2010) and Qin et al. (2014) bear similarity with ours and represent similar trends in gene expression as we have presented in **Manuscripts #4** and **#5**.

We found some interesting groups of genes which were differentially expressed in both the studies presented in **Manuscripts #4 and #5**. Families of transcriptional regulators were differentially expressed on exposure to the antimicrobials. Among these transcriptional regulators were SigS, LysR, and MerR family regulators.

SigS is involved in stress and virulence response in *S. aureus*; it is also a requisite for full virulence of *S. aureus* (Burda et al., 2014). The expression of *sigS* is modulated by factors involved in amino-acid biosynthesis, DNA-damage repair, and cell wall structure integrity

(Burda et al., 2014). However, *kdpD* and *lacR* are the central regulators of *sigS* expression; *lacR* modulates the expression of *sigS* indirectly whereas *kdpE* directly binds to the promoter region of *sigS* (Burda et al., 2014). *kdpDE* repress the expression of *sigS* (Burda et al., 2014). Oxidative stress also influences the expression of *sigS* and *sigS* mutants of *S. aureus* are more sensitive to ROS (Burda et al., 2014). We found *sigS* and the genes involved in oxidative stress response differentially expressed in our studies (in presence of the antimicrobials). In **Manuscript #4**, in presence of AGXX®, *sigS* was down-regulated at time-point 120 min by 2127 fold and the genes involved in oxidative stress response, namely, alkyl hydroperoxide reductase F and C (*ahpF*, and *ahpC*) were induced. *ahpF* showed an increase in expression levels at t12 (3.7 fold), t24 (5.7 fold), t80 (7 fold), and t120 (5 fold), and *ahpC* was up-regulated at t80 by 14.6 fold. The *kdpDE* genes were not significantly differentially expressed in presence of AGXX® or Ag, however, *kdpA* and *kdpB* which are also directly regulated by the *kdpDE* system were up-regulated on exposure to AGXX® at t120 by 43.6 and 11 fold, respectively. Both the *kdp* genes and oxidative stress response genes (*katA*, *trxA*, and *ahpF*) were also up-regulated in presence of GOX, AGXX® or GOX-AGXX® fibers in **Manuscript #5** (figure 6 and supplementary tables 6 and 9). The induced expression of genes involved in oxidative stress response in presence of AGXX® is indicative of formation of ROS which are lethal to bacterial cells. Taking together, the induction of oxidative stress response genes and the repression of *sigS* indicates that AGXX® might make *S. aureus* 04-02981 more sensitive to ROS (since *sigS* mutants are more sensitive to ROS) and may alleviate the virulence of this strain.

Another well-studied sigma factor is *sigB* which is an alternative transcription factor and a global stress and virulence regulator in *S. aureus* (Rachid et al., 2000). In our study, *sigB* was slightly down-regulated on exposure to AGXX® (**Manuscript #4**) as well as on exposure to cellulose, GOX, AGXX®, and GOX-AGXX® fibers (**Manuscript #5**). In **Manuscript #4**, *sigB* was found to be repressed at t24, t80, and t120 by 2.3, 4 and 5 fold, respectively. However, in **Manuscript #5**, the gene was found to be down-regulated in many samples, GOX-AGXX® at t0 (-3.1 fold), cellulose at t30 (-2.2), AGXX® at t120 (-2.4 fold), cellulose at t120 (-2.9), GOX at t120 (-2.2), and GOX at t180 (-2.4). The common trend observed in both the studies (documented in **Manuscripts #4 and #5**) is that *sigB* was differentially expressed only at the later time-points. Lack of *sigB* increases RNA III levels in *S. aureus*, and RNA III has antibiofilm effects (Lauderdale et al., 2009). Thus, *sigB* positively regulates and is important for biofilm formation in *S. aureus* (Rachid et al., 2000). Studies investigating the role of *sigB* in biofilm formation, attachment, and microcolony formation further confirm

this as *sigB* facilitates adherence to polystyrene microtiter plates and enhances bacterial microcolony formation (a phenomenon preceding biofilm formation) in *S. aureus* (Bateman et al., 2001). The repression of *sigB* in *S. aureus* 04-02981 in presence of the tested antimicrobials might suggest that these antimicrobials help in reducing biofilm formation.

The most abundantly found family of transcriptional regulators in *S. aureus* is the LysR family (Schell et al., 1993). LysR modulates the expression of several genes associated with metabolism, virulence, quorum-sensing, and motility (Maddocks et al., 2008). LysR was tremendously down-regulated in presence of AGXX® at t80 (-47600 fold) and at t120 (-11300 fold) in **Manuscript #4**. CidR transcriptional regulator belonging to the LysR family regulates the expression of the *cidABC* operon in response to acetate accumulation in the growth medium or the environment and displays a pro-death function in *S. aureus* (Yang et al., 2005; Chaudhary et al., 2016). The expression of *cidBC* in the exponential phase leads to the conversion of pyruvate to acetate. Excess acetate causes cytoplasmic acidification eventually leading to cell death (Thomas et al., 2014; Chaudhary et al., 2016). *cidAB* modulate cell death in the stationary phase of bacteria (Chaudhary et al., 2016). *cidA* acts as an effector of the induced cell lysis pathway and murein hydrolase activity and is responsible for cell lysis and biofilm formation (Chaudhary et al., 2016). In **Manuscript #4**, *cidA* was down-regulated in presence of Ag at t24 by 3.8 fold, and was up-regulated in presence of AGXX® at t80, and t120 by 3 and 8 fold, respectively. While *cidABC* is known to regulate murein hydrolase activity, the *lrgAB* operon affects antibiotic sensitivity in *S. aureus* (Yang et al., 2005). *lrgA* was up-regulated in presence of Ag at t6 by 6 fold, whereas the gene was repressed in presence of AGXX® at t12, t24, and t120 by 2.6, 3, and 4 fold, respectively (**Manuscript #4**). However, the differential expression of the genes *lrgAB* in **Manuscript #5** was time-dependent. Earlier time-points (0 and 30 min) showed down-regulation of both the genes, whereas at the later time-points, especially, at t180, the expression of both the genes was increased. Yang and co-workers demonstrated that the *cidR* gene product positively regulates the expression of the *cidABC* operon and increased levels of *cidABC* enhanced murein hydrolase activity. They propose that the *cid* and *lrg* operons encode holin and anti-holin-like proteins, respectively which regulate the murein hydrolase activity (Yang et al., 2005). Previous studies have shown that mutations in *cidA* diminished murein hydrolase activity and amplified antibiotic tolerance while mutations in *lrgAB* increased murein hydrolase activity and decreased antibiotic tolerance (Rice et al., 2005; Yang et al., 2005). The up-regulation of *cidA* and down-regulation of *lrgAB* genes in our studies may suggest that the antimicrobials increase the antibiotic sensitivity of *S. aureus* 04-02981. This might

further be supported by the fact that genes involved in methicillin resistance were affected by the tested antimicrobials. *mecA*, and *mecR1* were repressed in both our manuscripts. In **Manuscript #4**, *mecA* was repressed in presence of AGXX® at t12, and t24 by 2.3, and 5.5 fold, respectively. In **Manuscript #5**, *mecA*, and *mecR1* were down-regulated in presence of GOX-AGXX® at t120 by 10 and 14 fold, respectively. These data show that AGXX® individually as well as in combination with GOX increases the sensitivity of *S. aureus* 04-02981 to methicillin.

Bacteria alter intracellular concentrations of metals to adapt to environmental changes, maintain cellular homeostasis, and to protect themselves from excessive amounts of heavy metals causing cell damage (Jung et al., 2019). They have developed an efficient metal homeostasis system involving metal acquisition, metal efflux, sequestration, and detoxification via redox reactions regulated at transcriptional level (Jung et al., 2019). The MerR family of transcriptional regulators regulates heavy toxic metals such as arsenic, lead, mercury, and cadmium as well as essential metals such as zinc and copper (Jung et al., 2019). *merR* was down-regulated at t180 in presence of the AGXX® by 1500 fold (**Manuscript #4**). In **Manuscript #5**, a similar trend as in the expression of *lrgAB* genes was observed for *merR* (SA291_2138). The gene was repressed at earlier time-points in GOX-AGXX® at t0 (-3 fold), cellulose at t30 (-4 fold), GOX at t30 (-3 fold), and AGXX® at t30 (-2 fold), while the gene was induced at the later time-points in GOX-AGXX® at t120 (+3 fold), and in presence of cellulose at t180 (+2 fold). MerR also controls the expression of *copZA* through CueR – a MerR family transcriptional activator. In *S. aureus*, *copZA* expression is induced in presence of high concentrations of copper (Baker al., 2010). CueR directly regulates the expression of *copZA* which mediates copper induction (Gaballa et al., 2003). In *E. coli*, CueR also responds to silver and gold; CueR induces the expression of *copA* on addition of silver, copper, or gold (Stoyanov et al., 2001; Stoyanov and Brown, 2003). The *cop* operon (including the genes *copA, copY*, and *copZ*) is not only inducible by copper ions but also by silver ions as has already been shown in *Enterococcus hirae* (Odermatt et al., 1994). This seems logical since copper and silver ions both have the same charge, d^{10} electron configuration, and similar ionic radii (McQuillan and Shaw, 2014). Addition of AGXX® also induced the expression of *copA* and *copY* in *E. faecalis* (Clauss-Lendzian et al., 2018) as well as in MRSA in **Manuscript #4** (*copA*= 14 fold, and 20 fold up-regulated in presence of AGXX® at t12, and t80, respectively. *copZ*= 21 fold up-regulated in presence of AGXX® at t24) and in **Manuscript #5** (*copA*= up-regulated in presence of GOX-AGXX® at t0 andt120, and

AGXX® at t180 by approximately 4.5 fold). These data indirectly provide evidence for the toxic effect (toxic to the bacterium) of free silver ions (present in the cultures exposed to AGXX®) on *S. aureus* 04-02981.

In addition to the transcription regulators, we observed that siderophore biosynthesisgenes were highly induced in both the manuscripts. The details on differential expression are in supplementary tables 5 and 7 of **Manuscript #4**, and in supplementary table 8 of **Manuscript #5**. This could be connected to the ability of AGXX® to produce ROS. The highly oxidizing ROS may oxidize the metal binding Cys ligands in Fur ultimately leading to iron and zinc starvation (Loi et al., 2018b). Iron is a primary nutrient that *S. aureus* requires for colonization and pathogenesis (Hammer and Skaar, 2011). Vertebrate hosts trigger nutritional immunity on invasion by pathogenic bacteria. Nutritional immunity refers to sequestering iron, creating iron-deficient conditions for bacteria to mitigate bacterial pathogenesis (Hammer and Skaar, 2011). *S. aureus* overcomes nutritional immunity by inducing expression of *sbn* genes. AGXX® influences metal ion homeostasis as evident from up-regulation of the *sbn* genes in *S. aureus* 04-02981 in presence of AGXX®. Increased expression of the *sbn* genes in presence of GOX, AGXX®, and their combination is likely a stress response of *S. aureus* 04-02981 to overcome iron starvation.

Developing novel strategies to eradicate biofilms and to combat the problem of drug-resistance has led to several studies on testing various antimicrobial substances, especially, against *S. aureus*. Loi et al. (2018b) conducted a similar study like ours using AGXX®. They investigated the mechanism of action of AGXX® microparticles and its effect on the redox balance in *S. aureus* USA300 via phenotype analyses, RNA-seq, and redox biosensor measurements (Loi et al., 2018b). Their data showed an up-regulation of stress response regulons SigB and GraRS and a strong thiol-specific stress response of AGXX® (Loi et al., 2018b). In addition, they observed a strong induction of HypR, PerR, and QsrR regulons which are induced by H_2O_2, HOCl, and quinones in *S. aureus*, confirming oxidative stress imposed by the production of ROS and quinones in presence of AGXX® in *S. aureus* USA300 (Loi et al., 2018a).

Ruthenium was also used by other groups in the form of nanoparticles. Gopinath and co-workers synthesized ruthenium nanoparticles from the leaf extracts of *Gloriosa superba* and tested them against three Gram-negative and four Gram-positive bacteria. To compare the effect of ruthenium nanoparticles, they also tested vancomycin on the same bacteria. *Pseudomonas aeruginosa, Klebsiella pneumoniae*, and *Shigella dysenteriae* were resistant to

vancomycin. *Klebsiella pneumoniae* and *Shigella dysenteriae* were also resistant to ruthenium, but ruthenium inhibited the growth of *P. aeruginosa*, the zone of inhibition was 2.67 ± 0.33 mm. The zone of inhibition for *S. aureus* in presence of ruthenium nanoparticles was 3.33 ± 0.33 mm (Gopinath et al., 2014). Ruthenium nanoparticles worked more efficiently against Gram-positive bacteria than against Gram-negative bacteria. In a similar study, Vaidya et al. (2019) investigated the effect of rhodium, titanium, tantalum, zinc and ruthenium metal ions on Gram-positive and Gram-negative bacteria. Rhodium metal ions had the strongest inhibitory effect on the bacteria followed by ruthenium, *K. pneumoniae* (zone of inhibition = 11.5 mm, and 10.66 mm, respectively), *A. baumannii* (zone of inhibition = 12.5 mm, and 8 mm, respectively), and *E. faecium* (zone of inhibition = 7 mm for rhodium), in a concentration-dependent manner, with minimum inhibitory concentration of 1000mg/ L. At lower concentration of 50 mg/ L, none of the metal ions inhibited the growth of *E. faecium*. Rhodium and ruthenium metal ions showed synergistic antimicrobial activity against *E. faecium* (Vaidya et al., 2019).

In addition to using metals as antimicrobials, there have been studies where researchers have used metal-based antimicrobials in combination with another antimicrobial to enhance the efficiency. Such an investigation was carried out on MRSA using selenium nanoparticles in combination with antibiotics (ampicillin, oxacillin, and penicillin). Antibiotics with selenium nanoparticles inhibited the biofilm formation in MRSA by about 94%, whereas, antibiotics without selenium nanoparticles inhibited it only by 16%. Their data indicated that antibiotics had a higher growth inhibition effect on MRSA when used with selenium nanoparticles as compared with antibiotics without the nanoparticles (Cihalova et al., 2015). In another study, the response of *S. aureus* and *E. coli* to GO in combination with nanoparticles of titanium (Ti) and Ag was tested. Their data showed that Ti-GO-Ag nanoparticles act as efficient antibacterial agents (14 μg/ml MIC against *S. aureus*, and 4 μg/ml MIC against *E. coli*) and more efficiently than Ag or GO or Ti alone. The mechanism of these nanoparticles is by production of ROS (Jin et al., 2019) like in case of the antimicrobials GOX and AGXX® tested in the studies presented in this thesis.

4. Conclusion

In summary, AGXX® and GOX-AGXX® act as efficient antimicrobials and impose substantial stress on *S. aureus* 04-02981 as evident from the induction of siderophore biosynthesis, heat shock, copper stress and oxidative stress response genes. These

antimicrobials potentially inhibit biofilm formation in *S. aureus* 04-02981 and affect its survival in biofilms. The antimicrobial fibers also alleviate the virulence of *S. aureus* 04-02981. These multiple modes of action of GOX, AGXX®, and GOX-AGXX® give them a principal advantage over conventional antimicrobials in that they reduce the likelihood of developing resistance against these antimicrobials. This makes them suitable for various applications such as to coat medical devices like catheters, in wound dressings, and in water purification.

5. References

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Problematic Groups of Multidrug Resistant Bacteria and their Resistance Mechanisms

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Problematic Groups of Multidrug-Resistant Bacteria and Their Resistance Mechanisms

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Abstract

The occurrence of multidrug-resistant pathogenic bacteria is steadily increasing, not only in medical centers but also in food, animals and the environment, which is of primordial concern for health authorities worldwide. The World Health Organization (WHO) published a global pathogen priority list to encourage international interdisciplinary research initiatives on the occurrence, dissemination, and epidemiology of the most dangerous multiresistant pathogens with the aim to develop effective prevention strategies against the spread of these bugs and new therapeutic approaches to treat infections in agreement with the One Health concept. According to the WHO global pathogen priority list, the most critical resistant pathogens include carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* and carbapenem-resistant as well as third-generation cephalosporin-resistant *Enterobacteriaceae.* This critical group is followed by pathogens of high priority including vancomycin-resistant *Enterococcus faecium*, methicillin- and vancomycin-resistant *Staphylococcus aureus*, and clarithromycin-resistant *Helicobacter pylori*. Here, we summarize recent data on the occurrence and spread of these and other harmful resistant pathogens, on their resistance mechanisms as well as on the modes of resistance spread, as far as is known. We finish the chapter with an outlook on promising innovative strategies to treat infectious diseases caused by multiresistant pathogens – in combination with antibiotic therapy – as well as on approaches to combat the antibiotic resistance spread.

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Keywords
Antibiotic resistance · Bacterial pathogen · Biofilm · Horizontal gene transfer · Multidrug resistance · WHO pathogen priority list

Abbreviations

Agr	accessory gene regulator
BLNAR	ß-lactamase-negative ampicillin resistant
CDC	Centers for Disease Control and Prevention
COPD	chronic obstructive pulmonary disease
CRAb	carbapenem-resistant *Acinetobacter baumannii*
CRE	carbapenem-resistant *Enterobacteriaceae*
CRPa	carbapenem-resistant *Pseudomonas aeruginosa*
ESBL	extended spectrum ß-lactamase
EU	European Union
FDA	Food and Drug Administration
G-	Gram-negative
HGT	horizontal gene transfer
ICU	intensive care unit
IMP	active on imipenem
IS	insertion sequence
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	metallo-ß-lactamase
MDR	multidrug resistant
MRSA	methicillin-resistant *Staphylococcus aureus*
MSSA	methicillin-sensitive *S. aureus*
NDM	New Delhi MBL
OMP	outer membrane protein
OXA	oxacillinase
PBP	penicillin-binding protein
PMQR	plasmid-mediated quinolone resistance
PNSP	penicillin-non-susceptible *Streptococcus pneumoniae*
RND	resistance-nodulation-cell division
SCC*mec*	staphylococcal chromosome cassette *mec*
VIM	Verona integron-encoded MBL
VRE	vancomycin-resistant *Enterococci*
VREfm	vancomycin-resistant *E. faecium*
VRSA	vancomycin-resistant *S. aureus*
WHO	World Health Organization
XDR	extremely multidrug resistant

1 Introduction

Antibiotic drugs are unquestionably the most successful form of chemotherapy, and since people started to use them commercially, antibiotics have increased life expectancy in recent history by up to two decades (Shallcross 2014; Martens and Demain 2017). Nevertheless, modern mankind is facing the so-called antimicrobial resistance crisis (Barriere 2015; Martens and Demain 2017), annually accounting for an estimated two million antibiotic-resistant infections worldwide. It is proposed that, by 2050, 10 million deaths worldwide will be attributed to this issue (Robinson et al. 2016). In past times, the arsenal of new antibiotic drugs was satisfactory to manage the observed resistance in bacteria, but in recent years, overconsumption combined with the inappropriate prescription of antibiotics has resulted in the elevated occurrence of multidrug-resistant (MDR) and extremely multidrug-resistant (XDR) bacteria (Davies and Davies 2010; Banin et al. 2017). Beyond the abusive and not indicated use of antibiotics, poor infection control and substandard sanitation contribute to the resistance crisis. Widespread use of antibiotics in the agricultural industry has further accelerated this problem (Srinivas et al. 2017). For livestock applications, 50–80% of antibiotic drugs are administered (Cully 2014; Chang et al. 2015b), with a large fraction used at sub-therapeutic concentrations, aiming to promote growth and prevent diseases of livestock in several countries (Ter Kuile et al. 2016). Nevertheless, the European Union (EU) has banned the use of antibiotics as growth promotors. Further, countries outside the EU (such as the USA and Australia) have restricted the application of antibiotics in agriculture (Cogliani et al. 2011; Maron et al. 2013). Major mechanisms of how bacteria exert antibiotic resistance is, in addition to biofilm formation, also by acquiring new determinants via horizontal gene transfer (HGT) and mutations leading to suppressed antibiotic susceptibility (Blair et al. 2015). Bacterial biofilms in general show increased resistance to exsiccation, clearance by the immune system and lower susceptibility to antibiotics (Høiby et al. 2011). The increase in international mobility in the twenty-first century has had further strong effects on the spread of pathogenic bacteria throughout the world (Harvey et al. 2013; Laxminarayan et al. 2013; Shallcross 2014). The observed increasing rates of global antibiotic resistance has been accompanied with a decline in the number of companies developing new antibiotic drugs. Further, the number of approvals for new agents has significantly decreased (Chaudhary 2016; Sciarretta et al. 2016). This evolves as a major threat, as within a few years after the commercial introduction of new antibiotic drug, resistant strains are reported (Davies and Davies 2010; Smith et al. 2015). Since 1998, only two antibacterial agents that were approved by the Food and Drug Administration (FDA) have had a novel mechanism of action (Spellberg et al. 2004; Luepke et al. 2017). The problem of modified agents of known drug classes is, when widely applied, antibiotic-resistant bacterial strains might evolve more rapidly (World Health Organization 2001; Jensen et al. 2010). Thus, there is an urgent need for discovering new targets and designing new compounds. Recently, alternative therapeutics, such as phage therapy or antibodies, for the treatment of infections have been discussed

(Natan and Banin 2017; Pachón-Ibáñez et al. 2017; Tracanna et al. 2017; van der Meij et al. 2017).

Taking the alarming development and the imminence of antibiotic resistance into account, the WHO was asked to create a priority list of bacteria other than multiresistant *Mycobacterium tuberculosis*, in the hope it would support and focus research on the development of new antibiotic drugs effective against these pathogens. The introduced WHO global priority pathogen list aims to take a step forward in addressing this global crisis of antimicrobial resistance (World Health Organization 2017; Willyard 2017; Tacconelli et al. 2017). Thus, a multi-criteria decision analysis method was applied to prioritize resistant bacteria. Twenty bacterial species were selected and organized into three groups based on ten criteria. These three groups divided bacteria into critical, high-, and medium-priority pathogens (Fig. 1) (Tacconelli et al. 2017).

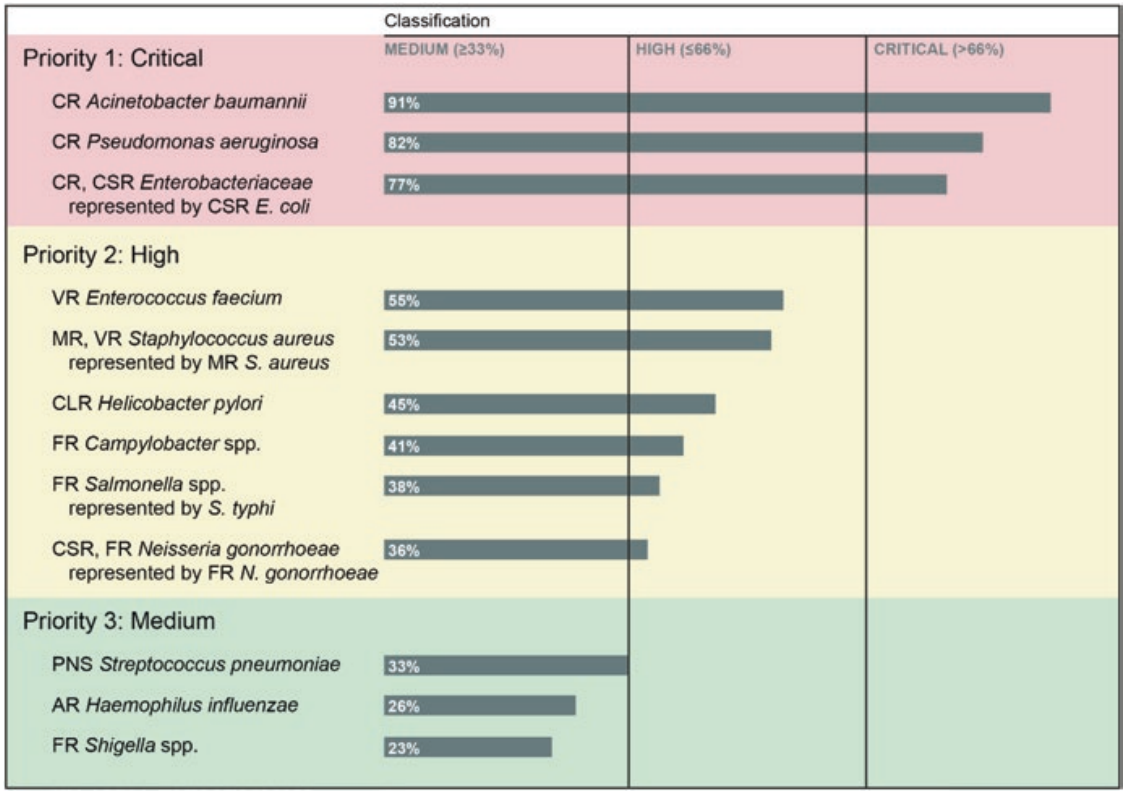

Fig. 1 Ranking of antibiotic-resistant bacteria according to the 10-criteria catalogue**.** Antibiotic-resistant bacteria were categorized according to ten criteria: treatability, mortality, healthcare burden, trend of resistance, prevalence of resistance, transmissibility, community burden, preventability in the healthcare setting, pipeline, and preventability in the community setting. 20 strains of drug-resistant bacteria were ranked and grouped according to the highest representative. Pathogens exhibiting more than 66% of the final weight were assigned to the priority 1 (critical) group, those between 33% and 66% were assigned to priority 2 (high), and bacteria less or equal 33% of final weight were ascribed to priority 3 (medium). *CR* carbapenem resistant, *CSR* 3rd-generation cephalosporin resistant, *VR* vancomycin resistant, *MR* methicillin resistant, *CLR* clarithromycin resistant, *FR* fluoroquinolone resistant, *PNS* penicillin non-susceptible, *AR* ampicillin resistant. (Figure adapted from Tacconelli et al. (2017))

In this review, we will give detailed information on bacterial species that, according to the WHO's global priority pathogen list, represent the most imminent dangers, further fueling the antibiotic resistance crisis. In addition to providing statistical information about their distribution, we will focus on the underlying mechanisms that have ultimately led to their emergence as antibiotic-resistant pathogens.

2 The Global Priority Pathogen List

2.1 Priority 1: Critical

2.1.1 Carbapenem-Resistant *Acinetobacter baumannii*

Acinetobacter are non-glucose-fermenting Gram-negative (G-) coccobacilli, primarily related with healthcare-associated infections. These bacteria harbor extensive intrinsic resistance determinants and have the capability to acquire new resistance factors (Peleg et al. 2008). *Acinetobacter baumannii*, an opportunistic pathogen, is associated with hospital-acquired infections and outbreaks worldwide, affecting particularly critically ill patients (Runnegar et al. 2010; Correa et al. 2017). The first reported *Acinetobacter* infections within an intensive care unit (ICU) date back to the 1960s (Stirland et al. 1969). Early *Acinetobacte*r-mediated infections were easily treatable with ß-lactams and sulfonamides (Stirland et al. 1969; Abrutyn et al. 1978), but these treatment strategies shortly evolved to be inefficient due to the rising resistance rates (Lecocq and Linz 1975). In the 1980s, carbapenems were used as therapeutics to treat infections caused by MDR bacteria, but resistances to these antibiotics in *Acinetobacter* were reported shortly after their commercial introduction (Paton et al. 1993; López-Hernández et al. 1998; Gonzalez-Villoria and Valverde-Garduno 2016). Carbapenems are broad-spectrum ß-lactam antibiotics, widely used as last-line antibiotics, especially for the treatment of critically ill patients and infections induced by antibiotic-resistant G- bacteria (Papp-Wallace et al. 2011).

A. baumannii colonization rates in healthy humans are low (about 1%) but higher in some Asian populations. Community-acquired infections caused by carbapenem-resistant *A. baumannii* (CRAb) are uncommon and most likely occur in patients with underlying pulmonary disease, renal failure, diabetes, or excessive alcohol abuse (Falagas et al. 2007a). Nosocomial outbreaks of *A. baumannii* are generally difficult to control, as this bacterium is able to survive on abiotic surfaces for extended periods of time. The hands of the hospital staff are a common mode of transmission, but the spread can also be caused by exposure to contaminated equipment and aerosolized water droplets (Dijkshoorn et al. 2007). Elderly people, especially those in long-term care facilities, were shown to be an important reservoir of MDR *A. baumannii* (Denkinger et al. 2013).

In the 1990s, multiresistant strains were first detected in Asia, where they developed as a great public health challenge (Kuah et al. 1994; Siau et al. 1996). In South and Southeast Asian hospitals, high rates of carbapenem resistance among G- pathogens, especially in *A. baumannii* isolates, were observed (Hsu et al. 2017). In some

Asian countries, including Malaysia, Thailand, Pakistan, India, and Taiwan, *A. baumannii* belongs to the group of most abundant nosocomial pathogens (Chawla 2008). In Korea, the resistance rate of *A. baumannii* to imipenem, a representative of carbapenems, had increased to 85% by 2015, thus representing an enormous health threat (Kim et al. 2017). A combination of factors involving non-indicated prescription of antibiotic drugs and international travel, including medical tourism, contributed to the accelerated rise and spread of *A. baumannii* in South and Southeast Asia (Hsu et al. 2017). Interestingly, the increased frequency of *A. baumannii* isolated in the clinical setting showed a high correlation with the observed rise in antibiotic resistance (Carlquist et al. 1982). In the USA, it was observed that when *A. baumannii* causes healthcare-associated infections, more than 60% of the isolates showed resistance to carbapenems (Sievert et al. 2013). Even though the occurrence of *A. baumannii* changed only marginally from 2000 to 2009 in the USA, an ongoing decrease concerning the susceptibility to most classes of antibiotic drugs was observed. Further, a threatening third of all isolates manifested combined resistances to carbapenems, aminoglycosides, and fluoroquinolones (Landman et al. 2007).

While uncomplicated urinary tract infections and other minor infections have low mortality, patients with bloodstream infections from CRAb showed mortality rates of more than 40% (Wisplinghoff et al. 2004; Munoz-Price et al. 2010). Between 2010 and 2014, 60 cases of bacteremia caused by CRAb from 7 states in the USA were studied. Catheter-related bloodstream infections were the most abundant infections observed, and nearly half of the patients died within 30 days of diagnosis (Olesky et al. 2017). *Acinetobacter* infections are generally associated with several risk factors, including the use of mechanical ventilation and previous antimicrobial therapy. Prior hospitalization, longer duration of hospital stay, especially in ICUs, but also preceding the prescription of carbapenems, and the use of invasive procedures were identified as potential risk factors (Sheng et al. 2010).

The ability of *A. baumannii* to form biofilms most probably contributes to the observed prolonged survival on abiotic surfaces leading to subsequent transmission (de Breij et al. 2010). Further, biofilm formation on urinary catheters, central venous catheters, and endotracheal tubes may also prompt infection (Longo et al. 2014).

Differing but complementary mechanisms leading to reduced carbapenem susceptibility have been described for *A. baumannii* (Vila et al. 2007; Tang et al. 2014). The mechanisms of resistance include various carbapenemases (most commonly oxacillinases, OXA, and metallo-ß-lactamases, MBLs), AdeABC efflux systems, modification of penicillin-binding proteins (PBPs), and modification of outer membrane proteins (porins) (Yoon et al. 2015). A major intrinsic resistance mechanism is facilitated by the reduced number and size of certain outer membrane proteins (OMPs), leading to a compromised bacterial permeability to antibiotics than when compared to other G- organisms (Vila et al. 2007). Three OMPs have been associated with carbapenem non-susceptibility (Poirel et al. 2011). Intrinsic resistance-nodulation-cell division (RND)-type efflux pumps such as AdeABC, AdeFGH, and AdeIJK further play a role in carbapenem non-susceptibility (Yoon et al. 2015). The main way for resistance is hydrolysis of the drugs by an arsenal of intrinsic and

acquired carbapenem-hydrolyzing ß-lactamases (carbapenemases). The acquirement of carbapenemases, such as Ambler class A carbapenemases, class B MBLs, and class D oxacillinases, leads to the observed increased emergence of carbapenem resistance. Molecular classes A, C, and D comprise ß-lactamases characterized by a serine in their active site, while class B ß-lactamases are metalloenzymes containing zinc in their active center. While rare-chromosomally encoded cephalosporinases (Ambler class C enzymes) may possess a slightly extended activity on carbapenems, they most likely play a minor role in the clinics. Carbapenemases with catalytic efficiency on carbapenems are mostly grouped into Ambler classes A, B, and D (Queenan and Bush 2007). Ambler class B carbapenemases comprise a broader spectrum than the other enzyme classes. They show a strong hydrolytic activity against most ß-lactam antibiotics and are not inhibited by ß-lactam inhibitors (Palzkill 2013). Ambler class D OXA-type ß-lactamases are native chromosomal oxacillinases and are encoded by several bla$_{OXA}$ genes, the most common are *bla*$_{OXA\text{-}23\text{-like}}$, *bla*$_{OXA\text{-}24\text{-like}}$, *bla*$_{OXA\text{-}51\text{-like}}$, and *bla*$_{OXA\text{-}58\text{-like}}$ genes. These enzymes and the presence of insertion sequences (IS), like IS*Aba1*, IS*Aba3,* IS*Aba4*, and IS*Aba9,* play an important role in the development of CRAb. While native chromosomal oxacillinases are generally expressed in low abundance, IS contribute to the mobilization and expression of the OXA-type-ß-lactamases, thus conferring carbapenem resistance. The IS*Aba1* sequence is the most prevalent and was described in *A. baumannii* isolates for the first time in 2001. This IS has been found to be associated with a number of OXA-type ß-lactamases (Evans and Amyes 2014). Today OXA-23 belongs to the most prevalent subgroup of oxacillinases worldwide (Mugnier et al. 2009; Poirel et al. 2011).

Many CRAb isolates were further shown to be MDR, carrying additional resistance determinants for several other groups of antibiotics like aminoglycosides, fluoroquinolones, and tetracycline (Doi et al. 2015), thus leading to a major threat to modern healthcare and significantly fueling the global resistance crisis.

2.1.2 Carbapenem-Resistant *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic pathogen frequently responsible for nosocomial infections (Rossi Gonçalves et al. 2017), especially in ICUs or in patients with predisposing conditions (Pirnay et al. 2009). This bacterium can be found ubiquitously in the hospital, not only associated with patients or hospital staff but also on abiotic surfaces (Tsao et al. 2017). *P. aeruginosa* is the causative agent of pneumonia, urinary tract infections, and infections of skin and soft tissue but is especially implicated in pneumonia of critically ill and/or immunocompromised patients. The pathogen is prevalently isolated from the respiratory tracts of patients with chronic lung disease, such as cystic fibrosis (Aloush et al. 2006; Gellatly and Hancock 2013). Delayed detection and treatment lead to rapid progression to respiratory failure, sepsis, and multi-organ failure, which are all associated with high mortality rates (Kang et al. 2003). *P. aeruginosa* is also often isolated from lakes, sewage, soil, animals, plants, and plant detritus (Pirnay et al. 2009), and resistant strains are detected in swimming pools and hot tubs in the USA (Lutz and Lee 2011). Carbapenem-resistant *P. aeruginosa* (CRPa) was also detected in wastewater

treatment plant effluent and in downstream rivers in Switzerland (Czekalski et al. 2012; Slekovec et al. 2012). These strains act as a potential reservoir for determinants of carbapenem resistance (Pappa et al. 2016).

The highest rates of carbapenem resistance in *P. aeruginosa* were observed in Eastern Europe, with Hungary, Slovakia, Poland, Lithuania, Croatia, Romania, Bulgaria, and Greece presenting resistance rates of >25% (European Centre for Disease Prevention and Control 2015). An extensive spread of carbapenemase-producing clones was observed in Belarus, Kazakhstan, and Russia, thus showing a gradient of resistance in Europe that rises from Northwest to Southeast (Edelstein et al. 2013). In Brazil, 43.9% of the isolates from patients with *P. aeruginosa* bacteremia, most of them from ICU residents, were carbapenem resistant. Among these patients, 31.2% received inadequate therapy, and the mortality rate was as high as 58.6% (Rossi Gonçalves et al. 2017). In Brazil, the high prescription rate of antibiotics, particularly of ß-lactams, carbapenems, and fluoroquinolones (Rodrigues Moreira et al. 2013) was described to be instrumental in *P. aeruginosa* developing resistance to various antibiotic agents during therapy. This was shown to occur either by mutation in chromosomal genes or by HGT (Zavascki et al. 2005; Xavier et al. 2010). The carbapenem resistance of *P. aeruginosa* in Brazil is mostly due to the production of MBLs (Rossi Gonçalves et al. 2017). In some hospitals, the resistance rates can be up to 60% (Kiffer et al. 2005; Baumgart et al. 2010). In Taiwan, 15.9% of the *P. aeruginosa* isolates from infected patients were carbapenem resistant. This study stated that the risk of infection with CRPa increased by 1% with each day in hospital (Tsao et al. 2017); thus, prolonged stays in healthcare settings were identified as a major risk factor leading to *P. aeruginosa*-mediated infections. Further risk factors include the preceding use of antibiotics, invasive procedures, comorbidities, and antecedent surgery. Mechanical ventilation, enteral/nasogastric tubes and inappropriate therapy are also associated with bacteremia by CRPa (Rossi Gonçalves et al. 2017). Infections caused by resistant *P. aeruginosa* are further frequently related with age, cancer, heart disease, diabetes, and invasive procedures like hemodialysis and tracheostomy (Aloush et al. 2006; Buehrle et al. 2017). The presence of a central venous catheter as a significant risk factor is a matter of debate, as some studies suggest that catheter exchange helps to prevent *P. aeruginosa* biofilm formation and thus significantly reduced infection risk (Jamal et al. 2014), whereas others did not identify these as priority risk factors (Rossi Gonçalves et al. 2017).

The capability of *P. aeruginosa* to form biofilms (Suárez et al. 2010) has enabled the bacterium to proliferate in water distribution systems and colonize central venous catheters (Fig. 2) (Wang et al. 2012; Jamal et al. 2014). As example, all strains analyzed in the Brazil study mentioned above were identified as strong biofilm producers (Rossi Gonçalves et al. 2017). Additionally, all MBL-positive *P. aeruginosa* isolates from Brazil showed the ability to form biofilms in vivo (Perez and Bonomo 2018). The severity of infections, especially associated with invasive procedures, might be more pronounced due to biofilm formation, as the antibiotic is inhibited from penetrating the cells by the surrounding polymeric matrix composed of polysaccharides, proteins, and DNA (Costerton et al. 1999; Hoiby et al. 2010).

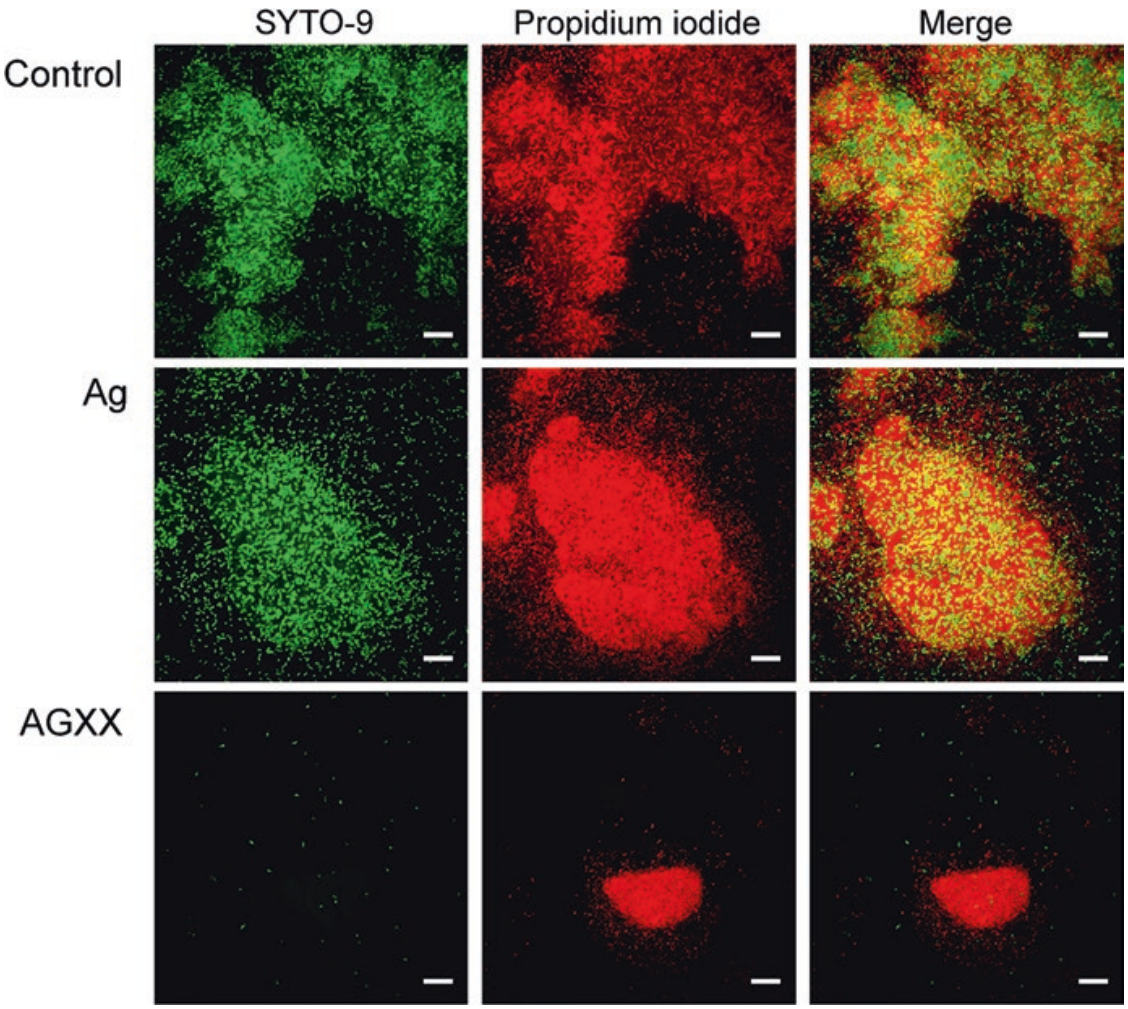

Fig. 2 Biofilm formation in *Pseudomonas aeruginosa*
Confocal images of biofilm formation by *Pseudomonas aeruginosa*. *P. aeruginosa* was grown on sterile coverslips for 24 h. The 24-h-old biofilms were exposed to silver (Ag) sheet or AGXX® sheet. The control panel refers to biofilm without any metal sheet. Biofilms were stained with SYTO9 (green) and Propidium Iodide (red) to visualize live and dead cells. Images show an average of Z-projections (500 nm spacing). Scale bars are 10 μm

The production of different enzymes, the lack of the outer membrane porin OprD, and the RND efflux pump systems MexAB-OprM and MexCD-OprJ, encoded on the genome, lead to the intrinsic resistance of *P. aeruginosa* to several classes of antibiotics. Resistance determinants, such as carbapenemase production, can also be acquired by HGT (Pirnay et al. 2009; Breidenstein et al. 2011; Poole 2011). Thus, *P. aeruginosa* has a great potential for developing a MDR phenotype (Schwartz et al. 2015).

Mutations in or lack of the porin OprD was shown to contribute to carbapenem resistance in clinical isolates of *P. aeruginosa* in Spain. OprD is a substrate-specific porin responsible for diffusion of amino acids (and also carbapenems) into the bacterial cell (Rojo-Bezares et al. 2014). A direct association between imipenem (a carbapenem) susceptibility and the levels of OprD expression was shown. Expression of OprD was not detected in imipenem-resistant isolates, whereas susceptible

bacteria showed close to normal expression levels (Dib et al. 1995). During imipenem treatment of *P. aeruginosa* infections in French hospitals, the most common mechanism of resistance was shown to be mutations in or loss of the porin OprD, with more than 85% of the isolates having lost the *oprD* gene (Fournier et al. 2013). Overproduction of chromosomally encoded AmpC ß-lactamases (also called cephalosporinase) and efflux pumps are further implicated in meropenem (a carbapenem) resistance in *P. aeruginosa* (Rodríguez-Martínez et al. 2009).

Expression/overproduction of RND efflux pumps further reduces carbapenem efficiency in *P. aeruginosa* (Choudhury et al. 2015; Pan et al. 2016). The MexAB-OprM efflux pump system plays a significant role in the intrinsic non-susceptibility of *P. aeruginosa* toward meropenem, quinolones, tetracycline, and chloramphenicol.

An important resistance mechanism of strains non-susceptible to ß-lactams is the expression of acquired carbapenemases. These isolates are usually resistant to all ß-lactams (Breidenstein et al. 2011; Poole 2011). Especially class B carbapenemases or MBLs are primarily encountered, with IMP-type (active on imipenem) enzymes predominantly encountered in Asia and VIM-type (Verona integron-encoded MBL) enzymes mostly found in Europe. Nevertheless, both enzymes are increasingly spreading globally (Walsh et al. 2005; Poole 2011). The most abundant carbapenemase is VIM; it can be plasmid-mediated and multiple copies lead to high-level meropenem resistance (San Millan et al. 2015), but it is usually integron-associated. IMP-6, another MBL, was demonstrated to be acquired from environmental bacteria by HGT (Xiong et al. 2013). Generally, MBLs occur as part of an integron structure on large genomic islands on the bacterial chromosome, but it was shown that they can also be encoded on transferable plasmids (Wright et al. 2015).

2.1.3 Carbapenem- and Third-Generation Cephalosporin-Resistant *Enterobacteriaceae*

Several representatives of G- *Enterobacteriaceae* are human pathogens, including *E. coli*, *Klebsiella* ssp., *Proteus* spp., *Enterobacter* spp., and *Serratia* spp. *Enterobacteriaceae* represent 50% of bacteremia cases, which are usually caused by redistribution of bacteria from their primary sites (Wilson et al. 2011). Infections with *Enterobacteriaceae,* most commonly arising from the gastrointestinal tract, involve high morbidity and mortality (Patel et al. 2008; Yamamoto and Pop-Vicas 2014). Even though infections caused by G+ pathogens are more common in healthcare settings, the highest mortality rate is associated with *Enterobacteriaceae* and other G- organisms (Wilson et al. 2011). *E. coli* and *Klebsiella pneumoniae* are the most abundant community – as well as hospital-acquired pathogens. These bacteria typically cause intra-abdominal infections, urinary tract infections, and primary bacteremia (Alhashem et al. 2017). Patient-to-patient transmission is comparably low, however, *K. pneumoniae* shows a higher rate of transmission than *E. coli* (Harris et al. 2007; Hilty et al. 2012).

Enterobacteriaceae are getting increasingly resistant to first- and second-line antibiotic drugs. Carbapenems are usually the treatment strategy for life-threatening infections by MDR *Enterobacteriaceae*, some of which produce extended spectrum

ß-lactamases (ESBLs). Infections with ESBL-producing G- bacteria and carbapenem-resistant *Enterobacteriaceae* (CRE) are increasing worldwide. Different geographical regions reveal carriage rates varying over time, but ESBL-producing *Enterobacteriaceae* occur globally nowadays, and carriage rates ranging from 8 to 28.8% have been reported in ICUs in Jerusalem and Korea, respectively (Friedmann et al. 2009; Kim et al. 2014). ESBL and AmpC enzymes together are responsible for the majority of the observed third-generation cephalosporin resistances in clinical isolates worldwide (Molton et al. 2013). In North American and European hospitals, those rates are around 10% for both *E. coli* and *K. pneumoniae*, while nosocomial ESBL rates as high as 80% and 60% were found in India and China, respectively (Livermore 2012). In the Indian community, *E. coli* resistance rates were as high as in the hospital environment. This might be due to the unregulated use of antibiotic drugs in agriculture and lower sanitation standards (Chaudhuri et al. 2011). In China, the rate of ESBL-positive strains among *E. coli* increased severely from 36.1% in 2002/2003 to 68.1% in 2010/2011 (Lai et al. 2014). For about three decades, a spreading of plasmid-mediated ß-lactamases in *Enterobacteriaceae* has been reported in Brazil. ESBL-producing strains, especially *K. pneumoniae* as the predominant pathogen, are widely distributed in healthcare settings (Sampaio and Gales 2016). In the USA, 18% of healthcare-associated infections in acute care hospitals and acute rehabilitation facilities can be attributed to ESBL-producing *Enterobacteriaceae* (Weiner et al. 2016).

The inadequate antibiotic prescription and inappropriate use of antibiotic drugs accelerated the spreading of CRE, leading to public concern. Selection pressure by the prescription of carbapenem antibiotics has been proposed to fuel the rapid spread of CRE (Yigit et al. 2001; Potter et al. 2016). In Europe, 17 countries reported increased dissemination or occurrence of CRE between 2010 and 2013 (Glasner et al. 2013). Infections caused by CRE especially affect severely ill patients with multiple comorbidities. ICU-resident patients revealed a notably high burden of infections with CRE and increased mortality when compared to non-ICU patients (Debby et al. 2012; Tischendorf et al. 2016; Papadimitriou-Olivgeris et al. 2017). Among ICU-resident patients in Israel, colonization with CRE was associated with at least a two-fold increase in the risk of infection by the colonizing strain (Dickstein et al. 2016). Recently, *E. coli, K. oxytoca*, and *Enterobacter cloacae* were frequently reported to harbor carbapenem resistance (Tzouvelekis et al. 2012; Gomez-Simmonds et al. 2016). Among the hospitalized patients, 3–7% are colonized by CRE in endemic areas, but these rates can vary between 0.3% and 50% depending on the healthcare setting, with the highest rates achieved in a Greek hospital (Banach et al. 2014; Bhargava et al. 2014; Papadimitriou-Olivgeris et al. 2012; Swaminathan et al. 2013; Vatopoulos 2008; Vidal-Navarro et al. 2010; Wiener-Well et al. 2010; Zhao et al. 2014). Greece has one of the highest rates of carbapenem-resistant G-bacteria globally. By 2008, carbapenem resistance had increased to 30% in hospitals and to 60% in ICUs (Walsh et al. 2005). A study in a tertiary hospital in China revealed that *K. pneumoniae* and *E. coli* were the most prevalent species. More than 70% of all nosocomial isolates exhibited high levels of resistance against ß-lactam antibiotics, while 64.9% of the strains harbored carbapenemase genes (Yang et al.

2017). CRE have also become widely distributed in the USA with 140,000 cases of nosocomial infections annually that show mortality rates between 26 and 44% (Centers for Disease Control and Prevention 2013; Falagas et al. 2014). Furthermore, *K. pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* became prevalent in Brazil in the last 10 years. KPC production is reported to be the most common resistance mechanism in carbapenem-resistant *K. pneumoniae* (Sampaio and Gales 2016).

Colonization of ICU patients with CRE is a massive risk factor for subsequent infection with *K. pneumoniae*. Almost 50% of the patients in a hospital in the USA developed an infection within 30 days after having been tested positive for colonization with the pathogens. Endoscopy and colonoscopy were shown to be risk factors for these infections (McConville et al. 2017). Further risk factors for increased susceptibility to CRE were the prescription of ß-lactam antibiotics within 30 days and receiving trimethoprim-sulfamethoxazole or glucocorticoids concomitant with an onset of bloodstream infection, as observed in a hospital environment in the USA (Bratu et al. 2005). Other risk factors were described to be comorbid conditions, prolonged hospital stay, critical illness, invasive medical devices, and mechanical ventilation (Falagas et al. 2007b; Gupta et al. 2011; Munoz-Price et al. 2013; Temkin et al. 2014). Long-term acute care hospital-resident patients experienced additional risk. For example, in Chicago 30.4% of patients in long-term facilities were colonized with KPC-producing *Enterobacteriaceae*, while only 3.3% of ICU patients from short-stay hospitals tested positive for colonization (Lin et al. 2013).

Genes encoding ß-lactamases on mobile genetic elements are one major mechanism contributing to the rapid dissemination of MDR G- bacteria worldwide. The most abundant mechanisms of ß-lactam resistance in *Enterobacteriaceae* were indeed described to be caused by the production of ESBLs, and a smaller proportion was due to altered efflux pump levels/activities or porin expression. ESBLs are mostly plasmid-encoded and can hydrolyze penicillins, broad-spectrum cephalosporins, and oxyimino-monobactams. These enzymes alone are not effective against cephamycins or carbapenems (Bradford 2001; Paterson and Bonomo 2005).

Enterobacteriaceae, harboring transmissible carbapenem resistance, have emerged as a big issue within the last two decades, and ß-lactamases present in these pathogens are a further driving force of resistance (Logan and Weinstein 2017). Major resistance mechanisms observed in CRE are the expression of high-level ESBLs or AmpC enzymes combined with mutations of porins, leading to decreased permeability to carbapenems or the acquisition of carbapenemase genes (Dai et al. 2013).

One resistance mechanism is mainly facilitated by plasmid-encoded ESBLs and AmpC cephalosporinases. AmpC activity in *Enterobacteriaceae* is mostly related with overproduction or derepression of chromosomal genes. Both enzyme types, when combined with mutations of porins, are described to confer resistance to carbapenems. Altered or completely lost porins can reduce diffusion into bacterial cells to rates that enable the action of ESBLs and AmpC enzymes (Paterson and Bonomo 2005; Bush and Fisher 2011). Further, drug efflux pumps and alterations in PBPs are associated with carbapenem non-susceptibility (Patel and Bonomo 2013).

KPC-producing *K. pneumoniae* was isolated in 1996 in the USA for the first time (Yigit et al. 2001). By 2015, KPC had spread globally and has become endemic in the Northeastern USA, Puerto Rico, China, Israel, England, Italy, Romania, Greece, Brazil, Argentina, and Colombia (Denisuik et al. 2013; Glasner et al. 2013; Rodríguez-Zulueta et al. 2013; Saito et al. 2014; Tängdén and Giske 2015; Chang et al. 2015a). KPC-producing *Enterobacteriaceae* can harbor variants of this gene; the most common are bla_{KPC-2} or bla_{KPC-3} on a Tn*3*-based transposon, Tn*4401* (Kitchel et al. 2009; Cuzon et al. 2011). The resistance level to carbapenem in KPC-producing strains can vary. This depends either on increased bla_{KPC} gene copy number, deletions upstream of the bla_{KPC} gene, and/or outer membrane porin loss (OmpK35 and/or OmpK36) (Kitchel et al. 2010; Patel and Bonomo 2013).

MBLs are categorized as class B enzymes, and VIM, NDM-1 (New Delhi MBL), and IMP are the most abundant representatives. The Indian subcontinent is the major reservoir for NDM-1-positive *Enterobacteriaceae* (Lascols et al. 2011), and low sanitation and hygiene levels lead to their wide occurrence in healthcare settings and in the community (Tängdén and Giske 2015). Most often, VIM and IMP MBLs are embedded in class I integrons on transposons or plasmids that lead to the spread. NMD-type MBLs are harbored on different plasmid incompatibility types. It has been proposed that the most abundant variant in *Enterobacteriaceae*, NDM-1, originated from *A. baumannii* (Dortet et al. 2012, 2014). More than two decades ago, the first transmissible carbapenemase gene, *IMP-1* MBL, was detected on an integron in *Serratia marcescens* in Japan. Shortly after the first description, a plasmid-mediated outbreak was observed in seven Japanese hospitals. Subsequently, dissemination of *Enterobacteriaceae* harboring the bla_{IMP-1} gene occurred throughout Japan (Ito et al. 1995). Further, Greece has been shown to be a hotspot for VIM-type *Enterobacteriaceae* and *K. pneumoniae* (Vatopoulos 2008; Logan and Weinstein 2017). In lower-income countries, NMD-1-type MBLs can spread via environmental sources in the community. In India, 4% of drinking water samples and 30% of seepage samples (water pools in streets or rivulets) contained bla_{NMD-1}-positive bacteria in 2011 (Walsh et al. 2011). Class D OXA ß-lactamases are a large group of oxacillinases and are frequently found in *Enterobacteriaceae* (Poirel et al. 2010; Carrër et al. 2010). A transferable plasmid harboring the bla_{OXA-48} gene is often associated with the spread of OXA-48-producing *Enterobacteriaceae*. The integration of the bla_{OXA-48} gene is facilitated by the acquisition of a Tn*199* transposon (Poirel et al. 2010, 2012a, 2012b; Carrër et al. 2010). OXA-48 enzymes reveal high activity on penicillins but low-level activity on carbapenems.

Intestinal carriage of *Enterobacteriaceae* harboring transmissible MDR also presents a major threat, as the intestine provides an environment where resistance determinants can be easily exchanged between bacterial strains. Strains encoding these genes often show additional acquired resistance to fluoroquinolones, aminoglycosides, and trimethoprim-sulfamethoxazoles, which have evolved as major threat to human healthcare.

2.2 Priority 2: High

2.2.1 Vancomycin-Resistant *Enterococcus faecium*

Enterococcus faecium is a G+ facultative anaerobic bacterium. *Enterococci* are capable of growing at hypotonic, hypertonic, acidic, and alkaline conditions. They hydrolyze bile esculin and pyrrolidonyl-B-naphthylamide, which inhibit the growth of most microorganisms (Huycke et al. 1998; Hollenbeck and Rice 2012). *Enterococci* are part of the normal gut flora and often used as indicators of fecal contamination (Boehm and Sassoubre 2014). They are found in human stool at up to 10^8 colony-forming units/g (Huycke et al. 1998; Mundy et al. 2000). *Enterococci* cause urinary tract infections, intra-abdominal and pelvic infections, surgical wound infections, bacteremia, neonatal sepsis, endocarditis, and rarely meningitis (Marothi et al. 2005). *Enterococci,* which are nosocomial pathogens, form biofilms, most likely contributing to their virulence and antibiotic resistance (Hollenbeck and Rice 2012; Hashem et al. 2017). These bacteria are responsible for about 12% of hospital-acquired infections (Hollenbeck and Rice 2012). *E. faecalis* and *E. faecium*, colonizing the gastrointestinal tract, can cause severe infections in immunocompromised patients (Miller et al. 2014). *Enterococci* are intrinsically resistant to cephalosporins, lincosamides, and nalidixic acid and are further not susceptible to low levels of aminoglycosides and clindamycin. They show acquired resistance to penicillin, vancomycin (a glycopeptide antibiotic), chloramphenicol, erythromycin, tetracycline, and fluoroquinolones and high-level resistance to aminoglycosides and clindamycin (Marothi et al. 2005).

The antibiotic resistance mechanisms of *E. faecium* include modification/inactivation of drug targets, overexpression of efflux pumps and a cell envelope adaptive response, assisting it to survive in the human host and in the nosocomial environment (Miller et al. 2014). *E. faecium* leads to biofilm-mediated infections in patients with medical devices. Atl_{Efm}, a major autolysin in *E. faecium,* contributes to stabilization of biofilms and surface localization of the virulence factor Acm, facilitating binding of Acm to collagen types I and IV. This presents Atl_{Efm} as potential target for treatment of *E. faecium* biofilm-mediated infections (Paganelli et al. 2013).

Nowadays, the majority of *E. faecium* isolates are resistant to ampicillin, vancomycin, and aminoglycosides (Arias et al. 2010). The emergence of vancomycin-resistant *Enterococci* (VRE) was first reported in 1986 in Europe, in 1993 in the USA, and in 1994 in Asia (Uttley et al. 1988; O'Driscoll and Crank 2015; Akpaka et al. 2017). Since then, the prevalence of vancomycin-resistant *E. faecium* (VREfm) has increased worldwide. VREfm causes 4% of healthcare-associated infections as per the reports from the National Healthcare Safety Network in America (Miu et al. 2016). The prevalence of VREfm has increased worldwide since 1986. A study on healthcare-associated infections in the USA reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention (CDC) found 80% of *E. faecium* isolates analyzed in 2006/2007 to be non-susceptible to vancomycin (Arias et al. 2010). In US hospitals, VREfm incidence had risen to 0.3% in 1989 and to 7.9% in 1993 (Schouten et al. 2000; Arias and Murray 2012). By 2002, 60% and in 2007 more than 80% of the *E. faecium* isolates in US hospitals revealed

vancomycin resistance (Arias and Murray 2012; Molton et al. 2013). By 2007, the prevalence of VREfm in Europe was higher than 30% in countries like Greece and Ireland, whereas Scandinavian countries reported very low rates (<1%) (Arias and Murray 2012). In Malaysia, the VREfm rate was 25.7% in 2006 (Getachew et al. 2009). In Canadian hospitals, the prevalence of VREfm increased from 1.8% in 2007 to 6.0% in 2013. Ninety percent of vancomycin-resistant isolates harbored the gene *vanA*. Interestingly, the prevalence of *vanB* vancomycin-resistant VRE in these medical centers decreased from 37.5% in 2007 to 0% in 2013 (Simner et al. 2015). A study conducted on hospitalized patients between 2009 and 2014 from seven Caribbean countries showed 90.9% of bacterial isolates to be *E. faecium*, and all of them were vancomycin resistant (Akpaka et al. 2017). In a study conducted in 30 hospitals in Argentina between 1997 and 2000, all *Enterococci* isolates were found to be non-susceptible to vancomycin. The incidence of *vanA*-positive VREfm was 98%, with minimal inhibitory concentrations (MICs) to vancomycin of 32–512 mg/l, while *vanB*-harboring strains revealed MICs to vancomycin of 16–32 mg/l (Corso et al. 2007).

Glycopeptides, like vancomycin, which interfere with the synthesis of peptidoglycan and thus inhibit bacterial growth, are commonly used in the treatment of enterococcal infections (Kristich et al. 2014). These antibiotics form complexes with C-terminal D-Ala-D-Ala peptide termini of peptidoglycan precursors on the outer surface of the cell. This prevents the cell wall biosynthetic enzymes (i.e., PBPs) from using them as substrates for transglycosylation and transpeptidation and hence leads to impairment of cell wall integrity (Kristich et al. 2014). In VRE, the C-termini of peptidoglycan precursors are exchanged to D-Ala-D-Lac or D-Ala-D-Ser, thus reducing the binding affinity of glycopeptides (such as vancomycin) to peptidoglycan by 1000-fold and sevenfold, respectively (Kristich et al. 2014; Ahmed and Baptiste 2017). This phenomenon disables glycopeptides to inhibit cell wall biosynthesis in bacteria (Kristich et al. 2014). Glycopeptide resistance is generally encoded on mobile genetic elements. However, some types of glycopeptide resistance are also chromosomally encoded (Kristich et al. 2014).

Genetic mechanisms of vancomycin resistance in *Enterococci* involve nine gene clusters conferring resistance to glycopeptides. The *van* gene cluster has components with various functions. A two-component signal transduction system consisting of VanRS (VanR is a response regulator/activator of vancomycin resistance and VanS a sensor kinase) recognizes glycopeptides and activates the expression of resistance genes in inducible *van* types. In the presence of vancomycin, the two-component system VanRS activates a promoter responsible for co-transcription of *vanA*, *vanH*, and *vanX* to regulate vancomycin resistance (Arthur and Courvalin 1993). VanH (a dehydrogenase converting cellular pyruvate to D-lactate) and VanA (a ligase forming D-Ala-D-Lac) produce modified peptidoglycan precursors, while VanX (cleaves D-Ala-D-Ala) and VanY (D,D-carboxypeptidases) remove unaltered peptidoglycan precursors (Kristich et al. 2014). Among the *van* gene clusters, *vanA* and *vanB* types of resistances are most common in hospitals and are found in enterococcal isolates from food, clinical, and veterinary samples (Hammerum 2012). *vanA* is generally carried on the transposon Tn*1546* and was first reported on

plasmid pIP816 in *E. faecium* BM4147 (Arthur and Courvalin 1993). *vanB* is harbored by Tn*5382*-/Tn*1549*-type transposons. These transposons are either plasmid- or chromosomally encoded (Kristich et al. 2014).

Infection control and antibiotic stewardship programs are important to prevent further development of antibiotic resistance and dissemination (Hollenbeck and Rice 2012). Control measures should include identification of patients colonized and infected by resistant *Enterococci*, strict adherence to hand hygiene, and active screening of high-risk patients (Faron et al. 2016).

2.2.2 Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*

Staphylococcus aureus is a G+ facultative anaerobic bacterium. It is part of the normal human microflora and is frequently found on the skin, in the respiratory tract, and in the nose. It is an opportunistic pathogen, accounting for about 80% of prosthetic infections. *S. aureus* forms strong biofilms and attaches firmly to medical devices and host tissues, causing chronic, difficult-to-treat infections (Kawada-Matsuo and Komatsuzawan 2012; Vaishampayan et al. 2018). *S. aureus* harbors a two-component regulatory quorum-sensing system, the accessory gene regulator (Agr), which plays an important role in biofilm-related infections (Qin et al. 2014).

Methicillin-resistant *S. aureus* (MRSA) is a leading cause of nosocomial infections. According to the reports from the National Healthcare Safety Network in America, MRSA is responsible for 8% of healthcare-associated infections (Miu et al. 2016). As per the recent US CDC report, among the 23,000 documented infections caused by antibiotic-resistant pathogens, almost half the cases were caused by MRSA (Hagras et al. 2017). MRSA lead to skin and soft tissue infections, respiratory tract infections, food poisoning, endocarditis, osteomyelitis, pneumonia, toxic shock syndrome, suppurative diseases, and fatal sepsis. Immunocompromised patients, patients with implants or diabetes or patients undergoing surgery, elderly people, and newborns are high-risk groups for MRSA infections (Ohlsen 2009).

In a study conducted in the USA, Canada, Latin America, Europe, and the West Pacific region from 1997 to 1999, 32 to 47% of skin and soft tissue infections were found to be caused by *S. aureus* (Schito 2006). The CDC reported 80,461 infections and 11,285 deaths caused by MRSA in 2011 (CDC 2013). The prevalence of MRSA is increasing globally, especially in developing countries. The occurrence of MRSA was reported to be 75% among hospital specimens in Hong Kong from 1997 to 1999, 53.1% in Bangladesh in 2004, 80% in Chile in 2006, 26% in Malaysia from 2006 to 2008, 92.4% in Columbia in 2009, 44.1% in Ethiopia in 2010 and 2011, and 43% in Indonesia in 2014 (Pandey 2017). However, the prevalence of MRSA in livestock is lower in some Asian countries compared to European countries, like in Japan 0.9%, Malaysia 1.4%, Korea 3.2%, China 11.4%, Sri Lanka 13.8%, and Taiwan 14.4% as compared to Poland 20.6% and Germany and the Netherlands with more than 35% (Jayaweera and Kumbukgolla 2017).

Methicillin is a β-lactam antibiotic belonging to the penicillin class. Methicillin resistance can be transferred via HGT (New et al. 2016). The penicillin-binding protein, PBP2, is a key molecule conferring resistance to β-lactams. Methicillin-sensitive *S. aureus* (MSSA) harbors four PBPs (PBP 1–4), and all of them are

inactivated by β-lactam antibiotics. In contrast, MRSA strains encode an extra PBP2', with low affinity to β-lactams, thus facilitating cell wall biosynthesis even in the presence of β-lactam antibiotics. The expression of PBP2' is controlled by the MecR1-MecI regulatory system (Kawada-Matsuo and Komatsuzawan 2012). In addition, three factors responsible for methicillin resistance in the presence of Triton X-100 have been recognized, namely, *fmtA*, *fmtB*, and *fmtC/mprF*. *fmtA* has been identified as a new PBP. Inactivation of *fmtA* reduces methicillin resistance, while mutation of *fmtB* reduces methicillin and oxacillin resistance (Kawada-Matsuo and Komatsuzawan 2012). FmtC/MprF is a membrane-associated protein and its inactivation diminishes methicillin resistance by decreased modification of phosphatidylglycerol with L-lysine. FmtC/MprF determines resistance against host defensive peptides and thus plays a role in virulence and pathogenicity of *S. aureus*. Its inactivation leads to increased negative charge of the membrane surface and increased binding of antibacterial peptides to the surface (Berger-Bächi and Rohrer 2002). Mutations in *fmtC/mprF* in *S. aureus* were shown to further cause a decrease in vancomycin and daptomycin resistance (Bayer et al. 2015; Lin et al. 2018a). Another methicillin-resistant mechanism involves the mobile cassette element SCC*mec* (staphylococcal chromosome cassette *mec*) that is integrated into a *S. aureus* gene of unknown function, *orfX* (Chambers and DeLeo 2009). This cassette carries both the *mecA* and *mecC* genes that encode a novel specific penicillin-binding protein (PBP2a) and the site-specific recombinase genes *ccrAB* and/or *ccrC*. The SSC*mec* cassette was first described in 1999 (Ito et al. 1999). SCC*mec* elements are divided into type I to XI based on the *mec* and *ccr* gene complexes and further classified into different subtypes (Liu et al. 2016).

Vancomycin, a last resort antibiotic, has been widely used in the treatment of MRSA. However, excessive use of the drug has led to the development of vancomycin-resistant *S. aureus* (VRSA) (Appelbaum 2006). In 2002, the first VRSA isolate with a MIC of higher than 100 μg/ml was reported in Michigan, USA (Gardete and Tomasz 2014). Until 2008, 11 VRSA clinical isolates, which were also resistant to methicillin, had been reported, out of which 9 cases were identified in the USA, 1 in Iran, and 1 in India. Out of the nine from the USA, seven were clinical isolates from Michigan (Périchon and Courvalin 2009). The US strains harbor a plasmid-borne Tn*1546* element, most probably acquired by conjugation from glycopeptide-resistant *E. faecalis* (Périchon and Courvalin 2009). The mechanism of resistance observed in VRSA is similar to that in *Enterococci* by alteration of peptidoglycan precursors. The C-terminal D-Ala-D-Ala is substituted by D-Ala-D-Lac, diminishing the binding of vancomycin, thus no longer inhibiting the cell wall synthesis in the bacterium (Schito 2006).

2.2.3 Clarithromycin-Resistant *Helicobacter pylori*

H. pylori is a G- microaerophilic, spiral organism (Yonezawa et al. 2013). It is a human gastric pathogen that causes peptic ulcers, gastritis, gastric adenocarcinoma, mucosa-associated lymphoid tissue lymphoma, chronic immune thrombocytopenic purpura in adults, and vitamin B12 deficiency (Shmuely et al. 2016; Alba et al.

2017). The route of transmission is commonly from person to person (Shmuely et al. 2016).

H. pylori forms biofilms, even on human gastric mucosa, reducing the susceptibility of the bacterium to different antibiotics including clarithromycin (but also metronidazole, erythromycin, amoxicillin, and tetracycline) (Yonezawa et al. 2015; Attaran et al. 2017). The incidence of clarithromycin resistance, and also the expression of efflux pump systems, is higher in biofilms compared to planktonic cells. Interestingly, the MIC of clarithromycin was increased by up to four-fold in 2-day-old biofilms and up to 16-fold in 3-day-old *H. pylori* biofilms (Yonezawa et al. 2013).

Clarithromycin is a macrolide, containing a 14-membered lactone ring with L-cladinose and D-desosamine groups of sugars (Alba et al. 2017). It binds to the 50S subunit of the bacterial ribosome and blocks the translation of peptides, thereby inhibiting bacterial growth (Yonezawa et al. 2013). The precise site of action of clarithromycin is the peptidyl transferase loop of domain V of 23S rRNA.

While clarithromycin is the first drug of choice to treat *H. pylori* infections, clarithromycin resistance in *H. pylori* has been linked to treatment failures, including poor compliance, resistance to antibiotics, and reinfection (Chey and Wong 2007; Shmuely et al. 2016). The incidence of clarithromycin-resistant *H. pylori* is higher in previously treated than in untreated patients (Shmuely et al. 2016). In developing countries, the annual occurrence of clarithromycin-resistant *H. pylori* is 4–15% higher than in industrialized countries, revealing rates of 0.5% (Gold 2001; Duck et al. 2004). A consistent increase in clarithromycin resistance has been reported in most countries. In Bulgaria, the resistance increased from 10% in 1996–1999 to 19% in 2003/2004. In the USA, the resistance was 6.2% in 1993 and the rate doubled in 9 years, to 12.9% in 2002. In Belgium, the rates increased from 6% in 1990 to 56% in 2009. In Japan, the resistance was 18.9% in 2002 and reached 27.7% in 2005. In a hospital in the USA, the resistance rate of *H. pylori* infections in patients between the ages of 3 and 19 years was as high as 50% (Shmuely et al. 2016). A meta-study compiling 87 studies on 52,008 *H. pylori* isolates from 2009 to 2014 gives a good overview of the prevalence of *H. pylori*. It included 43 Asian, 10 American, 5 African, and 29 European studies. There were 5.46% to 30.8% of *H. pylori* isolates resistant to clarithromycin, with the lowest rate in African and the highest rate observed in North American isolates. Among European countries, Norway showed the lowest resistance rate (5.9%), while Portugal showed the highest (42.4%). In Asian countries, the lowest resistance rates were observed in Malaysia (2.4%), while the highest rates were found in India (58.8%) (Ghotaslou et al. 2015). Recently, an increase in clarithromycin resistance among treatment failures showed 17.5% (primary resistance) to 63.2% after one eradication treatment failure (secondary resistance) and 75.4% after two eradication treatment failures (tertiary resistance) (Megraud et al. 2013; Selgrad et al. 2013).

Point mutations of the *23S* rRNA gene, mostly an adenine-to-guanine transition at positions 2142 and 2143, are the common mechanism of clarithromycin resistance, as they reduce the affinity of the drug to the ribosome (Megraud 1998; Yonezawa et al. 2013; Alba et al. 2017). Sporadic mutations in the translation

initiation factor IF-2, the ribosomal protein L-22, as well as in the efflux pumps, are other mechanisms of resistance (Alba et al. 2017). Excessive use of clarithromycin has led to the development of resistant strains of *H. pylori,* with the predominant mutations occurring in A2143G, A2142G, and A2142C in the *23S* rRNA gene, but T2182C, G2224A, T2215C, and C2694A in the V region of the *23S* rRNA gene have also been observed (Vianna et al. 2016; Alba et al. 2017). A2143G is the most frequently encountered mutation among the resistant strains in most European and Latin American countries (Vianna et al. 2016).

The latest Maastricht Guidelines recommend clarithromycin containing treatments against *H. pylori* infections in regions with low incidence of clarithromycin resistance. In regions with high levels of clarithromycin resistance, quadruple therapy with bismuth or the sequential therapy with 5 days of proton pump inhibitors and amoxicillin followed by 5 more days of proton pump inhibitors plus metronidazole and clarithromycin is recommended as the first-line treatment (Ghotaslou et al. 2015; Malfertheiner et al. 2012; Shmuely et al. 2016). In addition to the combinational use of antibiotics to treat infections, judicious use of antibiotics with the help of culture and antibiotic susceptibility testing of *H. pylori* and empiric eradication are essential to control further spread of antibiotic resistance (Boltin et al. 2015; Shmuely et al. 2016).

2.2.4 Fluoroquinolone-Resistant *Campylobacter* spp.

Campylobacter jejuni is a G- curve-shaped, thermophilic, and microaerophilic bacterium (Fernández and Pérez-Pérez 2016). It is a zoonotic, foodborne pathogen and causes about 500 million human infections worldwide annually (Bae and Jeon 2013; Bae et al. 2014). It is responsible for about 90% of the *Campylobacter* infections in humans (Iovine 2013) and is a leading cause of gastroenteritis since the late 1970s (Luangtongkum et al. 2009; Fernández and Pérez-Pérez 2016). *C. jejuni* has the ability to form biofilms on abiotic surfaces (Reuter et al. 2010; Bae et al. 2014) and can acquire antibiotic resistance genes in biofilms by natural transformation (Bae et al. 2014). The formation of biofilms likely increases the fluoroquinolone resistance among *Campylobacter* spp. (Bae and Jeon 2013). Gastroenteritis caused by *Campylobacter* is generally regarded as self-limiting. However, treatment is recommended in cases of a severe infection or infections in the immunocompromised elderly patients or in newborns and pregnant women (Fernández and Pérez-Pérez 2016). Fluoroquinolones such as ciprofloxacin are often used to treat *Campylobacter* infections. Spread of the bacteria from animals to humans often occurs via contaminated food. Poultry animals are especially seen as crucial reservoirs involved in this dissemination (Bae and Jeon 2013; Fernández and Pérez-Pérez 2016). The emergence of fluoroquinolone resistance in *Campylobacter* from food animals has evolved as a public health issue (Tang et al. 2017).

A study conducted among travelers returning to Finland from 1995 to 2000 showed that countries with especially high rates of ciprofloxacin-resistant *C. jejuni* were Spain with 22%, followed by Thailand and India, with 14%, and 6% of the isolates, respectively. The isolates were collected during two study periods (1995–1997 and 1998–2000). The study reported an increase in the incidence of resistance

among the investigated travelers between the two study periods from 40% to 60% within the study period (Hakanen et al. 2003). In 2000, the occurrence of ciprofloxacin-resistant *Campylobacter* spp. in clinical isolates (mostly *C. jejuni*) was 50% in Chile, 59.6% in Argentina, and 78% in Peru. In Argentina, 49.1% of the *Campylobacter coli* from a pediatric hospital were reported to be resistant to ciprofloxacin as well as to norfloxacin, another fluoroquinolone (Fernández and Pérez-Pérez 2016).

In Peru, an increase in ciprofloxacin resistance among *C. jejuni* and *C. coli* from 2001 to 2010 was reported. The highest rates of ciprofloxacin-resistant *C. jejuni* at the beginning and the end of the study were observed in Lima, with 73.1% and 89.8%, respectively, similar to resistance in *C. coli* (48.1% in 2001 and 88.4% in 2010) (Fernández and Pérez-Pérez 2016). A study conducted from 2003–2006 in Mexico reported ciprofloxacin-resistant *C. jejuni* isolates in chickens (85.8%), pigs (62.5%), cattle (39.8%), and humans (58.2%) (Zaidi et al. 2012). In Southern Ecuador, 90.9% of *C. jejuni* and 100% of *C. coli* strains, isolated from chicken liver for human consumption, were reported to be ciprofloxacin resistant (Fernández and Pérez-Pérez 2016). A recent study in the USA among feedlot cattle in 2012/2013 showed 35.4% of *C. jejuni* and 74.4% of *C. coli* to be fluoroquinolone resistant, a significant increase when compared to the 1.8% *C. jejuni* and 9% *C. coli* being non-susceptible to ciprofloxacin as reported earlier (Englen et al. 2005; Tang et al. 2017).

All fluoroquinolone resistance determinants reported in *Campylobacter* are chromosomally encoded. The frequency of emergence of fluoroquinolone-resistant mutants ranges from 10^{-6} to 10^{-8} per cell and generation (Luangtongkum et al. 2009).

The mechanisms of fluoroquinolone resistance in *Campylobacter* spp. are mainly due to mutations in *gyrA* and *parC* genes, encoding DNA gyrase and topoisomerase IV, respectively. Frequently, amino acid positions Thr-86, Asp-90, and Ala-70 of *gyrA* are mutated. Thr-86 mutations confer higher levels of resistance to ciprofloxacin as compared to Asp-90 and Ala-70. High-level ciprofloxacin-resistant *C. jejuni* isolates (MIC = 125 μg/ml) possess two mutations, in *gyrA* Thr-86 and in *parC* at Arg-139 (Engberg et al. 2001). Another mechanism of fluoroquinolone resistance in *Campylobacter* is the multidrug efflux pump CmeABC, consisting of a periplasmic protein acting as a bridge (encoded by *cmeA*) (Iovine 2013), an inner membrane drug transporter (encoded by *cmeB*), and an outer membrane protein (encoded by *cmeC*). CmeABC reduces the accumulation of the drug in the bacterial cell (Luangtongkum et al. 2009).

Regular and methodical surveillance of antibiotic resistance in *Campylobacter* spp. is an essential step in controlling the further spread of antibiotic resistance (Fernández and Pérez-Pérez 2016).

2.2.5 Fluoroquinolone-Resistant *Salmonella* spp.

Salmonella are G-, motile, zoonotic pathogenes that cause diseases like gastroenteritis, typhoid, paratyphoid, and bacteremia (Rushdy et al. 2013; Pribul et al. 2017). *S. enterica* is a human-restricted pathogen causing typhoid (González et al. 2018), a disease that is typically transmitted by the fecal-oral route (Schellack et al. 2018).

The bacterium resides in the gall bladder as the primary reservoir. Further, it forms biofilms on the gall bladder, which are recalcitrant to ciprofloxacin treatment (González et al. 2018). In 2010, 26.9 million new cases of typhoid fever and 200,000 deaths were determined worldwide (Abd-elfarag 2015; Adhikari et al. 2017; Ugboko and De 2014). A community-based prospective *Salmonella* surveillance study, conducted in Asia from 2001 to 2003, showed occurrence of *S. typhi*, namely, 37% in China, 65% in India, 84% in Pakistan, 85% in Indonesia, and 100% in Vietnam. In the same study, the prevalence of *S. paratyphi* was observed to be 63% in China, 34% in India, 14% in Indonesia and in Pakistan, and 0% in Vietnam (Khan et al. 2010). In the USA, 1.2 million cases of infection are reported annually (Boore et al. 2015). In 2016, 94,530 cases of salmonellosis were reported in the EU (European Food Safety Authority 2017).

Fluoroquinolones, specifically ciprofloxacin, are the drugs of choice to treat *Salmonella* infections. However, overuse of ciprofloxacin has resulted in increased resistance. Ciprofloxacin-resistant *Salmonella* was first reported in 1990 (Menezes et al. 2010). A study in Brazil conducted from 2009 to 2013 on isolates from food of animal sources and from environmental samples screened for fluoroquinolone resistance among the isolates. The most prevalent serotype obtained was *S. typhimurium* followed by *S. enteritidis*. The occurrence of resistance was highest for enrofloxacin (48%), followed by ciprofloxacin (43%) and ofloxacin (40%), and the lowest resistance was observed for levofloxacin (30%) (Pribul et al. 2017). Despite emerging ciprofloxacin resistance, this drug is recommended as the first-line therapy in children and adults (González et al. 2018).

The fluoroquinolone resistance in *Salmonella* is predominantly due to mutations in *gyrA* and *parC* genes, as also described for *Campylobacter* (Sjölund-Karlsson et al. 2014). The second mechanism of resistance is overexpression of the efflux system AcrAB-TolC (Rushdy et al. 2013). AcrAB-TolC belongs to the resistance-nodulation-division family and has three domains, a membrane fusion protein (AcrA), a drug efflux transporter (AcrB), and an outer membrane channel protein (TolC) (Kim et al. 2016). Overexpression increases the efflux of the antibiotic that acts synergistically with the alterations in outer membrane proteins which includes absence of some/all of these proteins, namely, Omp-A, Omp-C, Omp-D, and Omp-F (Rushdy et al. 2013).

Mechanisms of fluoroquinolone resistance in *Salmonella* food isolates were identified. Either the investigated isolates had only a single mutation in *gyrA* with S83T, S83F, and D87N being the most common amino acid substitutions or a pair of novel double mutations in *gyrA* resulting in H80N and S83T substitutions and a single *parC* mutation causing a Q91H substitution were identified (Lin et al. 2015). Another mechanism used by *Salmonella* is alteration of porin expression, thus reducing the penetration of fluoroquinolones into the bacteria (Rushdy et al. 2013). In addition to the mutations in *gyrA* and *parC* genes, and chromosomally encoded efflux pumps, a plasmid-mediated resistance mechanism encoded by *qnrA* has also been observed in *Salmonella* spp. (Sjölund-Karlsson et al. 2014).

It was recently suggested that fluoroquinolone-resistant *S. typhi* strains would occur in the future, even if the use of these drugs were diminished, as these

resistance mechanisms are not linked with fitness costs (Baker et al. 2013). This poses a great challenge to the public health. Surveillance of infections and epidemiology, as well as studying the genes responsible for antibiotic resistance in *Salmonella* spp., are imperative measures to control the spread of antibiotic resistance and to effectively treat infections (Nabi 2017).

2.2.6 Cephalosporin- and Fluoroquinolone-Resistant *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a G- pathogenic diplococcus with a special feature of antigenic variability, strengthening its survival in the human host (Patel et al. 2011). It inhabits mucosal surfaces of the urethra in male and the cervix in female (Patel et al. 2011) but can also be found in the rectal and the oropharyngeal mucosa (Costa-Lourenço et al. 2017). *N. gonorrhoeae* causes symptomatic and asymptomatic infections of the genital and extragenital tract (Patel et al. 2011). It is an etiological agent of gonorrhea and the second leading cause of sexually transmitted diseases (Costa-Lourenço et al. 2017). In men, it causes urethritis. Untreated infections may lead to epididymitis, reduced fertility, and urethral stricture. In women, the symptoms include abnormal vaginal discharge, dysuria, lower abdominal discomfort, and dyspareunia (Alirol et al. 2017). The risk of gonococcal infection is lowering with increasing age, as most cases occur in individuals under the age of 24 (Costa-Lourenço et al. 2017). *Gonococci* form biofilms in vitro and likely in vivo (Unemo and Shafer 2014). Approximately 62 million cases of *N. gonorrhoeae* infections occur every year worldwide (Patel et al. 2011).

Fluoroquinolones and cephalosporins are the drugs of choice to treat *N. gonorrhoeae* infections. Cephalosporins inhibit the growth of bacteria by inhibiting the cross-links of peptidoglycan in the bacterial cell wall by binding to PBPs. The cephalosporins, ceftriaxone and cefixime, are the most effective recommended treatment options against *N. gonorrhoeae* infections. However, resistance to these drugs has emerged in the past two decades.

Ciprofloxacin-resistant *N. gonorrhoeae* isolates were reported in the 1980s from many countries (Patel et al. 2011). By the end of 1992, the resistance rates in Japan were 40% (Patel et al. 2011). In India, the use of ciprofloxacin started in the 1990s, and by the end of 2000, most isolates were resistant (Patel et al. 2011). The resistance to ceftriaxone and cefixime was first reported in Japan and then spread all over the world (Unemo and Shafer 2014). Resistance to ceftriaxone in *N. gonorrhoeae* has been reported in several American countries since 2007 (Pan American Health Organization/World Health Organization 2018). In South Africa, among men attending healthcare clinics, the incidence of ciprofloxacin resistance in *N. gonorrhoeae* increased from 7% in 2004 to 32% in 2007. In Kenya, quinolone resistance increased since it emerged in 2007 from 9.5% to 50% in 2009 (Mehta et al. 2011). In Europe, 50,001 *N. gonorrhoeae* cases were reported in 2013, and 53% of the clinical isolates were resistant to ciprofloxacin and 4.7% to cefixime (Spiteri et al. 2014). In a report published by WHO-GASP-LAC in 2013, ciprofloxacin resistance rates in clinical *N. gonorrhoeae* isolates in Latin American countries stayed below 5% until 2004, increased to >15% in 2006, and reached >40% in 2010 (Dillon et al.

2013). The spread of these resistances is thought to occur through HGT (Hess et al. 2012). In 2014, the prevalence of gonorrhea disease in the southern part of the USA was 131 cases per 100,000 individuals (CDC 2014), and the CDC estimated 820,000 new cases annually. Thirty percent of the isolates were ciprofloxacin resistant in cases of men having sex with men and 12% in case of men having sex with women (CDC 2015).

The use of fluoroquinolones as a drug of choice to treat gonococcal infections was recommended in 1993. Already in 1997, the first strains resistant to fluoroquinolone were reported in Hong Kong and the Philippines. In 2004, fluoroquinolone was no longer recommended for treatment, but cephalosporins came into use as a treatment against gonococcal infections. In 2007, cephalosporin resistance was reported in Japan and Australia. A year later, reduced susceptibility to cephalosporins was identified in the USA. In 2011, the WHO and CDC revised the treatment guidelines, and ceftriaxone was included in the combination therapy to treat gonococcal infections. However, in 2012 the first cases of ceftriaxone resistance were reported from Japan (Buono et al. 2015).

Fluoroquinolone resistance in *N. gonorrhoeae* can be chromosomally as well as plasmid-mediated (Patel et al. 2011). As already stated for *Campylobacter* spp. and *Salmonella*, in cases of high-level fluoroquinolone resistance, mutations take place at positions 91 and 95 in *gyrA* and at positions 87 and 91 in *parC* (Kubanov et al. 2016) but also in genes associated with NorM efflux pumps that export fluoroquinolones (Golparian et al. 2014). The mechanism of cephalosporin resistance is primarily due to alteration of the structure and function of key proteins, such as PBP2, encoded by *penA*, and PorB1b showing porin activity (Ross and Lewis 2012; Golparian et al. 2014). Another strategy used by *N. gonorrhoeae* to combat cephalosporins is mutations in the MtrC-MtrD-MtrE efflux pump system, a member of the resistance-nodulation-division pump family (Golparian et al. 2014).

Gonococcal resistance to cephalosporins is severe due to limited alternatives to treat gonococcal infections. Thus, it is imperative to fill the gaps in the surveillance and MDR data to understand the epidemiology of gonococcal MDR (Wi et al. 2017). Additionally, strengthening of diagnosis of *N. gonorrhoeae* infections is recommended by the Pan American Health Organization and the WHO as a control measure (Pan American Health Organization/World Health Organization 2018).

2.3 Priority 3: Medium

2.3.1 Penicillin-Non-susceptible *Streptococcus pneumoniae*

S. pneumoniae is a G+ facultative anaerobic organism. It causes pneumonia, sinusitis, otitis media, upper respiratory tract infections, and bacteremia, resulting in morbidity and mortality in infants and children (Bogaert et al. 2000; Ahmadi et al. 2015; Diawara et al. 2017). *S. pneumoniae* also triggers meningitis, which is the most dangerous disease of the central nervous system (Ahmadi et al. 2015). The bacterium forms robust biofilms to survive in the human nasopharynx (Talekar et al.

2014) and is responsible for 11% of deaths worldwide (Ahmadi et al. 2015) with the highest mortality rates reported in Africa and Asia (Diawara et al. 2017).

The prevalence of penicillin-non-susceptible *S. pneumoniae* (PNSP) is increasing rapidly. The first PNSP was reported in Australia in 1967 (Hansman and Bullen 1967; Liñares et al. 2010). A study conducted in 11 pediatric tertiary care centers in Canada from 1991 to 1998 showed the emergence of two international clones of PNSP, serotype 9V and 14 related to the Spanish-French clone, and the 23-F Spanish-US clone (Greenberg et al. 2002). In the USA, an invasive PNSP clone 35B, which caused invasive infections in patients in ten different states from 1995 to 2001, was identified by the CDC and Prevention's Active Core Surveillance (Beall et al. 2002). The prevalence of PNSP in Canada increased from 2.5% in 1991 to 11.3% in 1998 (Greenberg et al. 2002). The occurrence of PNSP in hospitals was >70% in Korea, 45% in South Africa, 44% in Spain, and 21.8% in Brazil (Greenberg et al. 2002; Levin et al. 2003).

The prevalence of PNSP in some European countries was shown to be very high, 25–50% in Spain, France, and Greece; 10–25% in Portugal, Ireland, Finland, and Turkey, and 5–10% in Italy, and relatively low with 1–5% in the UK, Germany, Sweden, Austria, and Norway (EARSS Annual Report 2006; Reinert 2009). In Poland, the prevalence of PNSP among children (age 2 to 5 years) in 2011–2012 was 44.8% (Korona-Glowniak et al. 2016). The prevalence of PNSP in Argentina increased significantly from 15.8% in 1993 to 67.3% in 2002 (Bonofiglio et al. 2011), and in Morocco it was 22% in samples collected from 2007 to 2014 (Diawara et al. 2017).

The dissemination of antibiotic resistance in pneumococci is mainly clonal (Sjostrom et al. 2007). *S. pneumoniae* expresses six types of PBPs, namely, 1a, 1b, 2a, 2b, 2x, and 3. The mechanism of penicillin resistance involves modification within or in flanking regions of the amino acid motifs which form the active catalytic center of the PBPs. This alters the PBPs, namely, PBP2x, PBP2b, and PBP1a. These modified variants display a reduced affinity to ß-lactam antibiotics, while their enzymatic function is apparently unaffected (Hakenbeck et al. 2012; Reinert 2009; Schweizer et al. 2017; Zhou et al. 2016).

Detection of PNSP is crucial to prevent and treat infections caused by penicillin-resistant *S. pneumoniae*. Surveillance of the clonal distribution of PNSP in combination with epidemiological analyses will help in understanding the risk factors associated with them. Use of conjugate vaccines might also help in reducing non-susceptibility toward the antibiotic (Ahmadi et al. 2015; Hampton et al. 2018).

2.3.2 Ampicillin-Resistant *Haemophilus influenzae*

Haemophilus influenzae is a G- facultative anaerobic coccobacillus that can cause various diseases, with symptoms ranging from mild to severe (Baba et al. 2017). The bacterium is associated with a significant number of respiratory tract infections as well as serious invasive infections, like meningitis and sepsis (Kiedrowska et al. 2017). Further, community-acquired pneumonia, acute otitis media, acute epiglottitis, and sinusitis can be caused by *H. influenzae*. The bacterium is often part of the physiological bacterial flora of the upper respiratory tract but is frequently isolated

from the respiratory tract of COPD (chronic obstructive pulmonary disease) patients, where it can lead to severe symptoms (Finney et al. 2014; Garmendia et al. 2014).

Antibiotic treatment can give rise to the occurrence of resistant *H. influenzae* strains that are frequently non-susceptible to ampicillins, including ß-lactamase-negative ampicillin-resistant (BLNAR) strains. The highest rate of ß-lactamase production in strains of *H. influenzae* was observed in South Korea and Japan, where more than half of all isolates were tested positive (Tristram et al. 2007). High prevalence of BLNAR strains has evolved into major clinical concern. Over the last few years, a significant increase in the occurrence of BLNAR strains has been observed in many European countries and throughout the world (Sanbongi et al. 2006; Jansen et al. 2006; Tristram et al. 2007). In European countries, the prevalence of nosocomial BLNAR strains was reported to range between 15% and 30% (Jansen et al. 2006; Witherden et al. 2014).

Resistance of *H. influenzae* to ß-lactams can be either enzyme- (facilitated by ß-lactamases) or non-enzyme-mediated. Traditionally, the most commonly occurring ß-lactam resistance mechanism in *H. influenzae* is ß-lactamase production, with the gene encoded on plasmids (Tristram et al. 2007). Non-enzyme-mediated resistance (BLNAR) can be facilitated by increased expression of the AcrAB efflux pump (Kaczmarek et al. 2004). Further, in BLNAR strains, alterations in PBP3, encoded by the *fts1* gene, have been attributed to elevated resistance to ß-lactam antibiotics (Kaczmarek et al. 2004; Wienholtz et al. 2017). Distinct mutations in *fts1* led to decreased affinity for penicillins as well as cephalosporins (Thornsberry and Kirven 1974; Ubukata et al. 2001; Hasegawa et al. 2003). This has been proposed to be the main molecular mechanism of non-ß-lactamase-mediated resistance among BLNAR strains (Mendelman et al. 1984; Tristram et al. 2007; Skaare et al. 2014).

2.3.3 Fluoroquinolone-Resistant *Shigella* spp.

Shigella are G- facultative anaerobic, rod-shaped bacteria and are an important cause of acute diarrheal disease worldwide. The majority of cases occur among children under the age of five in developing countries (Kotloff et al. 2013; Khaghani et al. 2014). Generally, *Shigella* infections are restricted to the gastrointestinal tract, while extraintestinal infections, such as bloodstream infections, reactive arthritis, and neurological complications, are rare (Bhattacharya et al. 1988; Muthuirulandi Sethuvel et al. 2017). Infections caused by *Shigella* spp. in humans are easily transmittable from person-to-person or by contaminated food/water (Muthuirulandi Sethuvel et al. 2017). Shigellosis is endemic among poor populations in African and Asian countries. *Shigella* epidemics have been reported from Bangladesh, Sri Lanka, Maldives, Nepal, Bhutan, Myanmar, and the Indian subcontinent (Emerging and other Communicable Diseases and Control Organization 1994). Nowadays, global occurrence of multidrug-resistant *Shigella* spp. that reveal increased non-susceptibility to third-generation cephalosporins and fluoroquinolones has emerged as a critical health issue. This trend has been predominantly observed in Asia (Wang et al. 2006; Gu et al. 2012; Taneja and Mewara 2016). Nevertheless, reports of MDR lineages or strains with increased resistance to fluoroquinolones are piling up globally (Aggarwal et al. 2016; Nüesch-Inderbinen et al. 2016).

Resistance to fluoroquinolones in *Shigella* is based on two mechanisms occurring either singly or in combination: Alterations in the targets of these antibiotics and non-permeability of the membrane and/or overexpression of drug efflux pumps that lead to decreased drug concentrations inside the cell reduce antibiotic susceptibility. Mutations in *gyrA, a* subunit of the bacterial DNA gyrase complex, and *parC*, a subunit of the bacterial topoisomerase, have been identified as important determinants for fluoroquinolone resistance (Chu et al. 1998). Chromosomal mutations in these genes were shown to participate in the dissemination of fluoroquinolone-resistant *S. sonnei* isolates (Ma et al. 2018). Plasmid-mediated quinolone resistance (PMQR) factors seem to fulfill a minor but additive role in the reduction of the susceptibility to fluoroquinolones (Vinothkumar et al. 2017). The presence of PMQR genes can promote mutations within the quinolone resistance determining region, leading to fluoroquinolone resistance, but spread to other *Enterobacteriaceae* may occur (Nüesch-Inderbinen et al. 2016). Further, *qnr* genes on mobile genetic elements are also able to confer low-level resistance to fluoroquinolones (Ruiz 2003; Hooper and Jacoby 2015). These genes encode proteins protecting the bacterial DNA gyrase and topoisomerase from quinolone/fluoroquinolone inhibition, thus leading to low-level resistance (Tran and Jacoby 2002; Tran et al. 2005a, 2005b; Redgrave et al. 2014). These plasmids often also harbor other antibiotic resistance genes that can be transferred to other species by conjugation (Martínez-Martínez et al. 1998).

3 Conclusions and Perspectives

The occurrence of multidrug-resistant bacterial pathogens presents a global threat. Especially alarming is the increasing incidence of multiresistant pathogenic bacteria outside medical centers. For example, there is a rising incidence of multiresistant opportunistic or nosocomial pathogens in the population, in food animals, and also in wild animals, e.g., vancomycin-resistant *Enterococci* and carbapenem-resistant *P. aeruginosa* have been recently detected in migratory birds (Martins et al. 2018; Yahia et al. 2018). Thus, implementation of efficient antibiotic stewardship programs is urgently needed all over the world. In addition, alternative treatment options to cure and/or prevent severe infectious diseases caused by multiresistant pathogens are imperatively required to prevent the advent of the post-antibiotic era. Alternative options include among others antibacterial vaccines, herbal products, bacteriophages, and improved biosecurity measures, as summarized by Bragg et al. (2018). Our group has been working on the development of antibacterial vaccines targeting surface-exposed proteins involved in conjugative spread of antibiotic resistance genes among pathogens. One of these vaccine candidates directed to staphylococcal and enterococcal pathogens has been successfully tested in a mouse infection model (Laverde et al. 2017).

Treatment of infections by MDR bacteria is often aggravated by the formation of thick, robust biofilms on infected tissues. Therefore, alternative treatment approaches should include biofilm inhibitors, such as natural or engineered antimicrobial

peptides (Lin et al. 2018a; b) or extracts from medicinal plants (Mehta and Das 2018). It is well-known that biofilm formation is controlled by second messenger molecules, such as cyclic di-guanosine monophosphate (c-di-GMP), and by interbacterial cell-cell communication via quorum sensing systems. Recently, some progress has been made by detecting small-molecule inhibitors of c-di-GMP signaling (Opoku-Temeng and Sintim 2017). Another promising approach to successfully attack strong biofilm forming pathogens should be based on the continuous discovery of novel quorum sensing inhibitors which are often plant-based natural compounds (Defoirdt 2018; Mehta and Das 2018).

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Manuscript #2

Review

Broad-host-range Inc18 plasmids: Occurrence, spread and transfer mechanisms

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Review

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vanA

ABSTRACT

Conjugative plasmid transfer is one of the major mechanisms responsible for the spread of antibiotic resistance and virulence genes. The incompatibility (Inc) 18 group of plasmids is a family of plasmids replicating by the theta-mechanism, whose members have been detected frequently in enterococci and streptococci. Inc18 plasmids encode a variety of antibiotic resistances, including resistance to vancomycin, chloramphenicol and the macrolide-lincosamide-streptogramine (MLS) group of antibiotics. These plasmids comprising insertions of Tn*1546* were demonstrated to be responsible for the transfer of vancomycin resistance encoded by the *vanA* gene from vancomycin resistant enterococci (VRE) to methicillin resistant *Staphylococcus aureus* (MRSA). Thereby vancomycin resistant *S. aureus* (VRSA) were generated, which are serious multi-resistant pathogens challenging the health care system. Inc18 plasmids are widespread in the clinic and frequently have been detected in the environment, especially in domestic animals and wastewater. pIP501 is one of the best-characterized conjugative Inc18 plasmids. It was originally isolated from a clinical *Streptococcus agalactiae* strain and is, due to its small size and simplicity, a model to study conjugative plasmid transfer in Gram-positive bacteria. Here, we report on the occurrence and spread of Inc18-type plasmids in the clinic and in different environments as well as on the exchange of the plasmids among them. In addition, we discuss molecular details on the transfer mechanism of Inc18 plasmids and its regulation, as exemplified by the model plasmid pIP501. We finish with an outlook on promising approaches on how to reduce the emerging spread of antibiotic resistances.

1. Introduction

The ability of bacteria to acquire new characteristics has led to ever-changing bacterial genomes and to the dissemination of virulence and antibiotic resistance genes in clinical settings (Christie, 2016). Horizontal gene transfer (HGT) is one of the major contributors to these bacterial evolution and diversification processes. A major subclass of HGT is conjugative transfer of plasmids and integrative conjugative elements (ICEs). The process of conjugation was discovered in *E. coli* by Lederberg and Tatum in 1946 and the first steps towards elucidation of conjugative processes are summarized in (Lederberg and Tatum, 1953). Conjugation requires the expression of transfer genes involved in DNA processing, replication, translocation and mating pair formation (MPF) (Christie, 2016).

Plasmids can be described as autonomously replicating, extrachromosomal elements that do not encode any essential function for the bacterial host, but rather provide beneficial traits that can be advantageous and/or facilitate survival under distinct environmental conditions (Bañuelos-Vazquez et al., 2017) and are usually denoted either as narrow- or broad host-range plasmids. Factors leading to a broad host-range can be the presence of multiple origins and/or the origin structure. The host-range is further expanded, if the replication initiation is not dependent on host initiation factors but if all essential proteins are encoded on the plasmid itself (Jain and Srivastava, 2013). Plasmids can be subdivided in plasmid incompatibility groups (Inc-groups), denoted by the failure of co-resident plasmids to be stably inherited in the absence of selection (Novick, 1987).

The Inc18-family classification is premised on the specific

Abbreviations: CC, Clonal complex; CDS, coding DNA sequences; CHAP, cysteine-, histidine-dependent amidohydrolases/peptidases; GREF, glycopeptide resistant *Enterococcus faecium*; GRE, glycopeptide resistant enterococci; GSEF, glycopeptide sensitive *Enterococcus faecium*; G+, Gram-positive; G−, Gram-negative; HGT, horizontal gene transfer; Inc-group, incompatibility group; ICE, integrative conjugative element; MLS, macrolide-lincosamide-streptogramine; MPF, mating pair formation; MRSA, methicillin resistant *Staphylococcus aureus*; NTF2, nuclear-transport factor 2; PSK, post-segregation killing; PFGE, pulsed field gel electrophoresis; ST, sequence type; SLT, soluble lytic transglycosylase; *tra*, transfer; T4SS, type IV secretion system; VREfm, vancomycin resistant *Enterococcus faecium*; VRE, vancomycin resistant enterococci; VRSA, vancomycin resistant *S. aureus*

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maintenance of these plasmids, including post-segregational killing (PSK) systems (Cegłowski et al., 1993; Hernández-Arriaga et al., 2014; Zielenkiewicz et al., 2005) and replication that occurs via a theta mechanism (Bruand et al., 1991; Ceglowski et al., 1993; Le Chatelier et al., 1993). These plasmids exist in a few copies per cell and processes governing copy number control have been elucidated in detail for several Inc18-family representatives (Brantl and Behnke, 1992a; Le Chatelier et al., 1994; de la Hoz et al., 2000). Their replication requires the rate-limiting replication protein, Rep, and a replication origin downstream of the *rep* gene (Brantl, 2015; Brantl and Behnke, 1992b; Brantl et al., 1993, 1992; Palmer et al., 2010). All Inc18-family plasmids encode these trans-acting replication proteins that bilaterally recognize the replication origin of other family representatives (Brantl et al., 1990). *rep* genes have been frequently used as markers to identify Inc18-like plasmids (Limbago et al., 2014). Additionally, all plasmids belonging to the Inc18-family encode Cop proteins that are similar among family representatives and their binding regions were shown to be highly homologous (Brantl, 2015; Brantl and Wagner, 1997; Ceglowski et al., 1993; Licht et al., 2011; Swinfield et al., 1990). Most Inc18 plasmids harbour efficient stabilization systems represented by a locus coding for a toxin-antitoxin system (Camacho et al., 2002; Ceglowski et al., 1993; Meinhart et al., 2003) and further encode partitioning systems (Lioy et al., 2015). A representative of the Inc18-family, pSM19035, has evolved as excellent model to study plasmid maintenance mechanisms (Ceglowski et al., 1993; Dmowski and Kern-Zdanowicz, 2016; Lioy et al., 2010; Meinhart et al., 2003; Zielenkiewicz et al., 2005). The Inc18 group of plasmids comprises common conjugative plasmids with a broad host-range harboured by G+ bacteria with a low-GC content, for example enterococci and staphylococci (Brantl et al., 1990), which have been frequently isolated from bacterial genera responsible for nosocomial infections (Mikalsen et al., 2015; Palmer et al., 2010). pRE25, pAMß1 and pIP501 are further representatives of Inc18-family plasmids (Brantl, 2015; Grohmann et al., 2016; Jain and Srivastava, 2013; Luo et al., 2005; Palmer et al., 2010; Rosvoll et al., 2010) and characteristic features of these plasmids are presented in Fig. 1.

Inc18 plasmids encode resistance to macrolides, lincosamides and streptogramines (MLS group of antibiotics), vancomycin and pIP501 further confers resistance to chloramphenicol. Due to the plasmid-encoded transfer genes, antibiotic resistances can be disseminated to other G+ and in some cases to Gram-negative (G−) bacteria (Kurenbach et al., 2003; Palmer et al., 2010). Antibiotic resistance genes are commonly clustered together on mobile plasmids, leading to co-selection of resistance determinants. Further, bacterial virulence determinants are generally regarded as factors providing selective advantages and promoting gastrointestinal colonization (Hegstad et al., 2010). They include among others secreted toxins, distinct bacterial cell surface molecules involved in biofilm formation, proteins involved in stress response, transport systems and specific gene regulators and play a role in reducing the bacterial susceptibility to antibiotic drugs. Several virulence factors have been identified in clinically relevant strains of enterococci (Biavasco et al., 2007; Freitas et al., 2017, 2016; Hegstad et al., 2010).

Enterococci are considered as harmless commensals of the gastrointestinal tract of humans and animals but have emerged over the past 30 years as important nosocomial pathogens. HGT has allowed antibiotic and virulence traits to converge in hospital adapted *Enterococcus faecalis* and *Enterococcus faecium* lineages (Bortolaia et al., 2015; Jensen et al., 2010; McBride et al., 2007) and these strains frequently possess acquired resistance to last-line antibiotics (Hidron et al., 2008; Mikalsen et al., 2015; Saheed and Rothman, 2016). The dissemination of vancomycin resistance from enterococci to methicillin-resistant *S. aureus* (MRSA) strains facilitated by Inc18-like plasmids has been observed frequently. This development is of special concern as vancomycin is a last-line antibiotic for treating MRSA infections that get increasingly common in the clinic (Jensen et al., 2010; Singh et al., 2017; Zhu et al., 2010). Nevertheless, factors promoting transfer of Inc18 plasmids among G+ bacteria leading to the spreading of resistances encoded by them are not completely understood yet (Palmer et al., 2010).

This review will cover the occurrence and dissemination of Inc18-like plasmids in natural, as well as in hospital environments, focusing on plasmids conferring vancomycin resistance to a wide range of bacterial species. We will then concentrate on the transfer mechanism of the Inc18-family model plasmid pIP501 that due to its small size has been studied in-depth in recent years.

2. Occurrence and dissemination of Inc18-type plasmids

2.1. Occurrence of Inc18-type plasmids in the clinical setting

Conjugation mediated by conjugative plasmids is one of the most effective processes to spread genetic material among bacteria. An important group of conjugative plasmids belongs to the Inc18-family. They have a broad host-range and occur naturally in *Streptococcus* and *Enterococcus* spp. (Zhu et al., 2010). pSM19035, pVA797 (Zúñiga et al., 2003), and pAM830, a *vanA* plasmid from an *E. faecalis* isolate, belong to the Inc18 group (Flannagan et al., 2003) of which pIP501 (Horodniceanu et al., 1976), and pAMβ1 (Clewell et al., 1974) are the best-characterized members. Inc18 plasmids have been shown to be responsible for vancomycin resistance transfer to *S. aureus*. Most vancomycin resistant *S. aureus* (VRSA) are MRSA isolates that have acquired *vanA*-mediated vancomycin resistance from enterococci. VRSA strains, even though quite rarely, have been identified in surface waters and it is proposed that vancomycin resistance was acquired by uptake of the *vanA* gene from VRE strains (Icgen, 2016). Samples taken from dairy farms (including milk samples) in Ethiopia revealed that 73.3% of all identified *S. aureus* strains were phenotypically resistant to vancomycin. The exceptionally high resistance rates can most likely be explained by insufficient hygiene strategies (Beyene et al., 2017). Further, in India VRSA strains have been identified in milk samples as well, even though to a much lesser extent (Bhattacharyya et al., 2016). As vancomycin is used for the treatment of serious infections caused by MRSA, occurrence of vancomycin resistance in MRSA is a serious threat to healthcare (Zhu et al., 2013). VRSA isolates from patients in Michigan, USA, were studied to determine the factors involved in plasmid transfer. They harboured a *vanA* gene localized on a 57.9 kb plasmid. The plasmid was a pSK41-like *S. aureus* plasmid with a Tn*1546*-like insertion carrying the *vanA* operon (Zhu et al., 2008). It was demonstrated that *vanA* transfer from *E. faecalis* to *S. aureus* took place via this pSK41-like conjugative plasmid, most likely pWZ909 (Zhu et al., 2013; Albrecht et al., 2014).

Enterococci are a common cause of nosocomial infections (Rosvoll, 2012). Vancomycin resistant *E. faecium* (VREfm) have been frequently reported since the 1980s, especially in clinical settings (Freitas et al., 2016). Among the VRE, approximately 70%–90% of the isolates from USA and Canada harboured *vanA* and about 10%–25% *vanB*, while in Europe the VRE outbreaks were caused mainly by the spread of *vanA*-containing VRE (Acar et al., 2000; Simner et al., 2015; Werner et al., 2008). A nosocomial outbreak of VREfm was reported in a haemo-oncology ward in a hospital in the South of Spain in 2009. These isolates carried *vanA* on a Tn*1546*-like element on an Inc18-type plasmid (Valdezate et al., 2012). Freitas et al. identified VREfm strains from 27 countries and five continents that were responsible for hospital outbreaks from 1986 to 2012. As already shown in 1996 by Arthur et al., *vanA* was mainly located on pRUM-like plasmids in the American isolates and on the Inc18 plasmid pIP186 in the European isolates (Arthur et al., 1996; Freitas et al., 2016). In a study in a large hospital in China, 47 VRE isolates from clinical samples and intensive care unit patients were examined. They demonstrated for the first time insertion of *fosB* (encoding resistance to fosfomycin) in the *vanRS-vanH* intergenic region of Tn*1546* resulting in co-resistance to fosfomycin and vancomycin

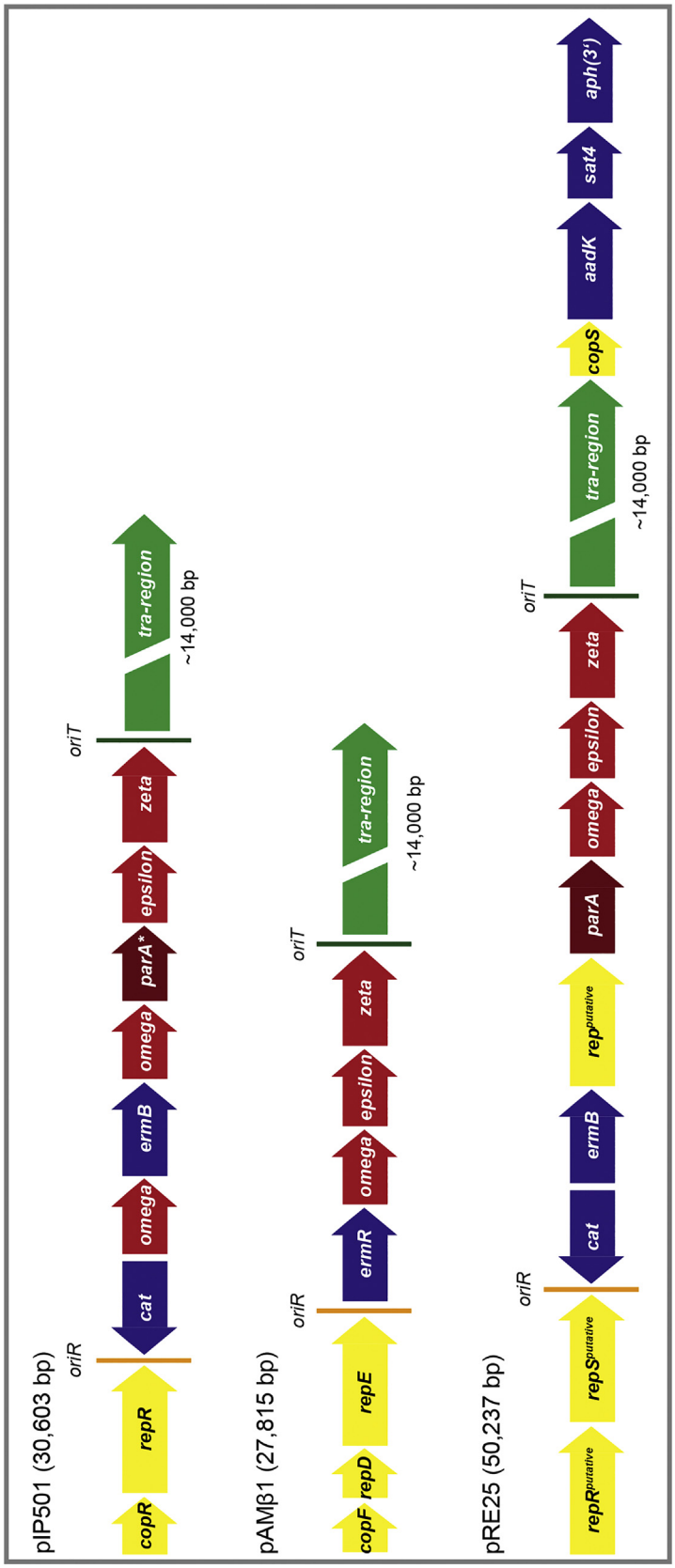

Fig. 1. Common features of selected Inc18-family plasmids. Genes involved in replication (yellow), coding for antibiotic resistances (blue), encoding transfer proteins (green) and maintenance systems (red) are depicted. The origin of replication (*oriR*) and the origin of transfer (*oriT*) are presented as vertical lines. Presented genes were either already annotated or identified by comparing with nearest homologues using BLAST search. *cat* = encoding resistance to chloramphenicol, *erm*B/R = encoding resistance to the MLS-group of antibiotics, *aadK* = encoding resistance to streptomycin, *sat4* = encoding resistance to streptothricin, *aph*(3′) = encodes resistance to aminoglycoside. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Qu et al., 2012). In addition, the Inc18 plasmid pRE25 containing a 30.5 kb segment highly similar to pIP501 (Schwarz et al., 2001) was found to be one of the most common plasmids among the VRE isolates (Qu et al., 2012). In a study on *E. faecium* isolates from blood cultures in Norway, gentamicin resistance increased significantly from 2003 to 2008. With 73%, *rep* from pRE25 was one of the most prevalent replicon types in these isolates (Rosvoll, 2012).

2.2. Occurrence of Inc18-type plasmids in environmental settings

Excessive use of antibiotics and growth hormones in animal farms and introduction of such pollutants in wastewater treatment plants creates a selection pressure, eventually making the bacteria resistant to these pollutants (Karkman et al., 2017). Avoparcin is a growth-promoting glycopeptide, structurally similar to vancomycin, which was used as a feed additive in animal farms in Europe in the 1970s (Acar et al., 2000). Avoparcin use was banned in Norway in 1995, in Germany in 1996 and in other European Union (EU) countries in 1997 (Garcia-Migura et al., 2008; Sørum et al., 2006). In Europe, VRE emergence was first reported in the 1980s (Leclercq et al., 1988). In farm and pet animals, VRE were always of *vanA* type (Acar et al., 2000). A connection between the use of avoparcin and the occurrence of *vanA* type VRE in farm animals was demonstrated in several studies (Aarestrup, 1995; Klare et al., 1995; Kruse et al., 1999). Avoparcin was not applied in the USA, but glycopeptide resistant enterococci (GRE) were detected in hospitals (Garcia-Migura et al., 2008). In the early and mid-1990s, several European countries suffered from the problem of *vanA*-type GRE with high level of vancomycin resistance (Johnsen et al., 2005). Rosvoll and co-workers showed that pIP501-like plasmids disseminated high-level *vanA*-type glycopeptide resistance (Rosvoll et al., 2010), mediated by Tn*1546* (Courvalin, 2006). In addition to being vancomycin resistant, some *vanA*- and *vanB*-type enterococci are also vancomycin-dependent, meaning they require vancomycin for their growth (Fraimow et al., 1994). These vancomycin-dependent strains lack a functional D-alanine-D-alanine ligase, which ligates D-alanine-D-alanine-lactate to D-alanine-D-alanine. In these strains, peptidoglycan synthesis is dependent on production of glycopeptide resistance proteins, and the production of these proteins depends on presence of vancomycin in the growth medium (Fraimow et al., 1994; Gholizadeh et al., 2001; Van Bambeke et al., 1999).

Several studies on GRE were conducted after the avoparcin ban in Europe. Sørum et al. investigated the prevalence of GRE in poultry farmers after three to eight years of ban on avoparcin in Norway. Faecal samples of poultry farmers from 29 farms, which had been previously exposed to avoparcin, were examined. GRE were detected in 18% of the faecal samples even after three years of ban (Sørum et al., 2006). The rate of GRE positive farmers did not change throughout the study period (27.6% in 1998 and 27.8% in 2003) (Sørum et al., 2006). Tn*1546* containing plasmids were found in most *E. faecium* isolates and a putative pRE25-like PSK system linked to Tn*1546* was detected in 97.1% of the isolates. Coding DNA sequences (CDS) 18 and 19 of pRE25 have been suggested to be structural and functional homologues of the PSK system from pSM19035 (Sørum et al., 2006). PSK occurs when daughter cells that do not inherit the plasmid encoding the toxin-antitoxin system die due to degradation of the unstable antitoxin. In this case, the stable toxin kills the plasmid-free cells (Ghafourian et al., 2014). PSK systems play a crucial role in maintaining antimicrobial resistance in reservoirs with negligible antimicrobial selection and may also play a role in stabilizing the plasmid-mediated *vanA*-type resistance (Sørum et al., 2006). Johnsen et al. investigated *E. faecium* isolates (from faecal samples from farmers and poultry) from two farms in Norway, which were previously exposed to avoparcin (Johnsen et al., 2005). Out of 222 isolates, 61% were glycopeptide-resistant *E. faecalis* (GREF), while the remaining 39% were glycopeptide sensitive *E. faecalis* (GSEF). 40% of the GREF isolates were shown to harbour a pSM19035-like PSK system (Johnsen et al., 2005).

GRE were still detected in the broiler sector in spite of a decade-long ban on the use of avoparcin by the EU. Garcia-Migura *et al.* found two main sites of Tn*1546* insertion in *E. faecium* isolates. Isolates from the UK, Norway and Denmark carried Tn*1546* in a sequence with 96% identity to a streptomycin adenyltransferase gene from a *Staphylococcus intermedius* plasmid (Garcia-Migura et al., 2008), while only isolates from the UK carried Tn*1546* inserted in an Inc18-like plasmid (Garcia-Migura et al., 2008).

Apart from the heavy use of avoparcin as a feed additive, antibiotics and quaternary ammonium compounds are widely used in hospitals and households as disinfectant (Silveira et al., 2015). This eventually contaminates the aquatic environments by disseminating the respective antibiotic resistance genes (Berglund, 2015). HGT events are common in aquatic environments and, as pointed out before, conjugative plasmids play a major role in HGT. The run-off from livestock facilities and wastewater contaminating the aquatic environments add to the environmental antibiotic contamination (Berglund, 2015). An *E. faecium* ST17 clone from hospital waste in Portugal was found to harbour a transferable Inc18-like plasmid conferring resistance to both antibiotics and quaternary ammonium compounds (Silveira et al., 2015). Spread of such pathogens resistant to antibiotics and disinfectants is a matter of concern as it makes treatment of infections even more challenging. Evaluating the risks associated with bacteria harbouring resistances typically encoded by Inc18-like plasmids in the environment and the prevention of novel drug resistant pathogens is a key to reduce the threat to human health (Bengtsson-Palme et al., 2018; Zhu et al., 2013).

2.3. Dissemination of Inc18-type plasmids

Enterococci and staphylococci are major causes of nosocomial infections. The occurrence of clinical VRE isolates in the USA is 80%, while > 30% of the enterococci in Greece and Ireland are resistant to vancomycin.

In most of these cases, an Inc18-like *vanA* plasmid, pWZ909, was involved (Zhu et al., 2010). The geographical dissemination of Inc18-like *vanA* plasmids in > 1600 hospital-adapted VRE isolates from three different collections in the US was investigated, namely from two institutions in Michigan and from other hospitals. 3.9% of the VRE isolates from Michigan harboured an Inc18-like *vanA* plasmid of which 12.5% were *E. faecalis* and 1% were *E. faecium*. 93% of the Inc18 plasmid positive isolates from Michigan had the same Tn*1546* insertion site as the Inc18-like plasmid associated with VRSA (Zhu et al., 2010). Of all isolates tested, in total 6% of the *E. faecalis* and 0.6% of the *E. faecium* isolates were positive for the plasmid. Among 21 *E. faecalis* isolates with Inc18-like plasmids, only three pairs of isolates had identical PFGE patterns, prompting that the Inc18-like *vanA* plasmid had rather spread among *Enterococcus* spp. by horizontal transfer than clonal spread of a single strain had occurred (Zhu et al., 2010). Sletvold et al. (2010) elucidated the genetic composition of the Inc18 plasmid pIP816, the first *vanA*-type plasmid, which was isolated from the clinical *E. faecium* strain BM4147 in France in 1986. pIP816 shares a 25 kb conserved DNA fragment with other *vanA* plasmids detected at the same time and mechanistically IS*1216*-facilitated composite transposition enabled *vanA* type glycopeptide resistance dissemination of all these plasmids (Sletvold et al., 2010). The spread of resistance plasmids like the Inc18-type plasmids needs to be monitored and prevented for the safety of healthcare. A schematic representation of putative dissemination routes of Inc18 plasmids is presented in Fig. 2.

Consequently, developing new approaches to cure infections caused by lethal, multidrug resistant pathogens is crucial (Kaye, 2016; Zhu et al., 2013).

2.4. Glycopeptide resistance reservoirs shared by humans and animals

Several studies have pointed towards a link between clonal complexes (CC) of *E. faecium* found in animals and humans (Freitas et al., 2011). Spread of GRE and GREF from poultry to farmers has been shown (Sørum et al., 2006), thus the existence of a common glycopeptide-resistance reservoir shared by animals and humans is likely (Johnsen et al., 2005).

A study focusing on the comparison of VRE isolates from three different sources, namely from pigs, healthy persons and clinical isolates was carried out by Freitas et al. (2011). It was based on wide surveillance studies carried out in Portugal, Denmark, Spain,

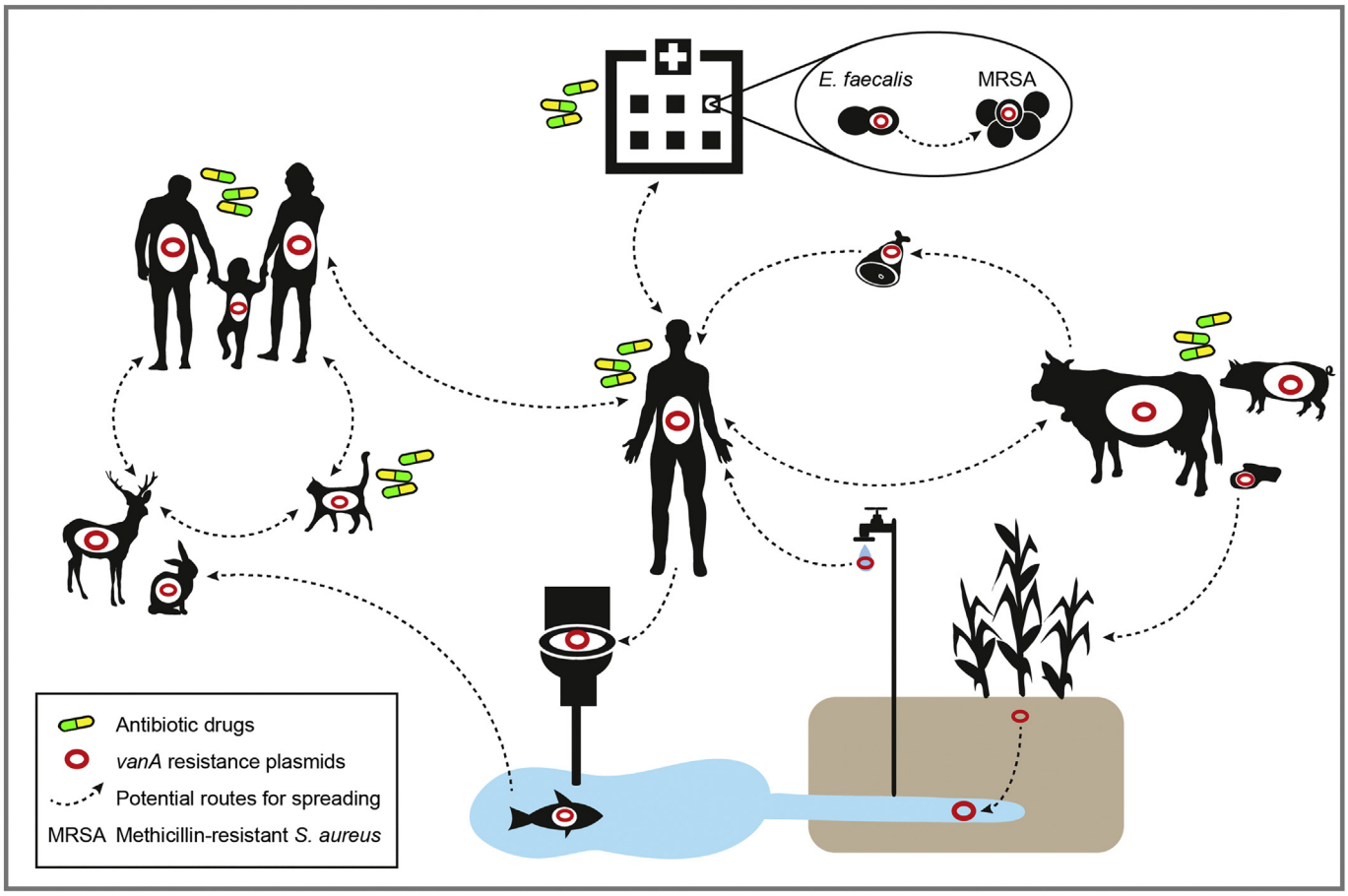

Fig. 2. Proposed spreading routes of Inc18-familiy *vanA* resistance plasmids. Inc18-like *vanA* resistance plasmids play a major role in the dissemination of multi-resistant bacteria. Transfer of these plasmids from *E. faecalis* to *S. aureus* resulted in the generation of vancomycin resistant *S. aureus* (VRSA) strains. Occurrence of Inc18-family plasmids in livestock can trigger further spreading of these plasmids among humans, either via direct consumption of animal products or via fertilization of crops with manure, which can lead to contamination of ground water. Wild animals are infected by water uptake and resistance plasmids can spread among humans either directly or via domestic animals as intermediate hosts.

Switzerland and the USA from 1986 to 2009. Interestingly, the PFGE profiles of the isolate *E. faecium* ST132 from pig manure and two clinical samples were identical with a 60-kb mosaic plasmid containing two replicons, rep_{Inc18} and rep_{pRUM}, and variations of Tn*1546*-IS*1216* (Freitas et al., 2011). A ST6 VRE isolate from pigs revealed to be highly similar to the multidrug-resistant clone ST6-CC2, widespread in hospitals in Italy, Portugal and Spain. This clone turned out to be resistant to vancomycin (*vanA*), teicoplanin, tetracycline, erythromycin, kanamycin, gentamicin, ciprofloxacin and chloramphenicol. These observations stipulate intra- and international spread of *E. faecalis* and *E. faecium* clones and their plasmids among humans and pigs (Freitas et al., 2011).

3. Transfer mechanism of Inc18-type plasmids: model plasmid pIP501

Conjugation systems can be found in nearly all bacterial species. They are responsible for the dissemination of mobile genetic elements, which contribute to the spread of fitness traits and, more problematically from a clinical perspective, multiple antibiotic resistances (Grohmann et al., 2017a, 2017b).

The major mechanism for horizontal plasmid transfer involves the translocation of a single stranded plasmid DNA via a type IV secretion system (T4SS) to a recipient cell (Christie, 2016; Grohmann et al., 2017b). Most mechanistic insights in the transfer process have come from the prototype T4SS, the VirB/D4 T-DNA transfer system of the plant pathogen *Agrobacterium tumefaciens*. Homologues of most Vir-proteins have been identified in T4SSs of G−, and, to some extent, in G+ genera. T4SSs are composite structures of functional protein modules: The relaxosome, the coupling protein and the T4SS machine, also termed MPF complex (Christie, 2016). Conjugative processes are activated by the establishment of donor-recipient contact and by dissemination of yet not understood signals. The DNA transport requires three reactions that act concerted in space and time: First, the relaxase and potential accessory proteins process the DNA molecule for transfer, followed by interaction with the coupling protein, finally resulting in the transfer of the DNA substrate across cell boundaries, facilitated by a multiprotein channel that is built up by MPF proteins (Bhatty et al., 2013). Even though G− and G+ bacteria differ significantly regarding their cell wall architecture, components homologous to VirB1, VirB4, VirB6, VirD4 and VirD2 have been identified in most G+ conjugative plasmids, including the Inc18-family plasmids pIP501, pAMß1 and pRE25, as well as the more host-specific pheromone-responsive plasmid pCF10 (Grohmann et al., 2003; Wallden et al., 2010). All identified G+ T4SSs encode a VirB4-like protein, represented by TraE of pIP501 or PrgJ of pCF10 (Grohmann et al., 2017b; Li et al., 2012). Furthermore, G+ T4SSs seem to code for a weak homologue of the lytic transglycosylase VirB1, such as TraG of pIP501 or PrgK from pCF10 (Arends et al., 2013; Kohler et al., 2017; Laverde Gomez et al., 2014).

Conjugative T4SSs of G− and G+ bacteria exhibit significant similarities with the first steps being almost identical. This involves processing of the plasmid DNA by the relaxase, e.g. TraA of pIP501 or PrgG from pCF10 (Grohmann et al., 2003). In contrast, the following steps seem to be considerably different.

Homologues of neither VirB7, VirB9 and VirB10, forming the outer membrane complex in G− systems (Wallden et al., 2010), nor VirB11-like ATPases have been found in G+ T4SSs yet (Bhatty et al., 2013; Grohmann et al., 2017b). Further, G+ T4SSs lack subunits building up the core complex. Only two of the conserved T4SS proteins were proposed to localize to the outside of the membrane, namely the VirB1-like cell wall hydrolase and the C-terminal domain of the VirB8-like protein. While G− VirB1-like proteins are secreted to the periplasmic space, G+ counterparts harbour a transmembrane domain and thus are anchored in the membrane (Alvarez-Martinez and Christie, 2009). G+ systems lack conjugative pili, but rather rely on surface adhesins ensuring the contact between donor and recipient, such as TraO from pIP501 and PrgC from pCF10 (Bhatty et al., 2015; Goessweiner-Mohr et al., 2014a; Grohmann et al., 2017b).

Inc18-family plasmids, like pIP501 and pRE25, and *Enterococcus* sex pheromone plasmids, just as pCF10, have been extensively studied to shed light on G+ conjugative transfer of single stranded DNA, while in-depth examination of *Streptomyces* plasmids helped to understand the divergent transfer mode involving double stranded DNA (Grohmann et al., 2017a, 2017b; Thoma and Muth, 2016; Wallden et al., 2010). Due to its small size and simplicity, pIP501 has become the paradigm to study conjugation of broad host-range plasmids in G+ bacteria with a low-GC content. As already indicated, this plasmid was originally isolated from a clinical *Streptococcus agalactiae* in 1975 (Evans and Macrina, 1983). pIP501 has been shown to be transferred to a wide variety of hosts, including streptococci, lactobacilli, lactococci, listeria, bacilli, clostridia, staphylococci and enterococci. The plasmid was further transferred to *Streptomyces lividans* and *E. coli* (Kurenbach et al., 2003). Inc18 plasmids can mobilize small, non-self-transmissible plasmids to a wide range of G+ genera (Langella et al., 1996; Thompson and Collins, 1988; Zúñiga et al., 2003). The transfer region responsible for conjugative transfer of pIP501 (Accession number: L39769.1; AJ505823.1) is conserved with those of the staphylococcal plasmids pGO1 and pSK41 and the lactococcal plasmid pMRC01 (Grohmann et al., 2003; Kurenbach et al., 2003). It is organised as a single operon of approximately 14 kb termed *tra*-operon, encoding the 15 genes *traA* to *traO*. A representative scheme of the pIP501 *tra*-operon is presented in Fig. 3. Three Vir-protein homologues of the pIP501 plasmid, VirB4-like TraE, VirB1-like TraG and VirD4-like TraJ, are also conserved in plasmid pRE25 from *E. faecalis* and in other related plasmids such as pSK41 and pGO1 (Wallden et al., 2010). pRE25 is considerably bigger than pIP501 with 58 CDS, five of them coding for resistances against 12 different antibiotics. This plasmid is transferred to a wide host-range and its *tra*-region appears to be very similar to that of pIP501 (Schwarz et al., 2001; Werner et al., 2013; Zhu et al., 2010). Overall, a 30.5 kb segment of pRE25 reveals remarkable homology to pIP501 and is flanked by two IS*1216V* elements (Schwarz et al., 2001). The identified pIP501-like region carries the putative minimal conjugative system comprising of an identical 49-bp *oriT*, followed by a gene 100% identical to *traA* of pIP501, coding for the relaxase. This homology suggests that the *tra*-region of pRE25 is made up by 15 genes downstream of the *oriT* region (Goessweiner-Mohr et al., 2014a; Teuber et al., 2003). pAMß1 was shown to present transfer rates comparable to that of pIP501 (Langella et al., 1996). pAMß1, as it is the case for pIP501, has a broad host-range and even was shown to transfer into *Clostridium* spp. (Berryman and Rood, 1995). As shown for pIP501, this plasmid has been demonstrated to transfer in the environment, as well as in animal's intestines. The relaxase of pAMß1 shows high sequence identity (96%) with TraA from pIP501 and further, the two plasmids comprise only slightly different *oriT* sequences (Clewell et al., 2014).

4. Regulation of transfer of Inc18-type plasmids (exemplified by the model plasmid pIP501)

Control of *tra*-gene expression is a general feature of mobile genetic elements of both G+ and G− origin. This ensures that the costly expression of multiple Tra-proteins only takes place when potential recipients and/or environmental conditions allow for efficient plasmid transfer (Grohmann et al., 2016).

For the pIP501 T4SS, it was demonstrated that the *tra*-genes were co-transcribed and that the mRNA levels remained unchanged until stationary growth phase (Kurenbach et al., 2006). As slightly lower transfer frequencies were observed in cultures with optical densities higher than OD_{600} of 1, it is speculated that transfer frequencies might decline in late stationary growth phase (Grohmann et al., 2016; Kurenbach et al., 2006).

One major component for conjugative transfer, the relaxase, is encoded by *traA*, the first *tra*-gene of the pIP501 *tra*-operon. TraA belongs to the IncQ-type DNA relaxases and can be classified into the MOB_Q family of relaxases, with $TraA_{pTI}$, $TraA_{p42a}$ and $MobA_{RSF1010}$ as further representatives (Garcillán-Barcia et al., 2009; Kurenbach et al., 2003). TraA was demonstrated to bind to the P_{tra}-promoter, thus negatively autoregulating the transcription of the *tra*-operon (Grohmann et al., 2016; Kopec et al., 2005; Kurenbach et al., 2006). This sophisticated autoregulation system has been described for several plasmids and is an efficient mechanism resulting in optimal balance between maximum transfer potential and the lowest burden for the cell (Grohmann et al., 2016). Due to detailed structural insights into the TraN protein-DNA complex and experimental evidence on the repression of the P_{tra}-promoter (unpublished data), it was proposed that the small cytosolic protein, TraN encoded as the second last *tra*-gene of the operon might have a role as an additional repressor of the pIP501 system, as will be described in later sections (Goessweiner-Mohr et al., 2014b).

5. Structure-function relationships of pIP501 transfer proteins

In this section, the pIP501-encoded transfer machinery will be described regarding functional and/or structural features, and the Tra-proteins will be subdivided in distinct functional classes: The relaxase and accessory proteins, motor proteins, peptidoglycan-hydrolysing proteins, MPF members and surface family-proteins. Until now, seven structural and/or functional homologues to the G− *A. tumefaciens* T4SS have been identified in the pIP501 conjugative system (Grohmann et al., 2016). The current model of pIP501 transfer and its putative regulation on transcriptional level are depicted in Fig. 4.

5.1. The relaxase and putative accessory proteins

TraA, the relaxase of the pIP501 T4SS, belongs to the group of RSF1010 relaxases, which are characterized by a conserved tyrosine in their active site at the N-terminus. *oriT* cleaving activity requires the coordination of the divalent cation Mg^{2+}, which is fulfilled by two histidines at amino acid positions 134 and 136 in TraA, separated by a hydrophobic residue (Kurenbach et al., 2002). Interestingly, TraA has

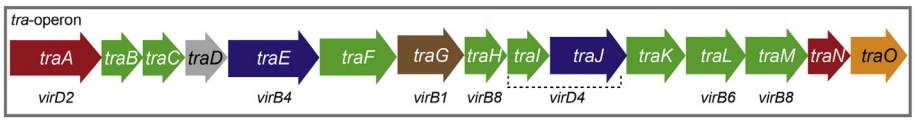

Fig. 3. Genetic organization of the transfer operon encoded on pIP501. The used colour code classifies the 15 *tra*-genes into the different functional groups as mentioned in the main text: Genes coding for the relaxase and accessory proteins (red), motor proteins (blue), peptidoglycan-hydrolysing proteins (brown), surface proteins (orange), mating pair formation proteins (green) and proteins of unknown function (grey). Homologues of the *A. tumefaciens* T4SS are depicted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

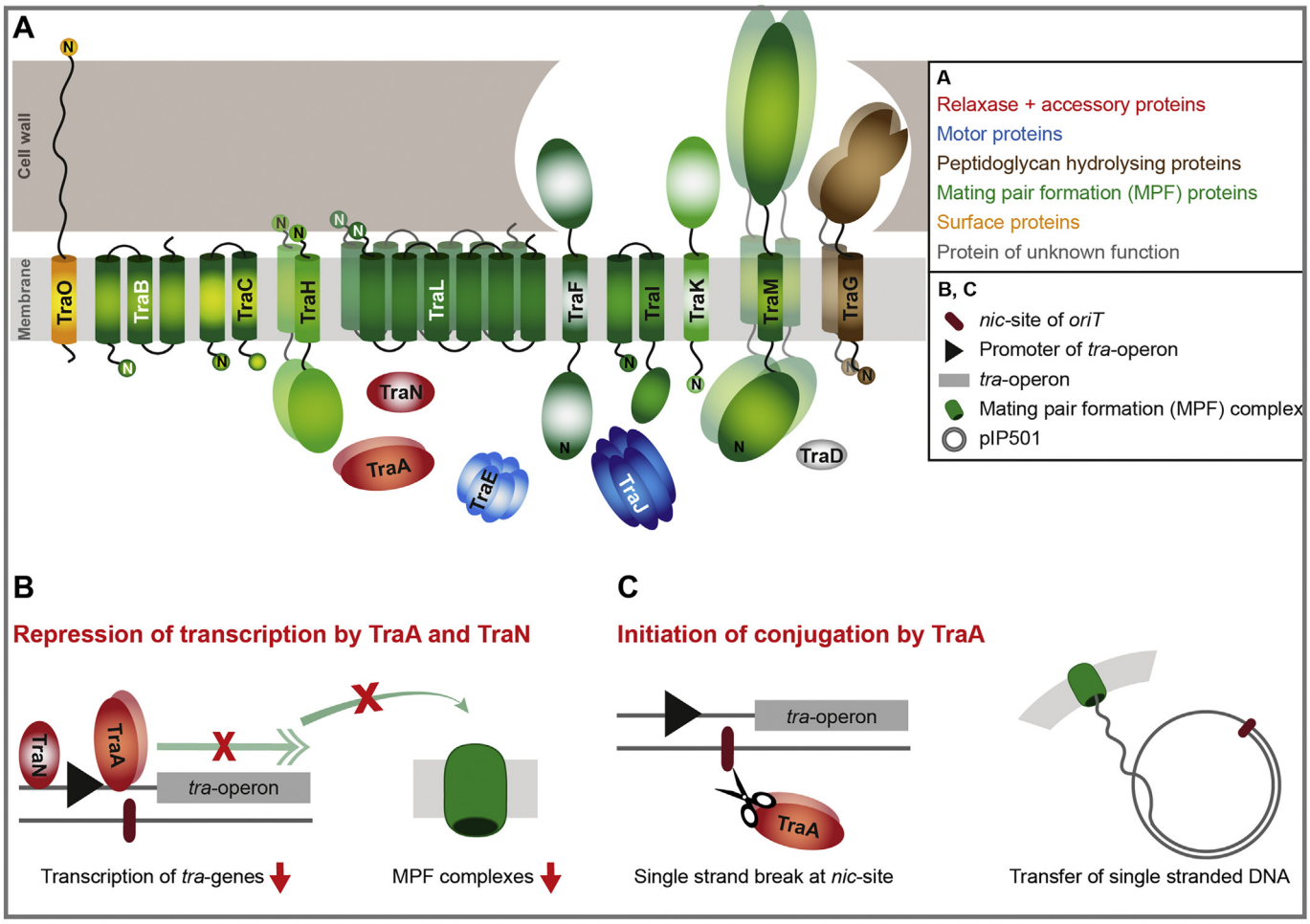

Fig. 4. Schematic drawing of the pIP501-encoded T4SS. Localization of the Tra-proteins in the G+ cell envelope (A), regulation of transfer (B) as well as DNA processing steps (C) are depicted.
(A) Local peptidoglycan digestion by TraG (in brown) allows for the assembly of mating pair formation (MPF) proteins (in green). TraO (in orange) most likely acts as a tether between donor and future recipient cell. The coupling protein TraJ and the ATPase TraE (in blue) are likely responsible for the generation of energy for different steps in the conjugative process.
(B) Binding of TraA to nucleotides within the promoter region and of TraN upstream of the promoter region leads to transcriptional repression of the *tra*-operon.
(C) Conjugative transfer is initiated with a single strand break at the *nic*-site of the *oriT* by the relaxase TraA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

been shown to form dimers and comprises a two-domain structure like other relaxases, with the function of the positively charged C-terminus remaining unknown (Kopec et al., 2005).

TraN is a small cytosolic protein, which shows specific DNA binding upstream of the pIP501 *nic*-site. Structural analysis of TraN revealed an internal dimer fold with antiparallel β-sheets in the centre and a helix-turn-helix motif on both ends. This fold suggests a DNA-binding mode, in which two adjacent DNA major grooves are occupied by TraN. Structural alignment of the two TraN halves led to a nearly perfect superposition. One half-site of TraN resembles the fold of excisionases (Xis) from transposons of G+ origin and bacteriophages as well as transcriptional regulators of the MerR family. Those proteins also recognize the DNA as a dimer (Goessweiner-Mohr et al., 2014b).

Interestingly, no C-terminal extension as found for MerR proteins, required for effector binding (Brown et al., 2003), was found in TraN or any TraN-like proteins on related T4SSs. TraN-like proteins all originate from the enterococcal plasmids pRE25, pAMß1, pVEF3 or are genomically encoded in *E. faecalis* and *E. italicus*. Even though the precise function of TraN is still under investigation, it has been proposed that TraN could act as an additional repressor. Either TraN directly inhibits the RNA polymerase from efficient transcription of the *tra*-genes by interaction with the relaxase TraA, thereby possibly increasing its binding strength or the fact that two proteins bind close to each other - TraA's binding site partially overlapping with the P_{tra}-promoter and TraN binding only 55 bp upstream of TraA - might lead to spatial problems for the transcriptional machinery. The concerted impact of these scenarios or one event by itself might lead to the increased repression in presence of TraN (Goessweiner-Mohr et al., 2014b; Grohmann et al., 2016).

5.2. Motor proteins

TraE and TraJ belong to the motor protein family. Both proteins contain Walker A and Walker B motifs, which are involved in NTP binding (Goessweiner-Mohr et al., 2014a). As both proteins were shown to be indispensable for conjugative transfer (Probst, 2017), TraE and TraJ very likely produce energy for different steps during the conjugative process (Grohmann et al., 2016).

TraE is a VirB4-like ATPase and showed self-interaction in a yeast-two-hybrid assay (Abajy et al., 2007). It is proposed that the protein forms oligomers and it might be involved in the DNA translocation process and/or assembly of the translocation machinery (Goessweiner-Mohr et al., 2014a). Interactions of TraE with TraD, TraG and TraN were predicted (Abajy et al., 2007).

TraJ is the potential VirD4-like coupling protein that is assumed to link the relaxosome with the MPF complex. A direct interaction with the relaxase TraA was shown by yeast-two-hybrid assays (Abajy et al., 2007), further strengthening TraJ's proposed function. Interestingly, unlike TraJ, most coupling proteins from G− as well as G+ genera possess a transmembrane domain to link them to the complex assembly sites (Christie, 2016). As TraJ seems to be a cytosolic protein without transmembrane helix and/or membrane anchor domains, it was hypothesized that the pIP501 T4SS could encode a two-partner coupling protein made up by TraJ constituting the enzymatic moiety and most likely the transmembrane protein TraI, operating as a tether to the membrane (Alvarez-Martinez and Christie, 2009; Goessweiner-Mohr et al., 2014a).

5.3. Peptidoglycan hydrolysing proteins

The VirB1-like protein TraG is a peptidoglycan hydrolysing enzyme that has been shown to be indispensable for the conjugative transfer of pIP501 (Arends et al., 2013). TraG shows a modular architecture with a transmembrane helix, likely responsible for its proper localization and two enzymatic domains, namely a soluble lytic transglycosylase (SLT) and a cysteine-, histidine-dependent amidohydrolases/peptidases (CHAP) domain. Furthermore, an *N*-acetylglucosamine binding site is located within the SLT domain, which has been proposed to act as linkage to the peptidoglycan. TraG-mediated cleavage activity was demonstrated *in vitro* on peptidoglycan from *E. faecalis* and *E. coli* (Arends et al., 2013). The central role of this protein appears to be local digestion of peptidoglycan at the sites of future MPF complex assembly. However, it might not only prepare the cell wall for insertion of the T4SS but seems to be directly involved in the assembly of the translocation channel. It was demonstrated that TraG interacts with the VirB8-like protein TraM, and correct localization of TraM at the cell periphery of *E. faecalis* cells required the presence of the transmembrane helix of TraG. Thus, TraG, besides its function as peptidoglycan hydrolysing enzyme, seems to further act as a scaffolding factor, which is crucially involved in the translocation complex assembly (Kohler et al., 2017).

5.4. MPF proteins

The VirB8-like protein TraM has been predicted to be part of the MPF complex. The C-terminus of TraM shows structural homology to VirB8. TraM is also structurally related to members of the nuclear-transport-factor 2 (NTF2) like superfamily. The N-terminal domain of TraM has been proposed to be cytoplasmic, whereas the C-terminal part faces towards the cell wall and appears to be surface exposed. TraM is a trimer in its biologically active form. This trimerisation is most likely facilitated by the N-terminal coiled-coil motif followed by a transmembrane helix. It has been proposed that G+ VirB8 homologues play a key role in cell wall spanning (Bhatty et al., 2013; Goessweiner-Mohr et al., 2014a). TraM might be an important part of the secretion apparatus, probably functioning as scaffolding protein or alternatively as additional attachment site for the potential recipient. Furthermore, TraM could also be actively involved in the morphogenesis of the actual T4SS MPF complex (Goessweiner-Mohr et al., 2013).

The NMR structure of the soluble C-terminal domain of TraH was solved recently (Fercher et al., 2016). Due to the similarity of TraH to VirB8-like proteins of G− T4SSs, we assumed that the protein plays a role in scaffolding of the MPF complex. TraH has been shown to be essential for the conjugative transfer and appears to localize to the cell wall via its N-terminal domain, whereas the C-terminus faces towards the cytoplasm. TraH, as the second VirB8-like protein of the pIP501 T4SS, comprises a NTF2-like fold, but shows with 6–19% only a very low sequence identity to other VirB8-like proteins. Interestingly, TraH revealed more resemblance to G− VirB8-proteins than to the C-terminal domains of the structurally characterized VirB8-like proteins from G+ origin, TraM from pIP501 or TcpC from the *C. perfringens* pCW3 T4SS, which both form trimers and comprise a N-terminal coiled-coil motif (Goessweiner-Mohr et al., 2013; Porter et al., 2012). It could be shown that the transmembrane helix of TraH is responsible for its homo-dimerisation (Fercher et al., 2016).

Even though TraH and TraM are both VirB8-homologues, they appear to have non-redundant functions, as respective single gene deletion resulted in a complete loss of conjugative transfer (Fercher et al., 2016; Goessweiner-Mohr et al., 2013). The reverse topology of TraH with its NTF2-like domain pointing inwards, suggests a role in scaffolding and/or assembly as a recruiting factor of the membrane-embedded T4SS complex at the cytoplasmic site. In contrast, the C-terminus of TraM might fulfil a similar task at the extracellular site and could act as attachment site for the recipient during conjugative transfer. The absence of any experimental evidence for interaction with other Tra-proteins further strengthens the role of TraH as nucleation factor, thereby interacting with other components in a weak and transient way (Fercher et al., 2016).

TraK contains a transmembrane helix and appears to localize to the cell envelope, even though it is not surface exposed. This suggests TraK as a member of the translocation channel but excludes a role in cell-to-cell attachment (Abajy et al., 2007). Interestingly, no significant sequence-based or structure-based relationships were detected except for T4SS proteins of closely related G+ T4SSs from *Enterococcus* and *Streptococcus*. These proteins all have a comparable length and a small N-terminal domain, predicted to be cytoplasmic. Interestingly, in plasmid pRE25, two putative T4SS proteins exhibit high sequence identity to TraK (Schwarz et al., 2001). It appears, though, that the two CDS have been generated from a single ancestor gene by a frame-shift mutation (Grohmann et al., 2003). As TraK-like proteins were only detected in *Enterococcus* and *Streptococcus* T4SSs, this suggests an exclusive role of TraK in these systems. Since both pIP501 and pRE25 exhibit an exceptionally broad host-range of conjugative dissemination, it is suggested that TraK might play a role in the adaptation of the respective T4SS to new hosts (Goessweiner-Mohr et al., 2014c).

The VirB6-like protein TraL is proposed to be a part of the MPF complex (Abajy et al., 2007), like other representatives of VirB6-like proteins from G+ as well as G− origin (Christie, 2016), which all show a broad variability in secondary structure motifs. TraL resembles VirB6-like proteins in size and in the predicted transmembrane helices. VirB6-like proteins comprise up to six transmembrane helices and might be central players in building up the MPF channel (Goessweiner-Mohr et al., 2014a).

Due to observed protein-protein interactions in yeast-two-hybrid and pull-down assays, it was predicted that TraC, TraF and TraI might also participate in the assembly of the MPF complex (Abajy et al., 2007).

5.5. Surface proteins

TraO is proposed to be the outermost portion of the pIP501 MPF complex (Abajy et al., 2007). TraO has a proline-rich domain with a highly repetitive sequence motif. Those repeats are also present in other G+ surface adhesins (Bhatty et al., 2013; Krishnan and Narayana, 2011), such as in the putative surface adhesin, PrgC, encoded by pCF10 (Bhatty et al., 2015; Wallden et al., 2010). TraO comprises a signal peptide at its N-terminus and shows an LPxTG cell wall anchor motif (Navarre and Schneewind, 1999) at the C-terminus, followed by a putative transmembrane helix. It is supposed that the N-terminus of TraO is exposed to the cell surface, thereby playing a role in establishing the contact to the potential recipient cell (Goessweiner-Mohr et al., 2014a).

6. Conclusions and perspectives

Emergence and spread of multiple drug resistant pathogenic bacteria present a serious drawback in antibiotic therapy in human and veterinary medicine. In this review, we summed up the current

knowledge of occurrence and dissemination of Inc18 multi-resistance plasmids, playing a major role in the generation of VRSA strains, which are resistant to the last-line antibiotic vancomycin. The molecular biology of the development of VRSA strains, in addition most of them are MRSA, has been elucidated. Inc18 plasmids with an integrated Tn*1546* played a major role in transfer of *vanA* from enterococci to MRSA.

pIP501 is the Inc18 plasmid with the broadest known host-range of conjugative resistance transfer. Due to its small size, it is a model to study broad-host-range plasmid transfer in G+ bacteria. Considerable advances were made in the last decade concerning i) structural-functional relationships of key components encoded by the pIP501 T4SS as well as on ii) the regulation of pIP501 transfer on the transcriptional level by the transfer factors, TraA and TraN.

Based on the increasing mechanistic understanding of resistance transfer promoted by Inc18 plasmids, potential prevention strategies have been developed by targeting surface exposed T4SS proteins, such as the pIP501 VirB8 homologue TraM. Anti-TraM antibodies were demonstrated to considerably reduce the survival of *E. faecalis* and *S. aureus* strains harbouring putative pIP501-like T4SSs in an *in vivo* infection model (Laverde et al., 2017). More efforts in this direction are urgently required to reduce the spread of dangerous multi-resistant pathogens in the clinic as well as in the environment, and even more importantly to prevent the development of novel multiple resistant superbugs.

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Manuscript #3

Mini-review

Multi-resistant biofilm-forming pathogens on the International Space Station

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Review

Multi-resistant biofilm-forming pathogens on the International Space Station

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The International Space Station (ISS) is a confined and closed habitat with unique conditions such as cosmic radiation, and microgravity. These conditions have a strong effect on the human and spacecraft microflora. They can affect the immune response of the crew-members, thus posing a threat to their health. Microbial diversity and abundance of microorganisms from surfaces, air filters and air samples on the ISS have been studied. *Enterobacteriaceae*, *Bacillus* spp., *Propionibacterium* spp., *Corynebacterium* spp., and *Staphylococcus* spp. were among the most frequently isolated bacteria. Microbial growth, biofilm formation, stress response, and pathogenicity are affected by microgravity. Increased resistance to antibiotics in bacteria isolated from the ISS has often been reported. *Enterococcus faecalis* and *Staphylococcus* spp. isolates from the ISS have been shown to harbor plasmid-encoded transfer genes. These genes facilitate the dissemination of antibiotic resistances. These features of ISS-pathogens call for novel approaches including highly effective antimicrobials which can be easily used on the ISS. A promising material is the antimicrobial surface coating AGXX®, a self-recycling material consisting of two noble metals. It drastically reduced microbial growth of multi-resistant human pathogens, such as staphylococci and enterococci. Further novel approaches include the application of cold atmospheric plasma for the sterilization of spacecrafts.

Keywords. Antimicrobial resistance; Biofilm; International Space Station; Pathogens; *Staphylococcus*

1. Introduction

The ISS is an isolated research station in closed and hostile space environment (Schiwon *et al.* 2013). It is characterized by high levels of cosmic radiation, microgravity and hypomagnetic conditions. These extreme conditions affect the immune response of the crew and alter their respiratory, gastrointestinal, and nasal bacterial flora thereby increasing the infection risk (Guèguinou *et al.* 2009; Taylor 2015). In-flight cross-contamination with *Staphylococcus aureus* and other pathogens in the upper respiratory tract of astronauts has been reported (Decelle and Taylor 1976).

Bacterial species associated with skin and mucous membranes of the crew dominate on the ISS (Novikova *et al.* 2006). Among the most frequently isolated bacteria are *Enterobacteriaceae*, *Bacillus* spp., *Propionibacterium* spp., *Corynebacterium* spp., and *Staphylococcus* spp. (Schiwon *et al.* 2013; Venkateswaran *et al.* 2014; Checinska *et al.* 2015; Urbaniak *et al.* 2018).

2. Microbial diversity and abundance on space stations

Diverse microbes have been isolated from various sites on the ISS. Potable water on the ISS was mainly contaminated by *Sphingomonas* spp. and *Methylobacterium* spp. (Castro *et al.* 2004; Novikova *et al.* 2006). Novikova and coworkers examined more than 500 air, water and surface samples from the Russian segment of the ISS for more than six years. More than 70 different species were isolated; half of them were of bacterial and half of them of fungal origin showing an exceptional biodiversity. *Staphylococcus* was the predominant genus. It was isolated from 84% of the air and surface samples. *Aspergillus* and *Penicillium* were the dominant fungi in air samples while *Aspergillus* and *Cladosporium* dominated on the surfaces (Novikova *et al.* 2006; Taylor 2015).

In the US segment of the ISS, from December 2011 to July 2012, allergic responses to cabin environment and dust in the Node 3 cabin were reported. This led to a study analyzing particulate and debris samples from the ISS which

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were collected in a vacuum cleaner bag during the respective time-period (Venkateswaran *et al.* 2014). This was the first study using Next Generation Sequencing (NGS) to examine the microbiome on the ISS. Culture-dependent approaches showed the presence of spore-forming bacteria and human commensals on the ISS. Using NGS, Firmicutes, Actinobacteria, Bacteroidetes, and Proteobacteria were the most abundant phyla. *Aspergillus* and *Penicillium* were the most prevalent fungi as detected by morphological and microscopic techniques (Venkateswaran *et al.* 2014). Whole metagenome analysis of microbes from ISS filter and dust samples revealed the presence of *Corynebacterium*, *Propionibacterium*, *Staphylococcus*, and *Penicillium* in dust and *Corynebacterium* and *Aspergillus* in the filter samples. *Corynebacterium ihumii* dominated the ISS filter material (Be *et al.* 2017). In another metagenomics study, metagenome sequences from eight ISS environmental locations within the US on-orbit segments in three consecutive flights were analyzed. Three hundred eighteen microbial species were detected. *Rhodotorula* and *Pantoea* dominated the ISS microbiome. A large variety of human pathogens, including *Acinetobacter baumannii*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Salmonella enterica*, *Shigella sonnei*, *S. aureus*, *Yersinia frederiksenii*, and *Aspergillus lentulus* were isolated. A continuous increase in the number of bacteria (as well as the number of antimicrobial resistance and virulence genes) was recorded from flight 1 to flight 3, while the number of fungi decreased successively (Singh *et al.* 2018a).

Microbial monitoring was also done in the Japanese experimental model Kibo on the ISS for four years. In September 2009, February 2011, and October 2012, samples were collected from the surface of the incubator, inside the door of the incubator, the air intake of the incubator, the air diffuser, and the handrail of the incubator. The samples mostly contained Proteobacteria and Firmicutes with Staphylococci and Enterobacteriaceae being the dominant bacterial groups in Kibo (Ichijo *et al.* 2016).

3. Effect of space flight on microbial physiology

Bacterial growth, biofilm formation capacity and virulence are enhanced under space flight conditions (Guèguinou *et al.* 2009; Taylor 2015; Urbaniak *et al.* 2018). It has been shown that catecholamine (observed in elevated levels in the plasma of individuals who have often been in space) enhances bacterial growth (Sonnenfeld 1999; Guèguinou *et al.* 2009) which might lead to contamination of the spacecraft and infection of crew-members (Guèguinou *et al.* 2009). Studies conducted on crew-members on the Mir space station from 1995 to 1998 revealed microbial infections including acute respiratory infections, conjunctivitis, and dental infections (Ball *et al.* 2001; Guèguinou *et al.* 2009).

Microgravity affects microbial physiology, response to stress, gene expression and pathogenicity (Guèguinou *et al.* 2009; Aunins *et al.* 2018). *E. coli* cells grown under low shear microgravity conditions showed increased resistance to general stress in exponential and stationary phase (Lynch *et al.* 2004). This indicates that low shear microgravity increases the stability of transcription factor σ^s thereby affecting σ^s-dependent regulatory processes like protein folding (Lynch *et al.* 2004). *Salmonella* Typhimurium grown aboard the Space Shuttle mission STS-115 showed increased virulence under low shear microgravity conditions compared to the same strain grown on Earth (Wilson *et al.* 2007). To study the effect of microgravity on *E. coli,* Aunins and coworkers analyzed the transcriptomic response of *E. coli* grown on the ISS with increasing gentamicin concentrations in comparison to the identical *E. coli* isolate grown on Earth with increasing gentamicin concentrations. Within two days, the isolate in space adapted to grow at higher gentamicin concentrations than the control-isolate on Earth. The isolate on the ISS showed up-regulation of 50 stress response genes suggesting microgravity induces stress responses associated with antibiotic stress and likely increases antibiotic stress tolerance in bacteria in space (Aunins *et al.* 2018).

4. Antibiotic resistance on space stations

Bacterial susceptibility to antibiotics is lowered under spaceflight conditions (Horneck *et al.* 2010). Antibiotic resistance among bacteria is an ever-increasing challenge and has also been observed in bacteria on the ISS and the Antarctic research station Concordia. 76% of the isolates from the Russian segment of the ISS showed resistance to one or more antibiotics. 83% of the isolates formed biofilms and 86% of them harbored putative conjugative plasmids facilitating the dissemination of antibiotic resistance (Schiwon *et al.* 2013). Antibiotic resistance was also reported in the US segment of the ISS. Five pathogenic *Enterobacter bugandensis* isolates from two locations were analyzed using whole genome analysis. Four of these isolates were from the waste and hygiene compartment and one isolate was from the Exercise platform of the ISS. All isolates were similar to two clinical *E. bugdanensis* strains. All of them harboured resistance genes conferring resistance to cefazolin, cefoxitin, oxacillin, penicillin, and rifampin and the multiple antibiotic resistance (MAR) locus which upregulates drug efflux systems rendering bacteria drug resistant. Additionally, 112 genes associated with virulence, and infectious disease were detected in these ISS isolates (Singh *et al.* 2018b).

Increase in the minimum inhibitory concentration (MIC) of antibiotics has been reported in microorganisms in spaceflight as compared to those on Earth. In 1982, the Cytos 2 experiment was conducted to determine the antibiotic susceptibility of *S. aureus* and *E. coli* from gastrointestinal and nasal microbiota of an astronaut aboard Salyut 7. The experiments were performed during orbital flight and compared to the ground controls. In case of *E. coli*, the MIC values for colistin and kanamycin were four times larger

than those of the ground controls. Similarly, for *S. aureus*, a two-fold increase in the MIC for oxacillin, chloramphenicol, and erythromycin was reported for the isolates on board as compared to ground controls (Tixador *et al.* 1985; Taylor 2015).

A range of different antibiotic resistances were identified in ISS filter and dust samples. These include resistance to aminoglycosides, beta-lactams, clindamycin, fluoroquinolones, lincosamides, streptomycin, and tetracycline (Be *et al.* 2017). *S. aureus* isolates from surfaces on the ISS and from the Permanent Multipurpose Module have been reported to be resistant to erythromycin, penicillin, and rifampin (Checinska Sielaff *et al.* 2016). In another study, among the *S. aureus* isolates recovered from antimicrobial coatings and the respective uncoated reference materials on the ISS over a period of 19 months (2013-2015), 89% were resistant to ampicillin and erythromycin, 33% to kanamycin, 11% to tetracycline and clindamycin, and all of them were resistant to sulfamethoxazole (Sobisch *et al.* 2019). Among the *S. hominis* isolates, 94% were resistant to sulfamethoxazole, 80% to erythromycin, 60% to ampicillin, 23% to clindamycin, 29% to tetracycline, 11% to kanamycin, 6% to meropenem, and 3% to tigecycline and doxycycline. All *S. epidermidis* isolates were resistant to sulfamethoxazole, 91% to erythromycin, 25% to ampicillin, and 8% to doxycycline. Among the *E. faecalis* isolates, all were resistant to kanamycin, gentamicin, and sulfamethoxazole, 67% to tetracycline and doxycycline, 67% to clindamycin, 33% to chloramphenicol, and 33% were resistant to meropenem (Sobisch *et al.* 2019).

Percentages of antibiotic resistances among bacteria isolated from diverse antimicrobial surfaces and uncoated reference materials on the ISS in this 19-months survey are depicted in figure 1 (modified from Sobisch *et al.* 2019). Thus, the most prevalent resistances were sulfamethoxazole and erythromycin resistance. In addition, ampicillin resistance was frequently detected, dangerously, often in combination with sulfamethoxazole and erythromycin resistance.

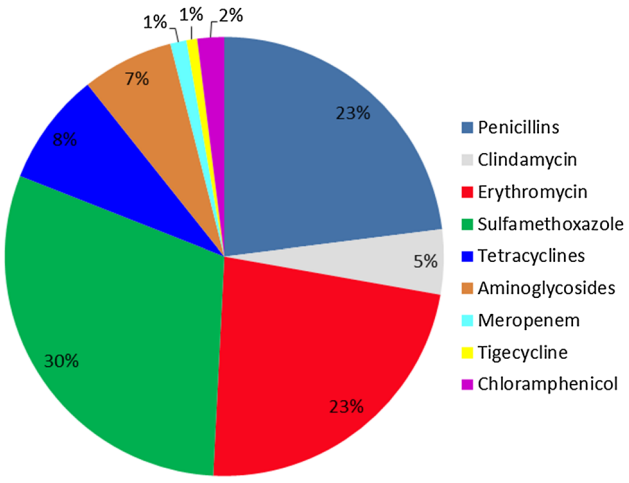

Figure 1. Antibiotic resistance on the ISS. Prevalence of antibiotic resistance (%) among all bacterial isolates recovered from surfaces on the ISS over a period of 19 months. Here, Penicillins comprise ampicillin and oxacillin. Aminoglycosides comprise kanamycin and gentamicin, and Tetracyclines include tetracycline and doxycycline.

5. Biofilm formation on space stations

Microbial contamination of indoor surfaces of spacecrafts may corrode the materials and decrease the efficiency of the equipment by biodegradation (Schiwon *et al.* 2013; Zea *et al.* 2018). Microbial biofilms have been observed on the ISS, and on Salyut/Mir (Soviet/Russian) space stations (Zea *et al.* 2018). On Salyut 6, biofilms were observed on the piping and equipment behind panels. On Salyut 7, electrical connectors, the thermal control system's radiator, the water recycling system, and rubber of hatch locks were contaminated by biofilms. On the Mir station, biofilms affected the navigation window, the air conditioning, the oxygen electrolysis block, the water recycling unit, the headphone of the EVA suit, and the thermal control system as well (Klintworth *et al.* 1999). On the ISS, biofilms were detected on rubber seals, viewing windows and on different hardware surfaces (Schiwon *et al.* 2013). A variety of pathogens isolated from the ISS formed strong biofilms. Most biofilm formers were staphylococci and enterococci (Schiwon *et al.* 2013; Sobisch *et al.* 2019). These biofilms can cause serious infections which are difficult to treat due to increased resistance to antibiotics and disinfectants. Therefore, it is imperative to search for novel approaches to inhibit or at least strongly reduce biofilm formation (Zea *et al.* 2018).

6. Countermeasures to mitigate microbial contamination in spacecrafts

Further culture-dependent and culture-independent approaches need to be performed to get a deeper insight in the bacterial resistome on the ISS. This knowledge will help in designing and developing mitigation strategies to control antibiotic resistance in bacteria (Urbaniak *et al.* 2018). In addition, development of new and effective procedures for the sterilization of spacecrafts or their components is crucial (Horneck *et al.* 2010). Cold atmospheric plasma is being studied as a sterilization procedure on board. It has been proposed to combat microbial infections, to inactivate antibiotic resistant bacteria such as MRSA (Morfill 2012), and to kill microbes in produce like tomatoes, peppers, and radishes, grown on the ISS (Hintze *et al.* 2017).

A large number of bacteria isolated from the ISS are human pathogens. Thus, it is imperative to use antimicrobials which are known to prevent growth and/or kill these pathogens. An antimicrobial against which no resistance has been reported so far is AGXX®, a surface coating consisting of silver and ruthenium. The coating was shown to be highly

effective against human pathogens such as MRSA (Vaishampayan *et al.* 2018), *E. faecalis* (Clauss-Lendzian *et al.* 2018), *Legionella* and *E. coli* (Guridi *et al.* 2015). Recently, the long-term antimicrobial effect of the coating was demonstrated by considerably reducing the microbial load on the ISS (Sobisch *et al.* 2019).

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Manuscript #4

Research Paper

A novel antimicrobial coating represses biofilm and virulence-related genes in methicillin-resistant *Staphylococcus aureus*

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A Novel Antimicrobial Coating Represses Biofilm and Virulence-Related Genes in Methicillin-Resistant *Staphylococcus aureus*

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Methicillin-resistant *Staphylococcus aureus* (MRSA) has become an important cause of hospital-acquired infections worldwide. It is one of the most threatening pathogens due to its multi-drug resistance and strong biofilm-forming capacity. Thus, there is an urgent need for novel alternative strategies to combat bacterial infections. Recently, we demonstrated that a novel antimicrobial surface coating, AGXX®, consisting of micro-galvanic elements of the two noble metals, silver and ruthenium, surface-conditioned with ascorbic acid, efficiently inhibits MRSA growth. In this study, we demonstrated that the antimicrobial coating caused a significant reduction in biofilm formation (46%) of the clinical MRSA isolate, *S. aureus* 04-02981. To understand the molecular mechanism of the antimicrobial coating, we exposed *S. aureus* 04-02981 for different time-periods to the coating and investigated its molecular response via next-generation RNA-sequencing. A conventional antimicrobial silver coating served as a control. RNA-sequencing demonstrated down-regulation of many biofilm-associated genes and of genes related to virulence of *S. aureus*. The antimicrobial substance also down-regulated the two-component quorum-sensing system *agr* suggesting that it might interfere with quorum-sensing while diminishing biofilm formation in *S. aureus* 04-02981.

Keywords: antimicrobial surface, MRSA, virulence, biofilm, quorum-sensing, RNA sequencing

INTRODUCTION

Staphylococcus aureus is an opportunistic pathogen commonly found in the human respiratory tract, nasal areas and skin. It colonizes the anterior nares of approximately 20–25% of the healthy adult population, while 60% are intermittently colonized (Kluytmans et al., 1997; Ellis et al., 2014). Methicillin-resistant *Staphylococcus aureus* (MRSA) is a crucial human pathogen causing infections ranging from skin and soft tissue infections to fatal sepsis (Marathe et al., 2015). It is one of the leading pathogens that cause nosocomial infections (Paniagua-Contreras et al., 2012; Lister and Horswill, 2014); it is resistant to methicillin and many other antibiotics (Marathe et al., 2015), and it is also known to produce thick biofilm (Paniagua-Contreras et al., 2012; Qin et al., 2014). MRSA was shown to cause catheter-associated and other medical devices-related

infections (Arciola et al., 2001; Paniagua-Contreras et al., 2012). Eighty percent of prosthetic infections are caused by Staphylococci (Kirmusaoglu, 2016). Its firm attachment to medical devices and host tissues, and its ability to form robust biofilms makes it a cause of chronic infections (Yarwood et al., 2004). *S. aureus* biofilms cause numerous infections in which the accessory gene regulator (*agr*) quorum-sensing system (QS) plays an important role (Yarwood et al., 2004). Around 90% of the infections caused by the bacterium are skin and soft tissue infections, and the *agr*QS system is associated with these infections (Sully et al., 2014).

Multiple drug resistance combined with a thick biofilm makes the treatment and eradication of *S. aureus* infections even more difficult. This entails the urge of development of novel antimicrobials, which could also be potential biofilm inhibitors. Virulence factors of *S. aureus* serve as targets for the newly developed class of biological anti-staphylococcal agents. These targets include, surface bound adhesins, immunoglobulin-binding proteins, surface-associated and secreted proteases, a family of immune-stimulatory exotoxins called 'superantigens' (SAgs), and potent leukocidal toxins (Sause et al., 2015).

Metals like copper and silver have been used as antimicrobials since a long time. The use of copper in human civilization is known since the 5th and 6th millennia B.C. Silver was officially approved for use as an antimicrobial agent in the 20th century (Chopra, 2007; Grass et al., 2011; Schäberle and Hack, 2014; Guridi et al., 2015). Copper and copper alloys have also been used as antimicrobials (Warnes and Keevil, 2013). These metals are known to kill bacteria and fungi by a phenomenon called contact killing (Grass et al., 2011) and can be used to coat medical devices as they inhibit biofilm formation of pathogens (Baker et al., 2010). In the 17th century, silver was described as an essential multipurpose medicinal product and the first scientific documentation of its medical use dates from 1901 (Maillard and Hartemann, 2013). However, in 1975, several patients died from a silver resistant *Salmonella* Typhimurium isolate in the Massachusetts General Hospital; this was the first report of silver resistant bacteria (Gupta et al., 1999). Excessive use of silver is questioned due to its toxicity to the environment as well as to the human body (Landsdown, 2010). Silver resistance, like antibiotic resistance in bacteria, prompts us to develop new strategies to control bacterial infections. One such novel, broad-spectrum antimicrobial agent is AGXX®.

AGXX® (Largentec GmbH, Berlin, Germany) is a combination of two transition metals, silver and ruthenium which can be galvanically electroplated on various carriers like V2A steel, silver sheets, Polydimethylsiloxane (PDMS), fleece, etc. The coating is conditioned by ascorbic acid and is active against many Gram-positive and Gram-negative bacteria (Guridi et al., 2015). It is not only an efficient antibacterial but also kills yeasts, viruses, and fungi (Landau et al., 2017a,b). The coating was used successfully for the decontamination of industrial cooling and process water (Landau, 2013). As it is only slightly cytotoxic (Bouchard, 2011), it can be incorporated into various medical applications. Although, the exact mode of action of the antimicrobial activity of the coating is not fully understood, it is known that the generation of reactive oxygen species (ROS) plays an important role in making it a potent antimicrobial. The formation of hydrogen peroxide and hydroxyl radicals has been detected by spectroscopic methods (Clauss-Lendzian et al., 2017). Putative formation of other ROS is under investigation. ROS can damage cellular components, including, DNA, lipids and proteins. Superoxide dismutase and catalase are involved in detoxification of ROS (Paraje, 2011).

In this study, we performed total RNA-sequencing of *S. aureus* 04-02981 (MRSA) to investigate differential gene expression after different times of exposure of the pathogen to the antimicrobials AGXX® or Ag. Our data demonstrate that AGXX® likely reduces biofilm formation and virulence in *S. aureus* 04-02981 by interfering with the QS, by down-regulating the expression of toxins like leukocidins (*lukE*) and gamma-hemolysins (*hlgA*), and of genes associated with surface adhesins and capsular polysaccharide.

MATERIALS AND METHODS

Preparation of Antimicrobial Metal Sheets

Silver sheets of 0.125 mm thickness were used as a base material to prepare the antimicrobial metal sheets. Both sides of the silver sheets were etched by immersing them in half-concentrated nitric acid, for 60 s. The silver sheets were cleaned with de-ionized water and galvanically plated with a 0.16 μm ruthenium coating on both sides for 40 s. Then, the sheets were cleaned with de-ionized water, conditioned with ascorbic acid, rinsed with de-ionized water and dried with a paper towel. Prior to use, AGXX®, and Ag sheets, used as reference material, were autoclaved at 121°C for 20 min.

Bacterial Strain and Culture Conditions

Staphylococcus aureus 04-02981 (Nuebel et al., 2010) was grown at 37°C in Tryptic Soy Broth [TSB] (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) with constant agitation at 150 rpm or on Tryptic Soy Agar [TSA] (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). Growth inhibition tests on agar surface were performed according to CLSI guidelines for disk diffusion test (Naas et al., 2006). For this assay, 0.25 cm^2 sheets of Ag and AGXX® were used.

For generation of growth curves, bacteria were pre-cultured overnight, diluted in TSB to an optical density at 600 nm (OD_{600}) of 0.05 and incubated for further 8 h either in presence of AGXX® or in the presence of silver (Ag), 24 cm^2 each in 30 mL medium to obtain a sheet surface to medium volume ratio (A: V) of 0.8. Cultures grown in the absence of a metal sheet served as controls. OD_{600} of the cultures was measured using the Genesys 10S UV-Vis spectrophotometer (Thermo Scientific, China). Colony forming units (CFU) per mL were determined hourly from 0 to 8 h post inoculation. Growth experiments were performed in triplicate with independent biological replicates.

Biofilm Screening Assay

To study the effect of Ag, and AGXX® on biofilm formation of *S. aureus* 04-02981, the Crystal Violet Assay was performed

without any metal sheet, in presence of Ag (24 cm^2 uncoated silver sheet) and in presence of AGXX® (24 cm^2 silver sheet coated with ruthenium for 40 s). The sheet surface: medium volume ratio (A: V) was 0.8 (24 cm^2 metal sheet: 30 mL medium). The overnight culture of *S. aureus 0*4-02981was diluted to an initial OD_{600} of 0.05. The culture was incubated at 37°C and 150 rpm for 4 h (mid- exponential phase, OD_{600}∼1.5). Then, it was transferred to the transparent 96-well plate (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) containing Ag, or AGXX®. The plate was incubated at 37°C for 24 h, then the antimicrobial metal sheets were carefully removed and OD_{600} of the cultures was measured. In addition, at this stage, the CFU per mL of the planktonic cultures and the biofilms in presence as well as in absence of the metal sheets were determined. Means of five values each and two biological replicates are given. The biofilm assay was performed according to Schiwon et al. (2013). *Enterococcus faecalis* 12030, a strong biofilm former was used as a positive control (Huebner et al., 1999), and Tryptic Soy Broth (TSB) as a negative control (Schiwon et al., 2013). Biofilm formation was measured in EnSpire Multimode Plate Reader 2300-0000 (PerkinElmer, Turku, Finland) at 570 nm. Normalized biofilm formation was calculated by dividing the biofilm measure at OD_{570} by the bacterial growth at OD_{600}. Following criteria were used for the interpretation of the results, ODc = negative control; OD ≤ ODc = non-adherent, ODc ≤ OD ≤ (2 × ODc) = weakly adherent, (2 × ODc) < OD ≤ (4 × ODc) = moderately adherent, (4 × ODc) < OD = strongly adherent, as described in Nyenje et al. (2013). Biofilm inhibitory rates of AGXX® and Ag were calculated using the following equation, as described by Qin et al. (2014).

$$\text{Inhibitory rate (\%)} = \frac{OD_{570}\,(\text{Control}) - OD_{570}\,(\text{Sample})^{*}100}{OD_{570}\,(\text{Control})}$$

Student's *t*- test was used to check if biofilm inhibition was statistically significant, using SigmaPlot version 11.0 (Systat software, Inc., San Jose, CA, United States[1]) (Wass, 2009).

Spinning Disk Confocal Microscopy

Staphylococcus aureus 04-02981 was grown in TSB overnight at 37°C, 150 rpm, then it was diluted to an OD_{600} of 0.05 and further incubated at 37°C for 4 h (mid-exponential phase, OD_{600} ∼1.5). Then, the culture was transferred to a μ-Dish (μ-Dish 35 mm, low, from ibidi GmbH, Martinsried, Germany) containing Ag, or AGXX® (sheet surface: medium volume ratio = 0.8) and incubated at 37°C for 24 h. The culture was removed from the μ-Dish, and the biofilm on the μ-Dish was washed three times with phosphate buffered saline (PBS). The biofilm was stained for 10 min in the dark with Hoechst 33342 (5 μg/mL) and propidium iodide (1 μg/mL) (Thermo Fisher, Eugene, OR, United States). The staining solution was then replaced with 50% glycerol to prevent movement of bacteria during imaging. Imaging was performed with a Nikon TiE-based Visitron spinning disk confocal microscope using a 100× NA1.45 objective. Fluorescent dyes were excited using 405 nm (Hoechst 33342) and 561 nm (propidium iodide) laser lines and fluorescent emission captured through appropriate filters onto an iXon888 EMCCD detector (Andor, Belfast, United Kingdom). Images were subsequently analyzed using Fiji (ImageJ) version 3.2.0.2.

Metal Stress and RNA Extraction

Overnight cultures of *S. aureus* 04-02981 were diluted as described above and grown until mid-exponential growth phase (4 h post dilution, OD_{600}∼1.5). The cultures were then subjected to metal stress by exposure to AGXX® or Ag sheets (sheet-surface to medium-volume ratio of 0.8) followed by further incubation for 6, 12, 24, 80, and 120 min at 37°C with constant agitation at 150 rpm. As a control, no metal sheet was added to the culture. Cells from 30 mL culture were harvested by centrifugation for 1 min at 10,000 rpm and 4°C in a Heraeus Multifuge X3R Centrifuge (Thermo Electron LED GmbH, Osterode am Harz, Germany). Cell pellets were immediately frozen in liquid nitrogen and stored at −80°C or directly used for RNA extraction using the ZR Fungal/Bacterial RNA MiniPrep™ Kit (ZymoResearch, Freiburg, Germany) following the manufacturer's instructions. To recover total RNA including small RNAs, 1.5 volumes of absolute ethanol were added in step 5. Finally, total RNA was eluted with 50 μl DNase- and RNase-free water and stored at −80°C. RNA quantity and quality were assessed with a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Osterode am Harz, Germany) as well as on bleach agarose gels. Residual contaminating DNA was eliminated with TURBO DNA-free™ Kit Ambion (Life Technologies, Darmstadt, Germany).

RNA Sequencing

Total RNA sequencing was done by PrimBio Research Institute, Exton, PA, United States. The protocol was performed in five steps; rRNA removal was done using the Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina, Cat# MRZMB126), followed by library preparation, and templating, enrichment and sequencing.

RNA-Sequencing Data Analysis

Raw sequencing reads were aligned to the reference genome of *S. aureus* 04-02981, using Bowtie2 (Langmead and Salzberg, 2012) version 2.2.3 with optimal settings for the IonProton™ Sequence. Post-processing of the SAM files into sorted BAM files was carried out with SAMtools (Li et al., 2009, version 1.2-207). The samples AGXX®, and Ag were normalized (AGXX®-Control, Ag-Control) against the control of the respective time-points. Length normalized confidence interval RPKM (=Reads per Kilobase of transcript per Million mapped reads) values were obtained with Cufflinks (Trapnell et al., 2010). Finally, statistical analysis was carried out using the T-REx RNA-Seq analysis pipeline (de Jong et al., 2015). A gene was considered significantly differentially expressed when the fold change was ≥|2.0| and the false discovery rate (FDR) adjusted *p*-value ≤ 0.05. The data presented in this paper have been deposited at NCBI, and are accessible through GSE103064[2].

[1] http://www.systatsoftware.com

[2] https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE103064

Reverse Transcription Quantitative PCR (RT-qPCR)

To verify the results obtained from RNA-sequencing, RT-qPCR was performed on five genes detected as highly differentially expressed via RNA-seq. To this end, RNA extracted from *S. aureus* 04-02981 cultures exposed to Ag or AGXX® for 24, and 80 min, was used. First strand cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis kit (Thermo Fisher Scientific Inc., Walham, Germany) as per the manufacturer's instructions using 120 ng total RNA as template and random hexamer primers. cDNA was diluted with DNase- and RNase-free water and amplified in a LightCycler®480 II (Roche Diagnostics GmbH, Mannheim, Germany).

The *agrC*, *lukE*, *sdrC, srrA*, and *cap5A* genes were selected to verify the data obtained through RNA-seq. The gene *gyrB* was used as a control. These genes were amplified using TaqMan chemistry according to the instructions provided in LightCycler® 480 Probes Master Kit (Roche Diagnostics). All RT-qPCR reactions were carried out in a total volume of 20 μL. The amplification step was performed with 'Quantification' analysis mode at 95°C for 10 s, with a ramp rate of 4.4°C/s, followed by annealing at the respective annealing temperature for 50 s, with a ramp rate of 2.2°C/s and finally an extension at 72°C for 1 s, with a ramp rate of 4.4°C/s. The amplification step was performed 45 times. All primers and probes used in the study are listed in **Supplementary Table S1**. All RT-qPCR experiments were done in triplicate and each experiment was repeated at least twice. Data were analyzed by LightCycler® 480 Software release 1.5.0 by using the 'Relative Standard Curve' method; the standard curves were constructed using genomic DNA from *S. aureus* 04-02981. Data represent expression ratios, calculated by normalizing to the *gyrB* gene and relative to the untreated culture of *S. aureus* 04-02981 which served as the calibrator, as described in 'Guide to performing Relative Quantitation of Gene expression using real time-quantitative PCR' by Applied Biosystems. Means of five *C*p values each were used to calculate the relative expression ratio.

Statistical Analysis

Statistical tests were performed to analyze the significance of the obtained data. Student's *t*-test was applied to the normalized target, and normalized control values (normalized concentration). The tests were performed and analyzed using SigmaPlot version 11.0 (Systat software, Inc., San Jose, CA, United States[3]) (Wass, 2009).

RESULTS

AGXX® Inhibits the Growth of *S. aureus* 04-02981

To analyze the effect of Ag, and AGXX® on the growth of *S. aureus* 04-02981, disk diffusion tests with Ag, and AGXX® were performed in accordance with NCCLS-CLSI guidelines (Naas et al., 2006). The agar plates were monitored at 24 h intervals for 5 days to check if Ag or AGXX® exhibited an inhibitory effect on the pathogen, in the form of a zone of inhibition on the agar plate. The diameter of the inhibition zones was measured in 'cm.' The mean diameter of the inhibition zone was calculated to be 1.2 cm for AGXX® while no zone of inhibition was observed for Ag.

To verify the inhibitory effect of AGXX® on *S. aureus* 04-02981 as demonstrated in the agar diffusion tests, experiments in TSB medium were performed measuring the CFU/mL every hour for a period of 8 h, using the A: V ratio (metal mesh: medium volume) of 0.8, as described in Section "Materials and Methods." As observed in the disk diffusion assay, Ag did not show a significant inhibitory effect on the growth of *S. aureus* 04-02981 in liquid cultures. In contrast, AGXX® had a profound inhibitory effect on this strain. The OD_{600} of *S. aureus* 04-02981 in presence of AGXX® was very low, (OD_{600} AGXX® at t8 = 0.149) as compared to Ag (OD_{600} Ag at t8 = 3.086) and the control (OD_{600} Control at t8 = 3.173) (**Supplementary Table S2**). The CFU/mL of *S. aureus* 04-02981 grown in the batch culture with AGXX® increased from 2.77×10^6 in the 1st hour to 3.99×10^{10} in the 4th hour, but then decreased to 1.08×10^7 in the 8th hour. The colony counts of *S. aureus* 04-02981 + AGXX® (after 8 h of growth) were much lower than that of the same strain with Ag (1.27×10^{11}) or without metal amendment (1.73×10^{11}) (**Table 1**). These data confirm the antimicrobial effect of AGXX® on *S. aureus* 04-02981.

AGXX® Strongly Reduces Biofilm Formation of *S. aureus* 04-02981

The effect of AGXX®, and Ag on biofilm formation of *S. aureus* 04-02981 was analyzed using the Crystal Violet assay. *E. faecalis* 12030, a strong biofilm former served as a positive control (Huebner et al., 1999), and TSB as the negative control (**Figure 1**). **Figure 1A** shows the biofilm formation by *S. aureus* 04-02981, measured at 570 nm, **Figure 1B** shows the biofilm formation (OD_{570}) normalized to the bacterial growth (OD_{600}) to take the antimicrobial effect of AGXX® into account.

To determine the bacterial killing activity of AGXX® under these conditions (after 24 h of growth, prior to adding crystal violet), we measured the CFU per mL of the planktonic cultures and the biofilms in the presence as well as in absence of the two different metal sheets.

The following values were obtained for the biofilms: For *S. aureus* 04-02981 without metal sheet (control), $2.34 \times 10^9 \pm 8.49 \times 10^7$ CFU per mL, for the strain in presence of Ag, $2.13 \times 10^9 \pm 2.40 \times 10^8$, and in presence of AGXX®, $1.80 \times 10^4 \pm 1.41 \times 10^3$. When we measured the CFU per mL in the respective planktonic cultures, for the control, $2.55 \times 10^8 \pm 2.12 \times 10^7$, and for the strain in presence of Ag, $2.00 \times 10^8 \pm 1.41 \times 10^7$ CFU per mL were obtained. However, no colonies were observed in presence of AGXX®. Thus, we conclude that in contrast to Ag, all planktonic bacteria were killed by AGXX® and after exposure to AGXX®, only a drastically reduced number of bacteria (1.80×10^4 CFU per mL) survived in the biofilm in comparison to Ag (2.13×10^9 CFU per mL).

In summary, the biofilm formation measures normalized to the bacterial growth show that AGXX® reduced biofilm

[3]www.systatsoftware.com

TABLE 1 | Colony forming units (CFU)/mL of *Staphylococcus aureus* 04-02981 (without sheet = control), in the presence of AGXX® or Ag.

Sample	0 h	1 h	2 h
Control	$1.27 \times 10^6 \pm 2.0 \times 10^5$	$7.63 \times 10^7 \pm 1.5 \times 10^6$	$8.71 \times 10^9 \pm 1.0 \times 10^9$
Ag	$1.23 \times 10^6 \pm 4.7 \times 10^5$	$6.80 \times 10^6 \pm 1.1 \times 10^6$	$6.23 \times 10^9 \pm 1.2 \times 10^9$
AGXX®	$7.67 \times 10^5 \pm 4.1 \times 10^5$	$2.77 \times 10^6 \pm 1.4 \times 10^6$	$4.93 \times 10^7 \pm 3.4 \times 10^7$
	3 h	**4h**	**5 h**
Control	$1.11 \times 10^{10} \pm 1.1 \times 10^9$	$1.26 \times 10^{11} \pm 7.5 \times 10^9$	$\mathbf{1.73 \times 10^{11} \pm 3.3 \times 10^9}$
Ag	$1.02 \times 10^{10} \pm 1.2 \times 10^9$	$1.25 \times 10^{11} \pm 1.0 \times 10^{10}$	$\mathbf{1.27 \times 10^{11} \pm 6.2 \times 10^8}$
AGXX®	$2.06 \times 10^8 \pm 4.7 \times 10^7$	$3.99 \times 10^{10} \pm 1.1 \times 10^9$	$\mathbf{1.71 \times 10^8 \pm 1.6 \times 10^7}$
	6 h	**7 h**	**8 h**
Control	$1.66 \times 10^{11} \pm 5.7 \times 10^9$	$1.26 \times 10^{11} \pm 3.5 \times 10^9$	$\mathbf{1.17 \times 10^{11} \pm 4.4 \times 10^9}$
Ag	$1.22 \times 10^{10} \pm 4.6 \times 10^8$	$1.12 \times 10^{10} \pm 1.4 \times 10^9$	$\mathbf{1.02 \times 10^{10} \pm 1.4 \times 10^9}$
AGXX®	$1.99 \times 10^8 \pm 7.1 \times 10^8$	$1.34 \times 10^7 \pm 1.4 \times 10^6$	$\mathbf{1.08 \times 10^7 \pm 1.8 \times 10^6}$

The values for 5th hour and 8th hour are bolded because after t = 5h, the CFU values of MRSA + Ag decreased. And until t = 8h, the CFU values for all the three samples (MRSA, MRSA + Ag, and MRSA + AGXX) decreased.

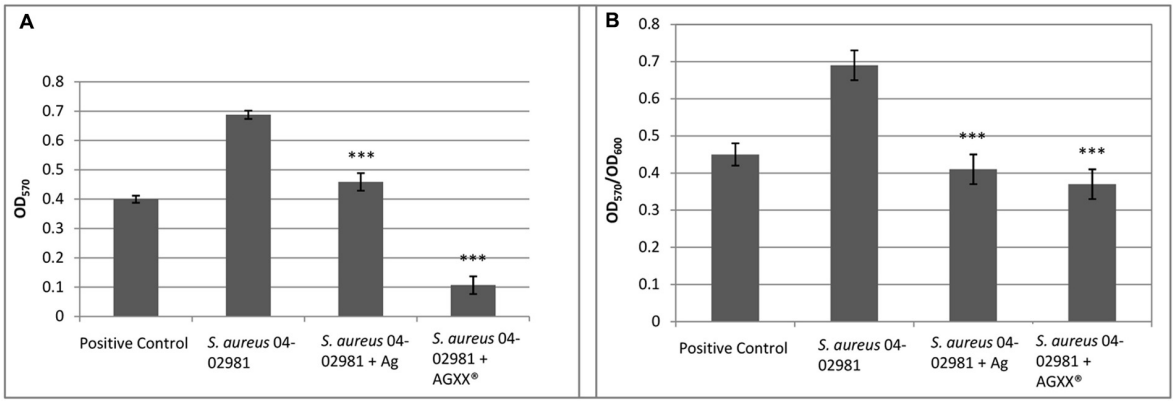

FIGURE 1 | AGXX® reduces biofilm formation of *S. aureus* 04-02981. Mean of five OD values of the biofilm assay with AGXX®, Ag, and without metal amendment with standard deviation are shown. **(A)** Shows the biofilm formation by *Staphylococcus aureus aureus* 04-02981, measured at 570 nm; **(B)** shows the biofilm formation (OD_{570}) normalized to the bacterial growth (OD_{600}) to take the antimicrobial effect of AGXX® into account. Asterisks indicate the *p*-values obtained from *t*-test using SigmaPlot (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$) representing the statistical significance of the data.

formation of *S. aureus* 04-02981 by 46%, whereas the inhibitory effect of Ag on biofilm formation was less pronounced (41%).

The strong reduction of biofilm formation by AGXX® was confirmed by Hoechst 33342/propidium iodide staining of biofilms grown for 24 h in presence of AGXX®, Ag and without antimicrobial sheet (**Figure 2**). The inhibitory effect of Ag was also clearly visible, although it was less distinct.

AGXX® Strongly Induces Stress Response and Represses Pathogenesis in *S. aureus* 04-02981

The raw RNA sequence data obtained were aligned to the *S. aureus* 04-02981 genome. High sequencing depth was achieved as a mean value of ∼12.4 million reads was obtained. The numbers of reads per sample ranged from ∼8.4 million reads (Ag_24) to 175 million reads (Control_120) (**Supplementary Table S3** and **Supplementary Figure S1**). From the data, it is clear that the antimicrobial coating has a strong impact on the transcriptome of *S. aureus* 04-02981. In total, 2864 genes were differentially expressed in *S. aureus* 04-02981 on exposure to AGXX® and Ag (**Supplementary Table S4**). The number of differentially expressed genes in presence of AGXX® or Ag at different time-points is presented in **Figure 3**.

From **Figure 3A**, it can be seen that the number of differentially expressed genes at t24, t80, and t120 was quite similar. The maximum impact of AGXX® on the transcriptome of *S. aureus* 04-02981 was reached already after exposure for 24 min (723 genes up-regulated and 823 genes down-regulated) and remained nearly the same after exposure for 80 min (716 genes up- and 822 genes down-regulated), and 120 min (726 genes up- and 836 genes down-regulated). The lowest number of genes was differentially expressed at t6.

The differentially expressed genes were categorized as per Gene Ontology (GO) using the GSEA_Pro option in the RNA-Seq analysis section in the T-REx RNA-Seq analysis pipeline (de Jong et al., 2015). Several GOs were obtained via

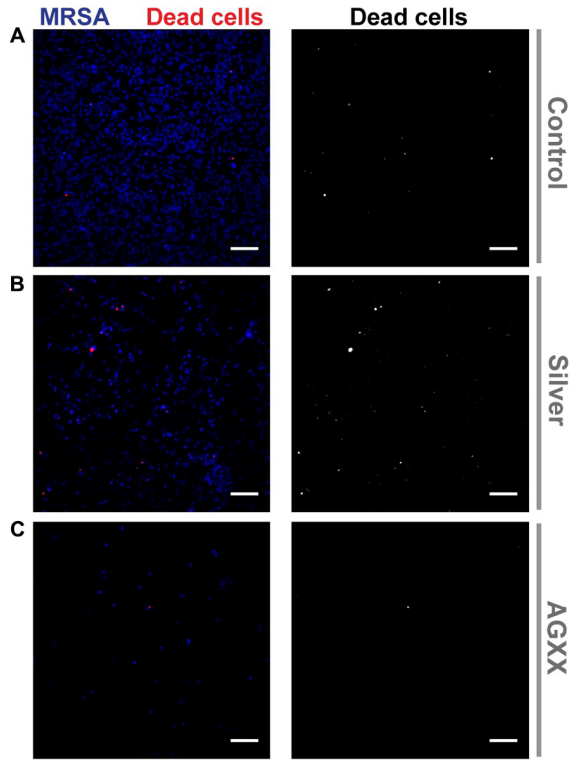

FIGURE 2 | Confocal images of biofilm formation by *S. aureus* 04-02981 (MRSA). The pathogen was grown on sterile cover slips for 24 h with the following conditions: **(A)** without antimicrobial sheet, **(B)** with a silver sheet or **(C)** with an AGXX® sheet. Biofilms were then stained with Hoechst 33342 (to mark out *S. aureus* 04-02981; blue) and propidium iodide (to identify dead cells; red) followed by acquisition of small *Z*-stacks (500 nm spacing) through the biofilms using a confocal microscope. Images show an average of Z-projection (average of 4–5 Z planes containing the biofilm) of the fluorescence signal through the biofilms with the propidium iodide staining shown alone in the images on the right (gray scale images). Scale bars = 10 μm.

GSEA_Pro, namely, oxidoreductase process, lipopolysaccharide synthesis, ATP binding, membrane transport, metabolism, metal binding, pathogenesis, transcription regulation, response to heat shock, iron-siderophore transporter activity, serine protease activity, etc. (**Supplementary Table S5**). In the GO "lipopolysaccharide synthesis," the *cap* genes mediating capsular polysaccharide synthesis (*cap*5A, *cap*A, and *cap*8C) were all down- regulated. Genes (*clp*B, *cts*R, *clp*C, and *groES*) involved in response to heat shock were up-regulated. Among the genes related to virulence (pathogenesis), 10 out of 11 genes were down-regulated, while only one gene was up-regulated at t120 (staphylokinase, a plasminogen activator). Among the responding transcriptional regulator genes, nine were up-regulated and 25 were down-regulated. **Figure 4** shows the differential expression of these GOs in *S. aureus* 04-02981 exposed to AGXX®.

hlgA (SA2981_RS09385) was the most differentially expressed gene associated with virulence, it was down-regulated at t24 (378 fold), at t80 (192 fold), and at t120 (16 fold). The protein encoded by *hlgA* functions as a two-component toxin along with leukocidins in the lysis of erythrocytes (Gouaux et al., 1997). Among the transcriptional regulators, the gene of the LysR family transcriptional regulator, *lysR* was the most significantly influenced one by AGXX®, being down-regulated about 4700 fold at t80, and about 11,000 fold at t120. One of the LysR family transcriptional regulators, HutR is involved in metabolic processes of *S. aureus* (Ibarra et al., 2013). AGXX® had the highest impact on the expression of *cap*A, of all the genes mediating capsular polysaccharide synthesis. *cap*A was down-regulated by 329 fold at t80. Among the most differentially expressed genes in response to heat shock was *clp*B. It is a member of the stress-induced multi-chaperone system and works with DnaK, DnaJ, and GrpE in the recovery of the cell from heat-shock damage (Frees et al., 2005). Among the genes in the GO families influenced by AGXX®, only those involved in enterotoxin (SA2981_RS09440), and staphylokinase production were also influenced by Ag, by -533 fold, and -2 fold, respectively, at t80 (**Supplementary Table S6**). In addition to the GO families, the effect of AGXX®, and Ag on the expression of operons in the pathogen was analyzed using the GSEA_Pro option on the T-REx pipeline. The results are presented in **Supplementary Tables S7**, **S8**, respectively.

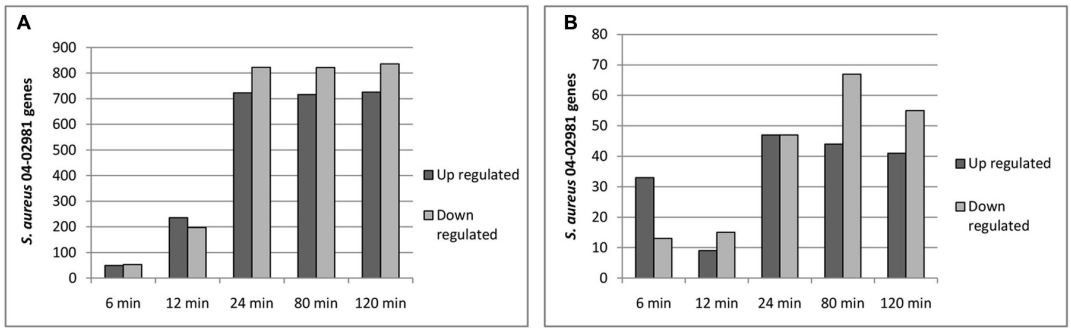

FIGURE 3 | (A,B) Show the number of differentially expressed genes in *S. aureus* 04-02981 on exposure to AGXX® **(A)** and Ag **(B)** for the indicated time-period, compared to control (*S. aureus* 04-02981 grown without a metal sheet).

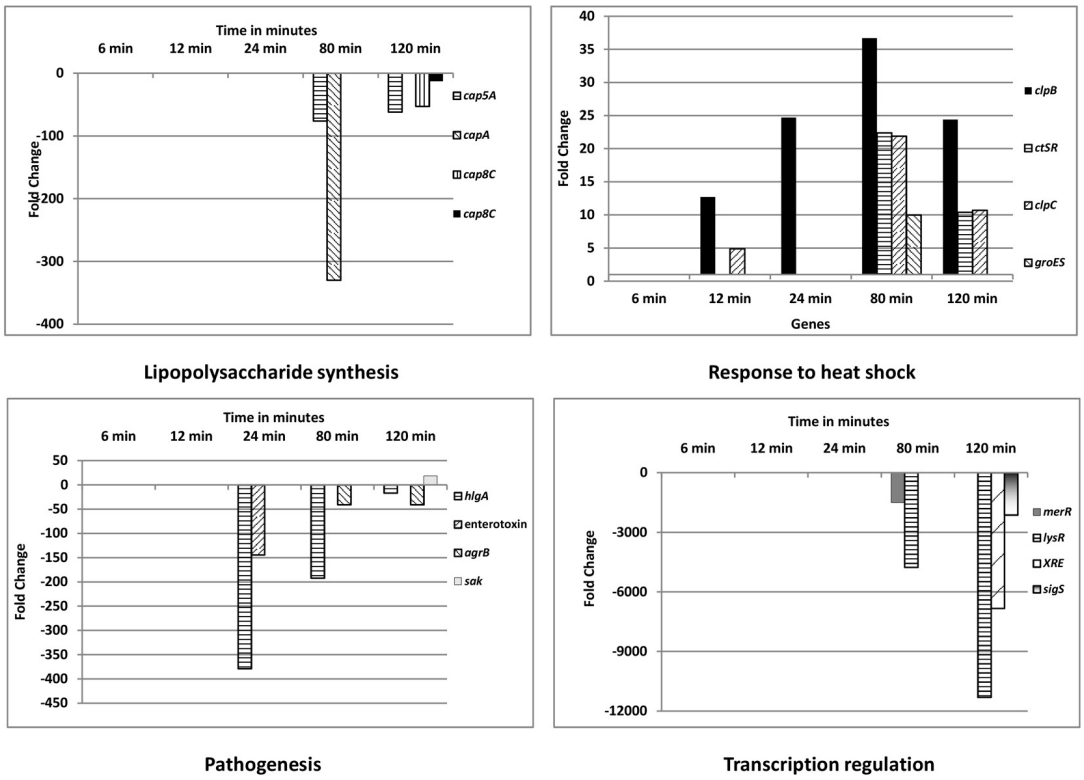

FIGURE 4 | Gene Ontology (GO) categories of differentially expressed genes of *S. aureus* 04-02981 exposed to AGXX® for different time-periods. Only significantly differentially expressed genes, which are likely relevant in the response of *S. aureus* to AGXX®, are presented. Details on the other genes are provided in the **Supplementary Table S5**, along with all of the other GOs, Gene IDs, and fold-change in gene expression, for each time-point. Capsular polysaccharide synthesis mediating *cap* genes are shown in 'Lipopolysaccharide synthesis.' Genes encoding chaperone ClpB (*clpB*), transcriptional regulator CtsR (*ctsR*), ATP-dependent Clp protease ATP-binding subunit ClpC (*clpC*), and chaperonin GroES (*groES*) are presented in the panel showing response to heat shock. In the pathogenesis panel, the genes for γ-hemolysin subunit A (*hlgA*), enterotoxin, accessory gene regulator subunit B (*agrB*), and staphylokinase (*sak*) are presented. Among the transcriptional regulator genes, that of the LysR family transcriptional regulator (*lysR*), MerR family transcriptional regulator (*merR*), XRE family transcriptional regulator (*XRE*) and RNA polymerase sigma factor SigS (*sigS*) are shown.

AGXX® Represses the Expression of Biofilm and Virulence-Associated Genes

We checked the effect of AGXX®, and Ag on the expression of genes associated with biofilm formation and virulence in *S. aureus* 04-02981. Many genes that are known to be crucial for biofilm formation and virulence were differentially expressed on exposure to AGXX® while Ag had an effect on just a few of them. The genes affected by AGXX® encode virulence factors, methicillin resistance, surface adhesins, capsular polysaccharide, two-component systems, and other biofilm- associated genes, as well as toxins (**Table 2**).

Upon exposure to AGXX®, the QS system genes *agrA*, *agrB*, *agrC*, and *agrD* of *S. aureus* 04-02981 were all down-regulated. Genes involved in the synthesis of capsular polysaccharide were also down-regulated. In general, the response of *S. aureus* 04-02981 to AGXX® was clearly visible after 24 min of exposure time. Genes encoding adhesins, *isdC, srtB,* and *sdrC* were also down-regulated. The *mecA* gene was down-regulated at t24. The up-regulation of genes inducing biofilm formation in *S. aureus*, such as *saeR* (2.3 fold at t120), *icaA* (36 fold at t24, 29 fold at t80 and 27 fold at t120), *icaB* (8 fold at t120) and *icaD* (55 fold at t12, and 6 fold at t120) was intriguing. The genes *icaB*, *icaA*, and *icaD* are involved in *ica*-dependent biofilm formation. In addition, other key genes associated with biofilm formation and virulence, such as, *codY*, *srrA*, *luxS*, and genes for toxins like leukocidins, enterotoxins, hemolysins, were all differentially expressed at least at one of the time-points (**Figure 5**). Description of all locus tags and Gene IDs shown to the right of the heatmap is given in **Table 2**.

In general, it was observed that AGXX® had a huge impact on the transcriptome of *S. aureus* 04-02981, in particular at the later time-points 24, 80, and 120 min. In contrast, the effect of Ag was much less pronounced as already visible in the growth kinetics and to a lesser extent in the biofilm assays. Although, quite a number of *S. aureus* 04-02981 genes were differentially expressed upon exposure to Ag, only very few belong to the group of biofilm or virulence-associated genes. Among those, which were significantly differentially expressed in the presence of Ag, were *fmtC*, which is associated with methicillin resistance (approximately 3 fold up-regulated at t80; in the presence of AGXX® it was 2 fold up-regulated at t24), transcriptional regulator *sarR* (approximately 3 fold down-regulated at t24;

TABLE 2 | Differential expression of biofilm, and virulence-associated genes in *S. aureus* 04-02981 on exposure to AGXX®.

Locus tag	Abbreviation	Description	6 min	12 min	24 min	80 min	120 min
SA2981_RS10640	*agrD*	Accessory gene regulator D			−8.9	−11.9	−17.5
SA2981_RS10645	**agrC*	Histidine kinase of the competence regulon ComD			−9.9	−7.7	−7
SA2981_RS10635	*agrB*	Accessory gene regulator B		−5.7	−18.3	−40.7	−40.8
SA2981_RS10650	*agrA*	Two-component system, LytR family, response regulator AgrA			−2.2		
SA2981_RS05970	PSM- β	Phenol-soluble modulin Beta				−10.5	−22.7
SA2981_RS05965	PSM- β	Phenol-soluble modulin Beta				−10.2	−12.4
SA2981_RS10825	*sigB*	RNA polymerase Sigma-B factor			−2.3	−4	−4.9
SA2981_RS07680	**srrA*	DNA-binding response regulator SrrA		−4.6	−9.1	−4.6	−5.3
SA2981_RS00190	*mecA*	mecA-Penicillin- binding Protein 2		−2.3	−5.5		
SA2981_RS12040	*sarR*	Transcriptional regulator SarR					
SA2981_RS06390	*codY*	GTP-sensing transcriptional pleiotropic repressor CodY			−2.3	2.4	3.2
SA2981_RS00550	*sarH1*	Staphylococcal accessory regulator A			−2.4		
SA2981_RS05325	*FmtA*	FmtA protein involved in methicillin resistance				−2.6	
SA2981_RS05940	*arcD*	Arginine/ornithine antiporter ArcD					2.4
SA2981_RS00770	*capF*	Capsular polysaccharide synthesis enzyme Cap8F			−4.8	−3.1	−2
SA2981_RS05275	*sspb*	Staphopain B precursor				−2.3	
SA2981_RS13390	*cidA*	Holin-like protein				2.8	8.2
SA2981_RS13925	*arcA*	Arginine deiminase	−2.6	−11.8	−114.7	−20	−4.6
SA2981_RS03620	*saeR*	two-component system, OmpR family, response regulator SaeR					2.3
SA2981_RS06960	*FmtC*	Protein involved in methicillin resistance/L-lysine modification of phosphatidylglycerol			2.3		
SA2981_RS05900	hemolysin II	Alpha-hemolysin precursor				3.8	3.7
SA2981_RS01335	*lrgA*	Antiholin-like protein		−2.6	−3.1		−3.7
SA2981_RS00745	**capA*	Capsular polysaccharide synthesis enzyme Cap5A			−34.9	−76.9	−61.9
SA2981_RS00750	*capB*	Tyrosine-protein kinase EpsD/capsular polysaccharide synthesis enzyme		2.4	−4.9	−29	−38.1
SA2981_RS00755	*capC*	protein-tyrosine phosphatase/capsular polysaccharide synthesis enzyme		2.8	−15	−20205	−52.9
SA2981_RS13940	*aur*	Zinc metalloproteinase precursor/aureolysin			−12.4		
SA2981_RS02875	**sdrC*	Serine-aspartate repeat-containing protein C			−13.4		−9.8
SA2981_RS02035	Exotoxin 6	Superantigen-like protein			−8.7	4	−12.6
SA2981_RS13920	*arcB*	Ornithine carbamoyltransferase			−16.3	−26.1	−8.6
SA2981_RS05715	*isdC*	NPQTN cell wall anchored protein IsdC				−5	−9.1
SA2981_RS05735	*srtB*	Sortase B				−8.3	−22.4
SA2981_RS14090	*icaD*	Polysaccharide intercellular adhesin (PIA) biosynthesis protein		54.7			5.6
SA2981_RS14085	*icaA*	Polysaccharide intercellular adhesin (PIA) biosynthesis *N*-glycosyltransferase			35.7	29.9	26.5
SA2981_RS14095	*icaB*	Polysaccharide intercellular adhesin (PIA) biosynthesis deacetylase				−102.4	7.8
SA2981_RS09385	**lukE*	Leukotoxin/leukocidin			−378.9	−192.2	

**Genes selected for validation via RT-qPCR.*

not differentially expressed in the presence of AGXX®), the gene of the holin-like protein CidA (approximately 4 fold down-regulated at t24; ∼2 and ∼8 fold up-regulated at t80 and t120, respectively, with AGXX®), the arginine deaminase gene *arcA* (approximately 6 fold down-regulated at t120 and 4.6 fold down-regulated with AGXX®), the hemolysin II gene (approximately 2 fold down-regulated at t24 and approximately 3 fold down-regulated at t120; ∼3.7 fold up-regulated with AGXX® at t80 and t120) and the gene of the antiholin-like protein lrgA (approximately 6 fold up-regulated at t6 with Ag, in the presence of AGXX®, it was ∼3- to 3.7 fold down-regulated at t24, t80, and t120).

Validation of RNA-Sequencing Data Using RT-qPCR

From the RNA-seq data, we observed that AGXX® affected genes encoding two-component systems, surface adhesins, capsular polysaccharides, and toxins. In total, five, highly

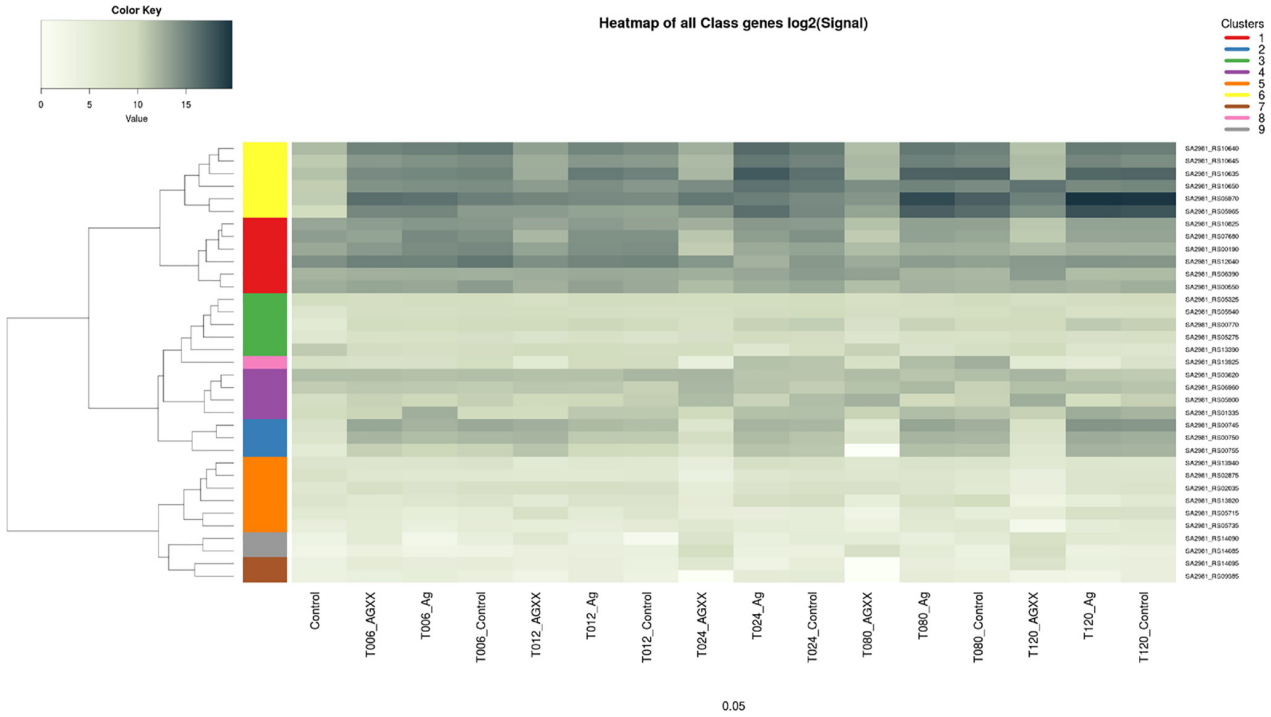

FIGURE 5 | Heatmap of differential expression of biofilm, and virulence-associated genes in *S. aureus* 04-02981. The genes are clustered as indicated by the dendrograms on the left side of the heatmap. Yellow represents genes *agrD, *agrC, agrB, agrA*, and *PSM*-β, red represents genes *sigB, *srrA, mecA, sarR, codY,* and *sarH1*. Green color is for genes *fmtA, arcD, capF, sspb, cidA*, pink represents *arcA* while purple is for *saeR, fmtC*, hemolysin II, and *lrgA* genes. Blue represents genes mediating capsular polysaccharide synthesis, namely, **capA*, *capB, capC*. *aur, *sdrC*, exotoxin 6, *arcB, isdC,* and *srtB* are shown in orange. Gray represents *icaD*, and *icaA* and brown color represents *icaB*, and **lukE* genes. *Indicates genes selected for RT-qPCR.

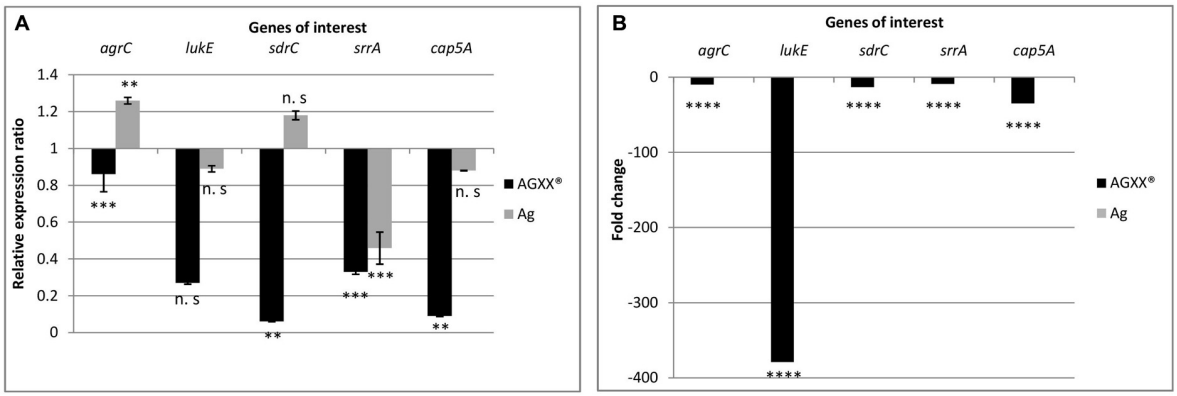

FIGURE 6 | Differential expression of *agrC*, *lukE*, *sdrC*, *srrA*, and *cap5A* in *S. aureus* 04-02981 on 24-min exposure to Ag or AGXX®, obtained via RT-qPCR **(A)**. Expression ratio of the genes of interest in *S. aureus* 04-02981 on exposure relative to control (untreated culture of *S. aureus* 04-02981) normalized to *gyrB*. **(B)** Shows differential expression of *agrC*, *lukE*, *sdrC*, *srrA*, and *cap5A* in *S. aureus* 04-02981 on 24-min exposure to Ag or AGXX®, obtained via RNA-seq as fold change. Error bars indicate standard deviation. Asterisks indicate *p*-values showing statistical significance. They were obtained from *t*-test using SigmaPlot 11.0 (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; n.s, not significant).

differentially expressed genes encoding these functions were selected to validate the RNA-seq derived transcriptional response of *S. aureus* 04-02981 to exposure to Ag or AGXX®. The validation experiment was performed on RNA extracted from *S. aureus* 04-02981 cultures exposed for 24, and 80 min to Ag or AGXX® since the selected genes were most differentially expressed at these time-points. The five selected genes were, *agrC*, and *srrA* which are part of the two-component systems AgrCA and SrrAB, respectively (Baker et al., 2010; Wu et al., 2015), *lukE* which encodes a toxin (Liu et al., 2016), *sdrC* specifying a surface adhesin (Barbu et al., 2014), and *cap5A* mediating the synthesis of capsular polysaccharides

(Qin et al., 2014). *gyrB* was used as the house-keeping gene (Smith et al., 2010; Cheung et al., 2011). **Figures 6**, **7** show the results of these experiments.

After exposure to AGXX® for 24 min, all five genes were down-regulated both in RNA-seq analysis and in RT-qPCR studies as can be seen in **Table 2**, and **Figure 6**. However, after exposure to AGXX® for 80 min, *sdrC* was down-regulated in RT-qPCR assays but it was not differentially expressed in RNA-seq. All the other genes were down-regulated in both approaches as seen in **Table 2** and **Figure 7**, respectively. On exposure to AGXX® for 24 min, *sdrC* was the most down-regulated gene followed by *cap5A*, *lukE*, *srrA*, and *agrC*, whereas after 80 min, *agrC* was the most down-regulated gene followed by *srrA, lukE, cap5A*, and *sdrC*. On exposure to Ag for 24 min, *srrA* was the most down-regulated gene, whereas *agrC* was the most up-regulated gene, and after 80 min, *cap5A* was the most down-regulated gene while *sdrC* was the only up-regulated gene, as observed in the RT-qPCR experiments.

DISCUSSION

Multiple drug resistant, biofilm forming nosocomial pathogens such as MRSA pose a severe threat to public health demanding the development of novel antimicrobials as well as potent biofilm inhibitors. AGXX® is an effective antimicrobial that is active against many Gram-positive and Gram-negative bacteria (Guridi et al., 2015). AGXX® has been demonstrated to kill *S. aureus* 04-02981 as shown here by disk diffusion assay and growth kinetics experiments. In addition, AGXX® inhibited biofilm formation of *S. aureus* 04-02981 by ∼46%. Moreover, for all time-points examined, the number of differentially expressed *S. aureus* 04-02981 genes was much higher upon exposure to AGXX® (in total 2391) than to Ag (317). For t120, the time-point showing the highest number of differentially expressed *S. aureus* 04-02981 genes, 1562 genes were differentially expressed in presence of AGXX®, while only 96 genes were affected by Ag.

Up-regulation of genes of Gene Ontology (GOs) groups "response to heat shock" and "oxidoreductases" involved in oxidative stress response, and down-regulation of genes of GOs "pathogenesis" and "lipopolysaccharide synthesis" involving genes mediating capsular polysaccharide synthesis important for biofilm formation, point to a role of AGXX® as an antimicrobial and potent biofilm inhibitor. Together with results of a recent study where we have shown that the QS system of *S. aureus* 04-02981, *agr* was completely repressed after 4 h of exposure to AGXX® (Probst et al., 2016), we propose that AGXX® acts as a potential biofilm inhibitor. In *S. aureus*, two main mechanisms of biofilm formation are known, namely *ica*-dependent biofilm formation, which involves the production of polysaccharide intercellular adhesin (PIA), and *ica*-independent biofilm formation (Kirmusaoglu, 2016). Here we show that, in the presence of AGXX®, *icaA, icaD* were up-regulated and *icaB* was down-regulated. *icaA* and *icaD* contribute to the production of PIA (polymer). *icaD* transfers PIA to the cell surface of the bacteria while *icaB* deacylates PIA by fixing PIA to the outer surface of the bacteria (Kirmusaoglu, 2016). In our study, intercellular adhesion biosynthesis *N*-deacetylase, *icaB* gene was down-regulated at t80 by ∼100 fold. The structural development of exopolysaccharide-based biofilm requires deacetylation of PIA (Arciola et al., 2015). Since *icaB* was strongly down-regulated at t80, deacetylation of PIA probably does not occur which would obstruct the development of an exopolysaccharide-based biofilm. Fitzpatrick et al. (2005) showed that biofilm formation was unaffected in an *icaADBC* operon-deleted MRSA strain, while the same mutation in a methicillin sensitive strain of *S. aureus* (MSSA) impaired biofilm formation, suggesting strain-specificity in *ica*-dependent biofilm formation.

A two-component system associated with *ica*-dependent biofilm formation is SrrAB that acts as an autoregulator of biofilm formation. Deletion of *srrAB* inhibited *S. aureus* biofilm formation under oxic as well as microaerobic conditions (Wu et al., 2015). In our study, *srrA* was down-regulated 4 to 5 fold after 24, 80, and 120 min of exposure to AGXX®.

Global regulatory systems such as the *agr QS* system are among the best-studied factors involved in *ica*-(PIA) independent biofilm formation. Other proteins involved in such biofilms are SasG, SasC, Protein A, FnbB, FnbA, ATLA or ATLE, SdrG, SdrC, SdrD, biofilm associated protein (Bap) and lipoteichoic acid (Kirmusaoglu, 2016). We observed that two of these genes were down-regulated when AGXX® was present, namely *sdrD* and *sdrC*, *sdrC* was down-regulated 13- to 10 fold at t24 and t120, while *sdrD* was down-regulated 2 to 3 fold at t24 and t80. Moreover, the expression of lipoteichoic acid synthase, an enzyme responsible for the synthesis of lipoteichoic acid (Karatsa-Dodgson et al., 2010) was down-regulated approximately 4 fold after 24, 80, or 120 min of AGXX® presence. These data suggest that AGXX® might be working in an *ica*-independent manner to inhibit biofilm formation.

The *agr* locus contains five genes, *agrA*, *agrB*, *agrC*, *agrD*, and *hld*. On exposing *S. aureus* 04-02981 to AGXX®, only *hld* was not differentially expressed at any time-point, while all the other four genes were significantly down-regulated. The *agr* gene cluster regulates the expression of virulence factors such as phenol soluble modulins (PSMs), proteins that are closely associated with human skin and soft tissue infections (SSTIs) (Sully et al., 2014). "AgrD is a precursor peptide of autoinducer peptide (AIP)" (Quave and Horswill, 2014), AgrB is a membrane protease, which is involved in proteolytic processing and export of AgrD. It is also involved in AIP production (Njoroge and Sperandio, 2009; Quave and Horswill, 2014). AgrBD produce and secrete AIPs. AgrC, a sensor histidine kinase is activated when AIPs bind to AgrC. As a consequence, AgrC undergoes phosphorylation to activate AgrA, which is a DNA-binding response regulator (Njoroge and Sperandio, 2009). In our study, the *agrB* gene was the most down-regulated, at t80, and t120 (approximately 41 fold in both cases), while *agrA* was differentially expressed only at t24 (2 fold down-regulated). At t12, only *agrB* was differentially expressed, approximately 6 fold down-regulated. None of the *agr* genes was differentially expressed at t6. PSMs are staphylococcal toxins playing a role in acute infection (Kirmusaoglu, 2016); they are required for maturation and detachment of biofilm (Ma

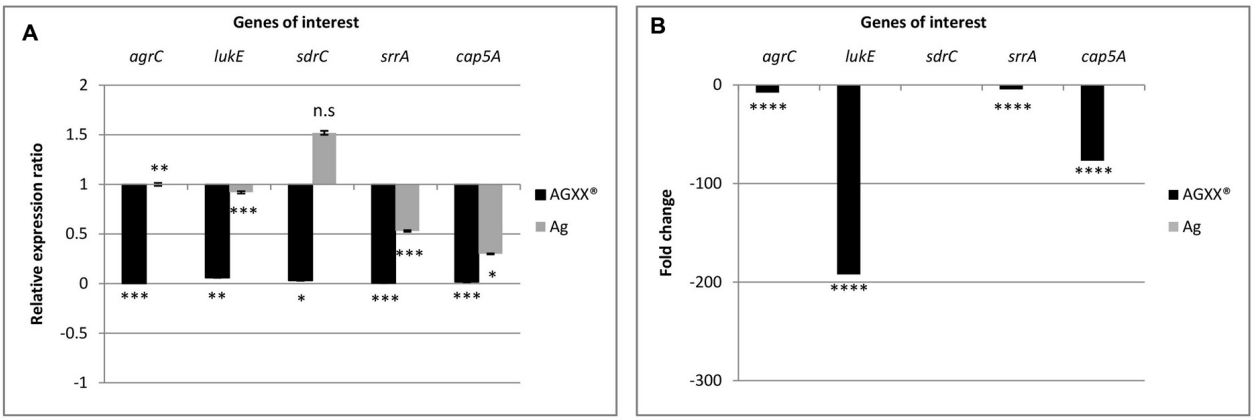

FIGURE 7 | Differential expression of *agrC*, *lukE*, *sdrC*, *srrA*, and *cap5A* in *S. aureus* 04-02981 on 80-min exposure to Ag or AGXX®, obtained via RT-qPCR. Expression ratio of the genes of interest in *S. aureus* 04-02981 on exposure relative to control (untreated culture of *S. aureus* 04-02981) normalized to *gyrB* are shown **(A)**. **(B)** Shows differential expression of *agrC*, *lukE*, *sdrC*, *srrA*, and *cap5A* in *S. aureus* 04-02981 on 80-min exposure to Ag or AGXX®, obtained via RNA-seq as fold change. Error bars indicate standard deviation. Asterisks indicate *p*-values showing statistical significance. They were obtained from *t*-test using SigmaPlot 11.0 (****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$; n.s, not significant).

et al., 2012). PSMs were also down-regulated in presence of AGXX® by ∼10 fold at t80, and by 12 and 23 fold at t120. *agr* also regulates the expression of *sspB* which encodes a cysteine protease. *sspB* is positively associated with biofilm formation (Ma et al., 2012). It was down-regulated by 2.3 fold at t80. Inactivation of the alternative sigma factor SigB decreases biofilm formation in *S. aureus* (Ma et al., 2012). In presence of AGXX®, *sigB* was down-regulated 2–5 fold at the longer exposure times (t24, t80, and t120). In summary, down-regulation of all of the genes mentioned in this paragraph will likely reduce biofilm formation by *S. aureus*.

The two component systems, AgrCA and SaeRS influence biofilm formation in *S. aureus*, by the production of PSMs and by suppressing the synthesis of extracellular proteases, respectively (Baldry et al., 2016). The extracellular proteases degrade proteins that are important for biofilm formation (Baldry et al., 2016). In *S. aureus*, the *saeRS* system regulates the production of many virulence factors such as leukocidins, superantigens, proteases, surface proteins, and hemolysins (Liu et al., 2016). The gene for LukE, which enables *S. aureus* evasion from phagocytic cells by damaging the phagocytes was strongly down-regulated at t24 (379 fold) and t80 (192 fold). SplA is a serine protease, which is directly controlled by the *saeRS* system. *splA* was down-regulated 135 fold after 80 min of AGXX® presence. Mutations in genes for extracellular proteases (*spl*ABCDEF) in *S. aureus* SH1000 induced an increase in extracellular protease activity, which was associated with a reduction in biofilm formation (Chen et al., 2013). These facts taken together with *saeRS* not being differentially expressed at any time-point in the presence of AGXX®, except for a slight 2.3 fold up-regulation of *saeR* at t120, might suggest that *saeR* is not expressed in the mid exponential phase of growth of *S. aureus* 04-02981.

Capsular polysaccharides are also possible targets of the *saeRS* system (Liu et al., 2016). They play an important role in the virulence of the organism (Tuchscherr et al., 2010). The synthesis of capsular polysaccharides is mediated by the *cap5ABCFG* genes (Qin et al., 2014). Among these genes, only *cap*G was not differentially expressed, all other genes were significantly down-regulated, especially at t24, t80, and t120, suggesting a role of AGXX® in repression of virulence in *S. aureus* 04-02981.

Another QS system, which significantly influences biofilm formation and virulence in Staphylococci is the *luxS* system. *lux*S impacts biofilm formation in a similar way as *agr* does, but by regulating different factors. *lux*S negatively regulates biofilm formation via cell-cell interactions based on autoinducer 2 secretion (Xu et al., 2006). The gene was 2.9 fold up-regulated at t24 in the presence of AGXX®.

In addition, the genes *isdC*, *srtB*, *sdrC*, encoding adhesins, were all down-regulated in the pathogen exposed to AGXX®. Iron regulated surface determinant IsdC is necessary for the primary attachment of *S. aureus* to surfaces such as polystyrene, as well as for the accumulation phase of biofilm formation; as such, it induces biofilm formation (Missineo et al., 2014). IsdC is anchored to the cell wall by sortase B (Hammer and Skaar, 2011). Serine-aspartate repeat containing protein C precursor (SdrC*)* assists bacteria in adhering to surfaces and promotes biofilm formation (Barbu et al., 2014). In *S. aureus* 04-02981 exposed to AGXX®, *isdC* was down-regulated by 5 and 9 fold at t80 and t120, respectively. The sortase B gene *srtB* was also down-regulated in cells treated with AGXX®, at t80 (8 fold) and t120 (22 fold). *sdrC*, too, was down-regulated some 10 to 13 fold at t24 and t120. Thus, we suggest that AGXX® inhibits biofilm formation in *S. aureus* 04-02981, also by repressing the expression of adhesins.

Reverse transcription quantitative PCR assays were performed on RNA extracted from *S. aureus* 04-02981 cultures exposed to Ag or AGXX® for 24 min, and 80 min to validate the RNA-seq data. In RT-qPCR, on exposure to AGXX® for 24 min, *agrC*, *sdrC*, *srrA*, and *cap5A* were statistically significantly down-regulated, whereas the down-regulation of *lukE* was not statistically

significant. In agreement with these data, the five genes were also significantly down-regulated in RNA-seq. By contrast, none of the five genes was significantly differentially expressed after 24 min in presence of Ag, as determined by RNA-seq, whereas RT-qPCR revealed a statistically significant down-regulation of *srrA* and a statistically significant up-regulation of *agrC*. The difference in expression of the other three genes *lukE*, *sdrC*, and *cap5A* was statistically not significant. When *S. aureus* 04-02981 was exposed to AGXX® for 80 min, all the five genes were down-regulated in RT-qPCR. The effect was statistically significant while in RNA-seq all genes were significantly down-regulated except *sdrC*. On exposure to Ag for 80 min, only *sdrC* was non-statistically significantly up-regulated. Thus, the trends in gene expression of *S. aureus* 04-02981 on exposure to AGXX® observed in RNA-seq and in RT-qPCR were similar.

In previous studies by others, differential gene expression of *S. aureus* in planktonic and biofilm mode has been examined. Resch et al. (2005) observed that in biofilms, genes encoding polysaccharide intercellular adhesin, and enzymes associated with cell envelope synthesis were significantly up-regulated (Resch et al., 2005). To combat biofilms, many metals have been tested for their capacity to inhibit bacterial biofilm formation. Specifically, silver nanoparticles have received much attention with respect to their antimicrobial nature. However, the minimum concentration of silver nanoparticles (AgNPs) required to eliminate biofilm formation is considered to have toxic effects on mammalian cells (Loo et al., 2016). They studied the effect of AgNPs and curcumin nanoparticles (Cur-NPs) on *S. aureus* and discovered that the combination of both nanoparticles was more effective than the individual AgNPs or Cur-NPs. Curcumin interferes with the QS system as was observed by the down-regulation of genes involved in QS, upon exposure to the substance (Loo et al., 2016). Ma et al. (2012) investigated the effect of two novel anti-virulence compounds on growth and biofilm formation of *S. aureus*. The compounds inhibited biofilm formation by repressing genes associated with biofilm formation such as *lrgA, sdrD, sspB, sigB, codY*, which were also down-regulated in our studies at least at one of the five time-points (Ma et al., 2012).

In summary, based on our findings, we conclude that AGXX® is an effective antimicrobial substance which might also act as a biofilm inhibitor based on our molecular data. The mechanism of inhibition is likely *ica*-independent without the production of PIA, by interfering with the QS system and by repressing genes associated with surface adhesin and lipopolysaccharide synthesis. In addition, the antimicrobial might also reduce pathogenesis of *S. aureus* 04-02981 by down-regulating the synthesis of toxins and virulence factors.

AUTHOR CONTRIBUTIONS

AV performed all the microbiological and molecular experiments, drafted the manuscript, and designed the figures. AdJ supervised and discussed bioinformatics analyses of RNA-seq, and prepared and deposited the RNA-seq data at NCBI. DW performed the confocal microscopy and analyzed the data. JK drafted part of the discussion and gave insightful suggestions on molecular biology of Gram-positive pathogens. EG designed the project and supervised all the experiments. All authors discussed and corrected the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00221/full#supplementary-material

FIGURE S1 | Library sizes of all the RNA samples (*S. aureus* 04-02981, *S. aureus* 04-02981 + Ag , and *S. aureus* 04-02981 + AGXX), at different time periods. The image indicates the read depth of each sample. The *X*-axis represents the experiment names as used in the factors file, and gene counts file during RNA-seq analysis via T-REx. The sample names comprise the metal sheet used, followed by the time of exposure. For example, sample 'AGXX_06' represents *S. aureus* 04-02981 exposed to AGXX for 6 minutes. The *Y*-axis represents the total number of mapped reads.

TABLE S1 | Primer and probe sequences used for RT-qPCR.

TABLE S2 | AGXX®-mediated growth inhibition of *S. aureus* 04-02981 in batch cultures.

TABLE S3 | Alignment rates of the RNA-sequences of *S. aureus* 04-02981.

TABLE S4 | Differentially expressed genes in *S. aureus* 04-02981 on exposure to Ag, and AGXX®.

TABLE S5 | Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 6 minutes. Rate = The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * −log2(adj-pvalue).

TABLE S6 | Gene Ontology assignments on exposing *S. aureus* 04-02981 to Ag for 80 minutes. Rate = The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * −log2(adj-pvalue).

TABLE S7 | Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 6 minutes. Rate = The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * −log2(adj-pvalue).

TABLE S8 | Expression of operons in *S. aureus* 04-02981 on exposure to Ag for 80 minutes. Rate = The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * −log2(adj-pvalue).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary information

A novel antimicrobial coating represses biofilm and virulence-related genes in methicillin-resistant *Staphylococcus aureus*

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Supplementary figure 1

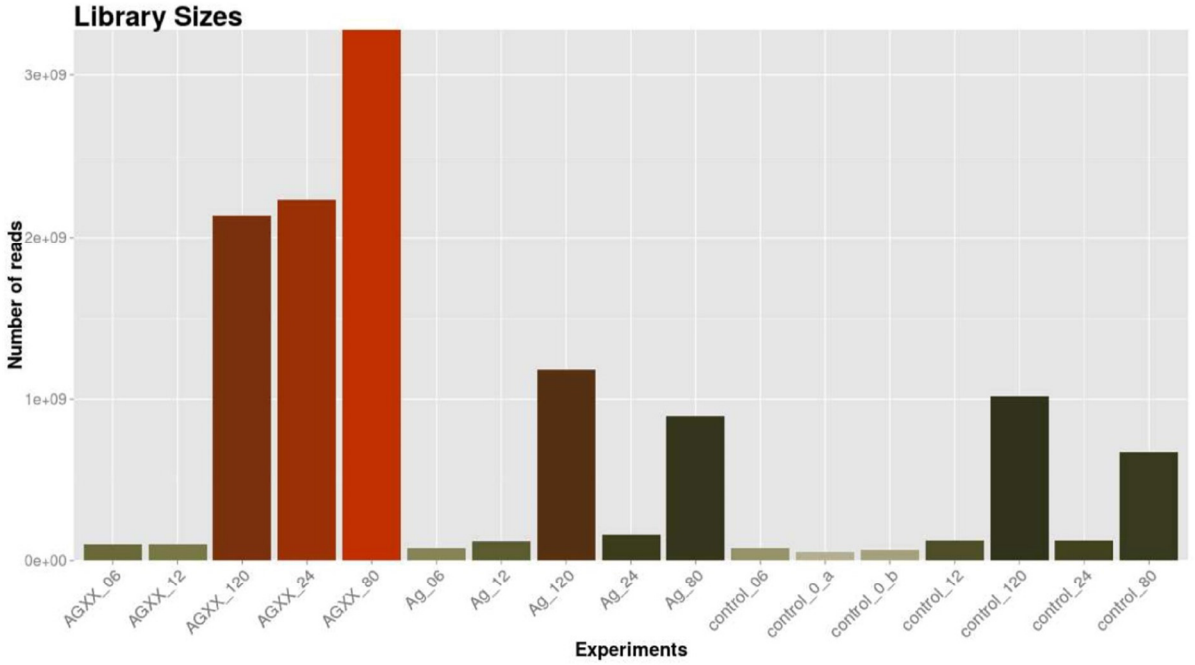

FIGURE S1 | Library sizes of all the RNA samples (*S. aureus* 04-02981, *S. aureus* 04-02981 + Ag, and *S. aureus* 04-02981 + AGXX), at different time periods. The image indicates the read depth of each sample. The *X*-axis represents the experiment names as used in the factors file, and gene counts file during RNA-seq analysis via T-REx. The sample names comprise the metal sheet used, followed by the time of exposure. For example, sample 'AGXX_06' represents *S. aureus* 04-02981 exposed to AGXX for 6 minutes. The *Y*-axis represents the total number of mapped reads.

Supplementary Table 1. Primer and probe sequences for RT-qPCR

Primers	Sequence (5' → 3')	Annealing temperature (°C)
gyrB-F	TTAGTGTGGGAAATTGTCGATAAT	
gyrB-R	AGTCTTGTGACAATGCGTTTACA	57
	6FAM-TACTTTGTATCCGCCACCGCCAAATT-TAMRA	
agrC-F	AAGATGACATGCCTGGCCTA	
agrC-R	TGTGCACGTAAAATTTTCGCAG	60
	6FAM-TGGTATCGAGAATCTTAAAGTACGTG-BHQ	
lukE-F	ACTACTGATGTTGGTCAAAC	
lukE-R	CCATCAGGCGTAACAAATTC	50
	6FAM-ATCTATAGGTGGCAATGGCTCATT-TAMRA	
sdrC-F	ATGAAGCTAAAGCGGCAGAA	
sdrC-R	GCTGTAGCGTTTTGTGGTGA	58
	6FAM-GCAAACTGCAACTGCAGATC-TAMRA	
srrA-F	CCAAGAGGCTTATGAACTTG	
srrA-R	TGACGATATAATCATCTGCACC	57
	6FAM-ATGGTATCCAGGTGGCAACTAAAT-TAMRA	
cap5A-F	TTAGATGAGGTGTCAAAGGAC	
cap5A-R	TATCCACACTCATAATCTTCGG	56
	6FAM-ACGCAACTTATCAACATCCAAGTT-TAMRA	

Supplementary Table 2. AGXX®-mediated growth inhibition of *S. aureus* 04-02981 in batch cultures

Sample	0 h	1 h	2 h	3 h	4 h
Control	0.05	0.125±0.012	0.471±0.129	1.009±0.081	1.457±0.046
Ag	0.05	0.121±0.003	0.414±0.10	0.971±0.026	1.425±0.006
AGXX®	0.05	0.084±0.005	0.095±0.001	0.104±0.005	1.111±0.003

Sample	5 h	6 h	7 h	8 h
Control	1.657±0.012	1.816±0.005	2.12±0.069	3.173±0.025
Ag	1.652±0.001	1.807±0.003	1.967±0.008	3.086±0.035
AGXX®	0.118±0.003	0.123±0.002	0.128±0.001	0.149±0.001

Supplementary Table 3. Alignment rates of the RNA- sequences of *S. aureus* 04-02981

Sample	reads	unpaired	% not aligned	% aligned 1x	% aligned >1x	Reads 1x
control_0_a	9.72E+06	100	1.71	77.57	20.72	7.54E+06
control_0_b	1.11E+07	100	1.28	74.4	24.31	8.28E+06
control_6	8.92E+06	100	1.13	70.31	28.56	6.27E+06
Ag_6	1.26E+07	100	1.28	75.3	23.42	9.52E+06
AGXX_6	1.06E+07	100	1.6	69.13	29.26	7.35E+06
control_12	1.31E+07	100	1.56	72.56	25.88	9.52E+06
Ag_12	1.10E+07	100	1.43	73.79	24.78	8.11E+06
AGXX_12	1.24E+07	100	1.27	69.69	29.04	8.67E+06
control_24	1.71E+07	100	1.48	59.08	39.44	1.01E+07
Ag_24	8.41E+06	100	1.91	60.97	37.12	5.13E+06
AGXX_24	1.22E+07	100	1.61	13.63	84.77	1.66E+06
control_80	1.26E+07	100	1.82	25.08	73.11	3.15E+06
Ag_80	1.25E+07	100	1.91	22.38	75.71	2.80E+06
AGXX_80	1.20E+07	100	1.88	13.43	84.69	1.61E+06
control_120	1.76E+07	100	1.78	25.05	73.17	4.41E+06
Ag_120	1.37E+07	100	2.22	23.66	74.12	3.24E+06
AGXX_120	1.60E+07	100	2.36	18.39	79.25	2.94E+06
Mean	**1.25E+07**		**1.660588235**	**49.6717647**	**48.66764706**	**5.90E+06**

Supplementary Table 4. Differentially expressed genes in *S. aureus* 04-02981 on exposure to Ag, and AGXX®

Contrast	Total number of genes	Up regulated	Down regulated
T006_AGXX-T006_Control	2864	49	53
T006_Ag-T006_Control	2864	33	13
T012_AGXX-T012_Control	2864	236	197
T012_Ag-T012_Control	2864	9	15
T024_AGXX-T024_Control	2864	723	823
T024_Ag-T024_Control	2864	47	47
T080_AGXX-T080_Control	2864	716	822
T080_Ag-T080_Control	2864	44	67
T120_AGXX-T120_Control	2864	726	836
T120_Ag-T120_Control	2864	41	55

T006_AGXX = *S. aureus* 04-02981 exposed to AGXX for 6 minutes
T006_Control = *S. aureus* 04-02981 without addition of any metal sheet, at 6 minutes time-point
T006_Ag = *S. aureus* 04-02981 exposed to Ag for 6 minutes

T012_AGXX = *S. aureus* 04-02981 exposed to AGXX for 12 minutes
T012_Control = *S. aureus* 04-02981 without addition of any metal sheet, at 12 minutes time-point
T012_Ag = *S. aureus* 04-02981 exposed to Ag for 12 minutes

T024_AGXX = *S. aureus* 04-02981 exposed to AGXX for 24 minutes
T024_Control = *S. aureus* 04-02981 without addition of any metal sheet, at 24 minutes time-point
T024_Ag = *S. aureus* 04-02981 exposed to Ag for 24 minutes

T080_AGXX = *S. aureus* 04-02981 exposed to AGXX for 80 minutes
T080_Control = *S. aureus* 04-02981 without addition of any metal sheet, at 80 minutes time-point
T080_Ag = *S. aureus* 04-02981 exposed to Ag for 80 minutes

T0120_AGXX = *S. aureus* 04-02981 exposed to AGXX for 120 minutes
T0120_Control = *S. aureus* 04-02981 without addition of any metal sheet, at 120 minutes time-point
T0120_Ag = *S. aureus* 04-02981 exposed to Ag for 120 minutes

Supplementary Table 5. Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 6 minutes

Locus Tag	GO	Rate	Gene product	Description	gene number	Fold change	p- value
SA2981_RS05375	GO:0005524	*	phosphoribosylaminoimidazolesuccinocarboxamide synthase	ATP binding	gene1075	-2.9	2.20E-02
SA2981_RS07055			peptide ABC transporter ATP-binding protein		gene1411	3.1	4.30E-02
SA2981_RS07825			DNA repair protein RecN		gene1526	-3.6	4.80E-05
SA2981_RS11280			iron ABC transporter ATP-binding protein		gene2256	6.4	1.40E-03
SA2981_RS11665			energy-coupling factor transporter ATPase		gene2333	2.8	4.80E-02
SA2981_RS05935	GO:0006525	*	carbamate kinase 1	Arginine metabolic process	gene1187	-3.9	8.20E-05
SA2981_RS07830			arginine repressor		gene1568	-4	2.80E-05
SA2981_RS07055	GO:0016887	*	peptide ABC transporter ATP-binding protein	ATPase activity	gene1411	3.1	4.30E-02
SA2981_RS11280			iron ABC transporter ATP-binding protein	SV40 T-antigen	gene2256	6.4	1.40E-03
SA2981_RS11665			energy-coupling factor transporter ATPase		gene2333	2.8	4.80E-02
SA2981_RS03100	GO:0055114	*	zinc-dependent alcohol dehydrogenase	Oxidoreductase process	gene619	-2.8	1.20E-02
SA2981_RS03195			cation:proton antiporter	Oxidation reduction process	gene638	3	2.80E-02
SA2981_RS04630			nitronate monooxygenase		gene926	2.8	1.80E-03
SA2981_RS11225			DNA starvation/stationary phase protection protein		gene2244	18.3	2.20E-08
SA2981_RS12575			nitrate reductase subunit alpha	with NarYV catalyzes the redu	gene2515	-2.9	2.80E-02

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Supplementary Table 6. Gene Ontology assignments on exposing *S. aureus* 04-02981 to Ag for 80 minutes

Locus Tag	GO	Rate	Gene product	Function	gene number	Fold change	p- value
SA2981_RS00680	GO:0006139	*	purine-nucleoside phosphorylase	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	gene135	-3.4	4.60E-02
SA2981_RS11700			adenylate kinase		gene2340	-2.3	3.70E-02
SA2981_RS09440	GO:0009405	*	enterotoxin	pathogenesis	gene1891	-533.5	2.30E-03
SA2981_RS10270			staphylokinase	virulence	gene2057	-2.3	2.30E-02
SA2981_RS12700			hypothetical protein		gene2540	-2.9	4.40E-03

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Gene Ontology assignments on exposing *S. aureus* 04-02981 to Ag for 120 minutes

Locus Tag	GO	Rate	Gene product	Function	gene number	Fold change	p- value
SA2981_RS04440	GO:0006355	*	transcriptional activator RinB	regulation of transcription, DNA-dependent	gene888	-4.7	1.10E-03
SA2981_RS10410			transcriptional activator RinB		gene2083	-25.2	6.60E-03
SA2981_RS11885			MarR family transcriptional regulator		gene2377	-475.3	4.40E-05
SA2981_RS00700	GO:0016020	*	phosphonate ABC transporter 2C permease protein PhnE	membrane	gene139	-15.7	2.70E-02
SA2981_RS03750			membrane protein		gene749	-2.8	1.30E-02
SA2981_RS12280			membrane protein		gene2456	-18.4	1.10E-05
SA2981_RS05900	GO:0051715	*	alpha-hemolysin	cytolysis of cells of another organism	gene1180	-2.9	3.00E-03
SA2981_RS12715			gamma-hemolysin component C		gene2543	-5.3	2.50E-02

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Supplementary Table 7. Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 6 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
SA2981_RS02170	**operon_0217**	*	membrane protein		-2.3	4.20E-02	gene433
SA2981_RS03100	**operon_0304**	*	(AdhP) zinc-dependent alcohol dehydrogenase	similar to zinc-dependent eukaryotic ADH enzymes and distinct from fermentative ADHs	-2.8	1.20E-02	gene619
SA2981_RS03115			(ArgS) arginine-tRNA ligase	catalyzes a two-step reaction 2C first charging an arginine molecule by linking its carboxyl group to the alpha-phosphate of ATP 2C followed by transfer of the aminoacyl-adenylate to its tRNA 3B class-I aminoacyl-tRNA synthetase			gene622
SA2981_RS03180	**operon_0312**	**	recombinase		8.9	7.00E-12	gene635
SA2981_RS03185			cation:proton antiporter	subunit A of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali	6.1	7.50E-10	gene636
SA2981_RS03190			cation:proton antiporter	subunit B of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali 3B in the case of S. meliloti it was proved to be involved specifically with K+ transport	5.2	4.70E-05	gene637
SA2981_RS03195			cation:proton antiporter		3	2.80E-02	gene638
SA2981_RS03200			cation:proton antiporter	subunit D of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali 3B contains an oxidoreductase domain 3B catalyzes the transfer of electrons from NADH to ubiquinone 3B in S. meliloti it is known to be involved specifically with K+ transport			gene639
SA2981_RS03205			cation:proton antiporter	subunit E of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali			gene640
SA2981_RS03210			cation:proton antiporter	subunit F of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali			gene641
SA2981_RS03215			cation:proton antiporter	subunit G of antiporter complex involved in resistance to high concentrations of Na+ 2C K+ 2C Li+ and/or alkali			gene642
SA2981_RS05930	**operon_0573**	***	ornithine carbamoyltransferas	catalyzes the formation of ornithine and carbamylphosphate from citrulline in the arginine catabolic pathway	-3.9	8.80E-05	gene1186
SA2981_RS05935			carbamate kinase 1		-3.9	8.20E-05	gene1187
SA2981_RS07825	**operon_0753**	***	DNA repair protein RecN		-3.6	4.80E-05	gene1567
SA2981_RS07830			arginine repressor		-4	2.80E-05	gene1568
SA2981_RS10275	**operon_0976**	***	autolysin		7	1.10E-02	gene2058
SA2981_RS10280			holin		241.5	4.80E-04	gene2059
SA2981_RS10540	**operon_0989**	**	transcriptional regulator				gene2109
SA2981_RS10545			ATP-dependent helicase		-2.7	3.50E-03	gene2110
SA2981_RS12545	**operon_1195**	*	DNA-binding response regulator				gene2509
SA2981_RS12550			sensor histidine kinase				gene2510
SA2981_RS12560			nitrate reductase subunit gamma				gene2512
SA2981_RS12565			nitrate reductase subunit delta		-5.8	1.50E-03	gene2513
SA2981_RS12570			nitrate reductase subunit beta (NarH)				gene2514
SA2981_RS12575			nitrate reductase subunit alpha (NarZ)		-2.9	2.80E-02	gene2515

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 12 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	gene number
SA2981_RS00230	**operon_0030**	**	membrane protein		26.7	3.40E-17	gene45
SA2981_RS00310	**operon_0038**	**	transposase		-15.9	6.40E-03	gene61
SA2981_RS00315			DNA- binding protein		-54.7	1.40E-02	gene62
SA2981_RS00320			transposase				gene63
SA2981_RS00410	**operon_0048**	***	membrane protein		15.9	6.20E-13	gene81
SA2981_RS00420	**operon_0049**	**	dihydroneopterin aldolase				gene83
SA2981_RS00425			Zn-dependent hydrolase				gene84
SA2981_RS00430			pyridine nucleotide-disulfide oxidoreductase				gene85
SA2981_RS01070	**operon_0110**	*	sugar ABC transporter ATP-binding protein				gene213
SA2981_RS01075			maltose ABC transporter substrate-binding protein				gene214
SA2981_RS01080			arabinogalactan ABC transporter permease				gene215
SA2981_RS01085			maltose ABC transporter permease		-2.7	2.00E-02	gene216
SA2981_RS01090			oxidoreductase				gene217
SA2981_RS01095			NADH-dependent dehydrogenase				gene218
SA2981_RS01100			xylose isomerase		-2.7	1.10E-03	gene219
SA2981_RS01130	**operon_0114**	**	formate acetyltransferase		-3.7	4.40E-04	gene225
SA2981_RS01135			pyruvate formate-lyase-activating enzyme		-4.2	2.20E-04	gene226
SA2981_RS01175	**operon_0119**	***	glutaryl-CoA dehydrogenase		141.5	1.40E-05	gene234
SA2981_RS01180			long-chain-fatty-acid-CoA ligase		5.1	3.30E-02	gene235
SA2981_RS01185			acyl CoA:acetate/3-ketoacid CoA transferase		17.8	1.10E-15	gene236
SA2981_RS01325	**operon_0133**	**	sensor protein LytS				gene264
SA2981_RS01330			DNA-binding response regulator		-3	2.50E-03	gene265
SA2981_RS01335			antiholin-like protein LrgA		-2.6	2.50E-03	gene266
SA2981_RS01340			antiholin-like protein LrgB	in conjunction with LrgA this protein inhibits the expression or activity of extracellular murein hydrolases	-7	2.70E-09	gene267
SA2981_RS01725	**operon_0168**	**	glyoxalase		5.6	1.70E-09	gene344
SA2981_RS01730			luciferase		4.6	6.40E-08	gene345
SA2981_RS01735			FMN reductase		2.3	6.50E-03	gene346
SA2981_RS02675	**operon_0261**	**	transcriptional regulator CtsR		6.6	3.70E-08	gene535
SA2981_RS02680			excinuclease ABC subunit B		6.3	1.30E-08	gene536
SA2981_RS02685			protein arginine kinase		4.3	3.70E-06	gene537
SA2981_RS02690			ATP-dependent Clp protease ATP-binding subunit ClpC		4.9	3.20E-06	gene538
SA2981_RS02860	**operon_0281**	***	tRNA-specific adenosine deaminase		-2.6	1.50E-02	gene572
SA2981_RS02865			haloacid dehalogenase		-2.5	1.80E-02	gene573
SA2981_RS02870			FMN-dependent NADPH-azoreductase		-2.5	3.20E-03	gene574
SA2981_RS03035	**operon_0298**	**	dihydrolipoamide dehydrogenase		55.1	3.00E-28	gene607
SA2981_RS03040			transcriptional regulator		141.3	5.00E-49	gene608
SA2981_RS03100	**operon_0304**	*	zinc-dependent alcohol dehydrogenase (adh	similar to zinc-dependent eukaryotic ADH enzymes and distinct from fermentative ADHs	-3.9	4.30E-05	gene619
SA2981_RS03105			hypothetical protein	frameshifted	-12.5	2.10E-12	gene620
SA2981_RS03110			hypothetical protein				gene621
SA2981_RS03115			arginine-tRNA ligase (ArgS)	catalyzes a two-step reaction 2C first charging an arginine molecule by linking its carboxyl group to the alpha-phosphate of ATP 2C followed by transfer of the aminoacyl-adenylate to its tRNA 3B class-I aminoacyl-tRNA synthetase			gene622
SA2981_RS03180	**operon_0312**	**	recombinase		8.3	3.50E-12	gene635
SA2981_RS03185			cation:proton antiporter		6.9	5.80E-12	gene636
SA2981_RS03190			cation:proton antiporter		5.5	2.70E-06	gene637
SA2981_RS03195			cation:proton antiporter		6.8	1.00E-06	gene638
SA2981_RS03200			cation:proton antiporter		3.5	6.90E-04	gene639
SA2981_RS03205			cation:proton antiporter		3	7.90E-03	gene640
SA2981_RS03210			cation:proton antiporter		2.8	1.00E-02	gene641
SA2981_RS03215			cation:proton antiporter		4.9	9.90E-07	gene642
SA2981_RS04610	**operon_0435**	**	XRE family transcriptional regulator				gene922
SA2981_RS05365	**operon_0514**	*	phosphoribosylaminoimidazole carboxylase				gene1073
SA2981_RS05370			N5-carboxyaminoimidazole ribonucleotide synthase		-2.7	3.20E-03	gene1074
SA2981_RS05375			phosphoribosylaminoimidazolesuccinocarb oxamide synthase		-4.5	5.80E-05	gene1075
SA2981_RS05380			phosphoribosylformylglycinamidine synthase				gene1076
SA2981_RS05385			phosphoribosylformylglycinamidine synthase subunit PurQ				gene1077
SA2981_RS05390			phosphoribosylformylglycinamidine synthase subunit PurL				gene1078
SA2981_RS05395			amidophosphoribosyltransferase		-2.3	7.40E-03	gene1079
SA2981_RS05400			phosphoribosylformylglycinamidine cyclo-ligase		-2.2	1.90E-02	gene1080
SA2981_RS05405			phosphoribosylglycinamide formyltransferase		-2.3	2.80E-02	gene1081
SA2981_RS05410			bifunctional phosphoribosylaminoimidazolecarboxamid e formyltransferase/inosine monophosphate cyclohydrolase (PurH)				gene1082
SA2981_RS05415			phosphoribosylamine-glycine ligase		-2.2	1.20E-02	gene1083

SA2981_RS05715	**operon_0551**	*	iron-regulated surface determinant protein C				gene1143
SA2981_RS05720			hypothetical protein				gene1144
SA2981_RS05725			heme uptake system protein IsdE		6.1	4.00E-02	gene1145
SA2981_RS05730			hemin ABC transporter permease				gene1146
SA2981_RS05735			SrtB family sortase				gene1147
SA2981_RS05740			monooxygenase IsdI	iron regulated 3B catalyzes the release of heme from hemoglobin allowing bacterial pathogens to use the host heme as an iron source	-2.8	4.80E-02	gene1148
SA2981_RS05745			hypothetical protein				gene1149
SA2981_RS05930	**operon_0573**	**	ornithine carbamoyltransferase	catalyzes the formation of ornithine and carbamylphosphate from citrulline in the arginine catabolic pathway	-4.9	3.00E-07	gene1186
SA2981_RS05935			carbamate kinase 1		-3.6	6.70E-05	gene1187
SA2981_RS05985	**operon_0581**	*	bacillithiol biosynthesis cysteine-adding enzyme BshC				gene1197
SA2981_RS05990			division/cell wall cluster transcriptional repressor MraZ		-3.3	1.10E-02	gene1198
SA2981_RS05995			ribosomal RNA small subunit methyltransfe	catalyzes the methylation of the N4 position of C1402 on the 16S rRNA			gene1199
SA2981_RS06000			cell division protein FtsL		-2.6	1.10E-02	gene1200
SA2981_RS06005			penicillin-binding protein				gene1201
SA2981_RS06710	**operon_0640**	*	phage head morphogenesis protein		83.1	4.70E-02	gene1342
SA2981_RS06815	**operon_0650**	**	gamma-aminobutyrate permease				gene1363
SA2981_RS07805	**operon_0752**	*	2-oxoglutarate dehydrogenase E2		-3.3	4.20E-05	gene1563
SA2981_RS07810			2-oxoisovalerate dehydrogenase subunit beta		-2	2.60E-02	gene1564
SA2981_RS07815			2-oxoisovalerate dehydrogenase subunit alpha				gene1565
SA2981_RS07820			dihydrolipoyl dehydrogenase	E3 component of the branched-chain alpha-keto acid dehydrogenase complex 3B catalyzes the oxidation of dihydrolipoamide to lipoamide	-2.4	6.30E-03	gene1566
SA2981_RS07825	**operon_0753**	**	DNA repair protein RecN		-3.7	5.00E-06	gene1567
SA2981_RS07830			arginine repressor		-4.7	1.30E-07	gene1568
SA2981_RS07885	**operon_0756**	**	membrane protein		-2.3	6.90E-03	gene1579
SA2981_RS10100	**operon_0951**	**	nitric oxide synthase oxygenase		-2.9	3.70E-04	gene2023
SA2981_RS10105			prephenate dehydratase		-3	1.90E-04	gene2024
SA2981_RS10340	**operon_0981**	*	phage tail protein				gene2069
SA2981_RS10355			phage head-tail adapter protein				gene2072
SA2981_RS10360			phage head-tail adapter protein				gene2073
SA2981_RS10370			phage capsid protein				gene2075
SA2981_RS10375			peptidase				gene2076
SA2981_RS10380			phage portal protein				gene2077
SA2981_RS10385			terminase				gene2078
SA2981_RS10395			HNH endonuclease				gene2080
SA2981_RS10540	**operon_0989**	**	transcriptional regulator				gene2109
SA2981_RS10545			ATP-dependent helicase		-2.9	4.90E-04	gene2110
SA2981_RS10560	**operon_0990**	**	glycosyl transferase family A		-4.4	3.80E-07	gene2113
SA2981_RS10570			integrase				gene2115
SA2981_RS10575			sphingomyelin phosphodiesterase				gene2116
SA2981_RS12965	**operon_1238**	**	antibiotic MFS transporter				gene2593
SA2981_RS12970			peptide ABC transporter ATP-binding protein				gene2594
SA2981_RS12975			peptide ABC transporter ATP-binding protein				gene2595
SA2981_RS12980			peptide ABC transporter permease				gene2596
SA2981_RS12985			nickel ABC transporter permease		5.9	4.30E-06	gene2597
SA2981_RS12990			nickel ABC transporter 2C nickel/metallophore periplasmic binding protein		7.5	2.60E-07	gene2598
SA2981_RS12995			hypothetical protein		8.2	1.70E-02	gene2599
SA2981_RS13000			hypothetical protein		15.7	8.10E-09	gene2600
SA2981_RS13005			diaminopimelate epimerase		12.9	8.10E-09	gene2601
SA2981_RS13435	**operon_1293**	**	ferrous iron transporter B		7.5	3.30E-02	gene2687
SA2981_RS13440			ferrous iron transporter A		29.2	8.10E-10	gene2688
SA2981_RS13555	**operon_1308**	**	TetR family transcriptional regulator		8.6	8.80E-09	gene2711
SA2981_RS13570	**operon_1311**	**	glyoxalase		4.2	6.30E-07	gene2714
SA2981_RS13620	**operon_1314**	**	type II secretion protein		-6	6.30E-06	gene2724
SA2981_RS13630			type VII secretion protein				gene2726
SA2981_RS14160	**operon_1373**	**	polysaccharide deacetylase				gene2832
SA2981_RS14165			GNAT family acetyltransferase		5.6	3.20E-04	gene2833
SA2981_RS14170			cobalt ABC transporter permease		2.9	4.30E-02	gene2834
SA2981_RS14175			heme ABC transporter ATP-binding protein		5.3	3.80E-04	gene2835
SA2981_RS14180			membrane protein		5.1	5.50E-07	gene2836
SA2981_RS14185			DNA-directed RNA polymerase subunit delta		7.7	5.40E-05	gene2837

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 24 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
SA2981_RS00090	**operon_0013**	**	tRNA-Glu		42.5	2.90E-06	gene17
SA2981_RS00095			tRNA-Asp		26.1	8.20E-06	gene18
SA2981_RS00230	**operon_0030**	**	membrane protein				gene45
SA2981_RS00310	**operon_0038**	**	transposase		-754.5	1.10E-07	gene61
SA2981_RS00315			DNA-binding protein				gene62
SA2981_RS00320			transposase		-131.5	4.80E-03	gene63
SA2981_RS00410	**operon_0048**	**	membrane protein		15.9	8.60E-14	gene81
SA2981_RS00420	**operon_0049**	**	dihydroneopterin aldolase		84.3	1.70E-38	gene83
SA2981_RS00425			Zn-dependent hydrolase		44.3	1.20E-34	gene84
SA2981_RS00430			pyridine nucleotide-disulfide oxidoreductase		3.9	2.40E-06	gene85
SA2981_RS00500	**operon_0056**	*	AraC family transcriptional regulator				gene99
SA2981_RS00570	**operon_0067**	**	siderophore biosynthesis protein SbnA		16	2.70E-04	gene113
SA2981_RS00575			2 2C3-diaminopropionate biosynthesis protein SbnB		19.5	1.30E-03	gene114
SA2981_RS00580			siderophore biosynthesis protein SbnC		30.6	4.40E-06	gene115
SA2981_RS00585			siderophore biosynthesis protein SbnD		20.7	9.30E-05	gene116
SA2981_RS00590			siderophore biosynthesis protein SbnE		15.9	9.50E-06	gene117
SA2981_RS00595			siderophore biosynthesis protein SbnF		4.9	1.50E-02	gene118
SA2981_RS00600			siderophore biosynthesis protein SbnG		6.2	1.70E-04	gene119
SA2981_RS00605			diaminopimelate decarboxylase		4	8.90E-03	gene120
SA2981_RS00610			siderophore biosynthesis protein SbnI		8.6	1.20E-08	gene121
SA2981_RS00860	**operon_0087**	*	sulfonate ABC transporter ATP-binding protein		52.1	6.60E-39	gene171
SA2981_RS00870			sulfonate ABC transporter permease				gene173
SA2981_RS00875			butyryl-CoA dehydrogenase		15.9	2.10E-22	gene174
SA2981_RS01260	**operon_0129**	*	PTS galactitol transporter subunit IIC (Gatc)	with GatAB forms a phosphoe	12.5	1.10E-02	gene251
SA2981_RS01265			sorbitol dehydrogenase				gene252
SA2981_RS01270			hypothetical protein		785.3	6.80E-04	gene253
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase		20.4	1.00E-12	gene254
SA2981_RS01445	**operon_0146**	*	virulence factor EsxA				gene288
SA2981_RS01450			protein EsaA		-3.3	1.30E-02	gene289
SA2981_RS01455			protein EssA				gene290
SA2981_RS01460			protein EsaB		-480.8	5.10E-06	gene291
SA2981_RS01465			protein EssB				gene292
SA2981_RS01470			protein EssC				gene293
SA2981_RS01475			protein EsaC				gene294
SA2981_RS01480			virulence factor EsxB				gene295
SA2981_RS02590	**operon_0256**	*****	5S ribosomal RNA		19.1	4.20E-08	gene518
SA2981_RS02595			tRNA-Val		24.7	4.20E-08	gene519
SA2981_RS02600			tRNA-Thr		21.6	8.10E-10	gene520
SA2981_RS02605			tRNA-Lys		15.2	5.50E-12	gene521
SA2981_RS02610			tRNA-Gly		33	9.10E-08	gene522
SA2981_RS02615			tRNA-Leu		37	1.70E-12	gene523
SA2981_RS02620			tRNA-Arg		60.7	3.30E-12	gene524
SA2981_RS02625			tRNA-Pro		25.1	1.50E-14	gene525
SA2981_RS02630			tRNA-Ala		11.1	8.00E-05	gene526
SA2981_RS02635			16S ribosomal RNA		6.1	8.50E-12	gene527
SA2981_RS02640			tRNA-Ile		9	4.70E-04	gene528
SA2981_RS03035	**operon_0298**	**	dihydrolipoamide dehydrogenase		158.1	1.00E-39	gene607
SA2981_RS03040			transcriptional regulator		174.8	5.30E-53	gene608
SA2981_RS03775	**operon_0373**	*	iron ABC transporter permease		6	8.90E-04	gene754
SA2981_RS03780			iron ABC transporter permease		9.6	1.10E-04	gene755
SA2981_RS03785			iron ABC transporter ATP-binding protein		17.2	7.80E-10	gene756
SA2981_RS04065	**operon_0401**	**	membrane protein		-2621	8.60E-20	gene813
SA2981_RS04070			acetyltransferase				gene814
SA2981_RS04990	**operon_0474**	**	ABC transporter substrate-binding protein		7.8	6.50E-04	gene998
SA2981_RS04995			peptide ABC transporter ATP-binding protein		33.4	3.00E-09	gene999
SA2981_RS05000			peptide ABC transporter substrate-binding protein		30.1	9.30E-07	gene1000
SA2981_RS05005			peptide ABC transporter permease		25.1	3.00E-07	gene1001
SA2981_RS05010			peptide ABC transporter permease		2.8	3.70E-02	gene1002
SA2981_RS05155	**operon_0488**	**	tRNA-Ser		33.2	4.00E-08	gene1031
SA2981_RS05160			tRNA-Asn		27.7	2.20E-09	gene1032
SA2981_RS05165			competence protein ComK				gene1033
SA2981_RS05715	**operon_0551**	*	iron-regulated surface determinant protein C				gene1031
SA2981_RS05725			heme uptake system protein IsdE				gene1033
SA2981_RS05730			hemin ABC transporter permease				gene1034
SA2981_RS05735			SrtB family sortase				gene1035
SA2981_RS05740			monooxygenase IsdI	iron regulated 3B catalyzes the release of heme from hemoglobin allowing bacterial pathogens to use the host heme as an iron source	-2207.3	2.20E-17	gene1036
SA2981_RS06740	**operon_0643**	*	antibiotic ABC transporter ATP-binding protein		-249.2	1.40E-04	gene1348

SA2981_RS06745			multidrug ABC transporter permease		13.8	4.10E-12	gene1349
SA2981_RS06750			two-component sensor histidine kinase		2.7	6.20E-03	gene1350
SA2981_RS06755			DNA-binding response regulator				gene1351
SA2981_RS06995	**operon_0676**	*	anthranilate synthase subunit I	with component II 2C the glutamine amidotransferase 2C catalyzes the formation of anthranilate from chorismate and glutamine			gene1399
SA2981_RS07000			glutamine amidotransferase		13.2	8.00E-04	gene1400
SA2981_RS07005			anthranilate phosphoribosyltransferase				gene1401
SA2981_RS07010			indole-3-glycerol-phosphate synthase		5.5	3.70E-02	gene1402
			phosphoribosylanthranilate isomerase	catalyzes the formation of 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate from N-(5-phospho-beta-D-ribosyl)-anthranilate in tryptophan biosynthesis			
SA2981_RS07015					-130.7	4.00E-03	gene1403
SA2981_RS07020				catalyzes the formation of L-tryptophan from L-serine and 1-(indol-3-yl)glycerol 3-phosphate			
			tryptophan synthase subunit beta		5.9	2.70E-02	gene1404
SA2981_RS07025			tryptophan synthase subunit alpha		7.7	5.70E-10	gene1405
SA2981_RS07915	**operon_0760**	*	shikimate kinase				gene1585
SA2981_RS07925			competence protein ComGF		-6.1	6.60E-03	gene1587
SA2981_RS07930			competence protein ComGE		-486.3	2.60E-05	gene1588
SA2981_RS07940			competence protein ComGC		-495.1	4.80E-05	gene1590
SA2981_RS07945			competence protein ComGB				gene1591
SA2981_RS07955			hydroxyacylglutathione hydrolase		-7.6	2.80E-13	gene1593
SA2981_RS07965			glucokinase		-3	4.30E-05	gene1595
SA2981_RS07975			rhomboid family intramembrane serine protease		-12.5	2.50E-19	gene1597
SA2981_RS07980			5-formyltetrahydrofolate cyclo-ligase		-21.3	2.80E-24	gene1598
SA2981_RS08000	**operon_0762**	*	transcriptional repressor		183.3	2.10E-43	gene1602
SA2981_RS08005			iron ABC transporter permease		10.8	2.00E-17	gene1603
SA2981_RS08010			zinc ABC transporter ATP-binding protein		9.4	8.90E-15	gene1604
SA2981_RS08015			endonuclease		12.2	8.70E-20	gene1605
SA2981_RS08020			DEAD/DEAH box family ATP-dependent RNA helicase		7.3	1.20E-10	gene1606
SA2981_RS08025			Nif3-like dinuclear metal center hexameric protein		6.2	7.20E-11	gene1607
SA2981_RS08030			tRNA methyltransferase		14.1	6.90E-16	gene1608
			RNA polymerase sigma factor SigA	sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released 3B primary sigma factor of bacterium			
SA2981_RS08035					-2.1	1.90E-02	gene1609
SA2981_RS08110			tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase MtaB				
	operon_0768	*			8.1	2.20E-14	gene1624
SA2981_RS08115			16S rRNA (uracil(1498)-N(3))-methyltransferase		12.2	7.30E-19	gene1625
SA2981_RS08120			ribosomal protein L11 methyltransferase		14.1	1.30E-21	gene1626
			molecular chaperone DnaJ	chaperone Hsp40 3B co-chaperone with DnaK 3B Participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins and by disaggregating proteins 2C also in an autonomous 2C			
SA2981_RS08125				dnaK-independent fashion	25	3.90E-25	gene1627
			molecular chaperone DnaK	heat shock protein 70 3B assists in folding of nascent polypeptide chains 3B refolding of misfolded proteins 3B utilizes ATPase activity to help fold 3B co-chaperones are DnaJ and GrpE 3B multiple copies in some bacteria			
SA2981_RS08130					3.2	4.50E-04	gene1628
			nucleotide exchange factor GrpE	with DnaK and DnaJ acts in response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins 3B may act as a thermosensor			
SA2981_RS08135					2.5	3.40E-03	gene1629
SA2981_RS08140			HrcA family transcriptional regulator		2.4	6.10E-03	gene1630
SA2981_RS08145			coproporphyrinogen III oxidase				gene1631
SA2981_RS08515	**operon_0795**	**	transposase		-1656.7	1.30E-13	gene1706
SA2981_RS08520			transposase				gene1707
SA2981_RS08525			transposase				gene1708
SA2981_RS08535			peptidase A24		-501.4	8.70E-09	gene1710
SA2981_RS09415	**operon_0892**	**	enterotoxin				gene1886
SA2981_RS09420			enterotoxin		-82.2	1.00E-02	gene1887
SA2981_RS09425			enterotoxin		-144.2	2.60E-03	gene1888

SA2981_RS09445	**operon_0895**	*****	tRNA-Ser		30.9	9.20E-08	gene1892
SA2981_RS09450			tRNA-Glu		28.6	5.30E-20	gene1893
SA2981_RS09455			tRNA-Asn		28.7	8.70E-10	gene1894
SA2981_RS09460			tRNA-Gly		41.2	6.50E-10	gene1895
SA2981_RS09465			tRNA-His		66.3	1.50E-11	gene1896
SA2981_RS09470			tRNA-Phe		16	1.30E-05	gene1897
SA2981_RS09475			tRNA-Asp		24.5	9.80E-06	gene1898
SA2981_RS09480			tRNA-Met		24.5	5.40E-06	gene1899
SA2981_RS09655	**operon_0918**	*****	tRNA-Gly		11.4	1.10E-05	gene1934
SA2981_RS09660			tRNA-Cys		52.8	5.90E-12	gene1935
SA2981_RS09665			tRNA-Gln		30.4	8.40E-15	gene1936
SA2981_RS09670			tRNA-His		77.4	2.50E-13	gene1937
SA2981_RS09675			tRNA-Trp		30.3	1.50E-07	gene1938
SA2981_RS09680			tRNA-Tyr		30.3	8.90E-11	gene1939
SA2981_RS09685			tRNA-Thr		24.7	1.80E-09	gene1940
SA2981_RS09690			tRNA-Phe		16	1.90E-05	gene1941
SA2981_RS09695			tRNA-Asp		24.6	1.10E-05	gene1942
SA2981_RS09700			tRNA-Met		25	2.60E-06	gene1943
SA2981_RS09705			tRNA-Ser		32.5	4.60E-09	gene1944
SA2981_RS09710			tRNA-Asp		24.3	1.20E-05	gene1945
SA2981_RS09715			tRNA-Ser		42.5	5.00E-10	gene1946
SA2981_RS09720			tRNA-Met		10.5	1.10E-12	gene1947
SA2981_RS09725			tRNA-Met		13.1	4.30E-06	gene1948
SA2981_RS09730			tRNA-Ala		12.1	3.30E-05	gene1949
SA2981_RS09735			tRNA-Pro		22.1	2.00E-13	gene1950
SA2981_RS09740			tRNA-Arg		11.6	5.20E-12	gene1951
SA2981_RS09745			tRNA-Leu		37.4	1.50E-14	gene1952
SA2981_RS09750			tRNA-Gly		32.9	9.60E-08	gene1953
SA2981_RS09755			tRNA-Leu		27.2	1.10E-11	gene1954
SA2981_RS09760			tRNA-Lys		22.7	2.80E-10	gene1955
SA2981_RS09765			tRNA-Thr		19.9	5.60E-09	gene1956
SA2981_RS09770			tRNA-Val		31.1	8.70E-09	gene1957
SA2981_RS09775			5S ribosomal RNA (Rrf)		18.7	5.10E-09	gene1958
SA2981_RS09780			23S ribosomal RNA		7.7	1.30E-13	gene1959
SA2981_RS10680	**operon_1003**	**	oxidoreductase		34.1	6.30E-29	gene2137
SA2981_RS10685			membrane protein		14.8	7.30E-18	gene2138
SA2981_RS10735	**operon_1010**	*****	dihydroxy-acid dehydratase	catalyzes the dehydration of 2 2C3-dihydroxy-3-methylbutanoate to 3-methyl-2-oxobutanoate in valine and isoleucine biosynthesis	32.8	5.50E-18	gene2148
SA2981_RS10740			acetolactate synthase 2C large subunit 2C biosy	catalyzes the formation of 2-acetolactate from pyruvate 3B also known as acetolactate synthase large subunit with IlvI catalyzes the formation of 2-acetolactate from pyruvate 2C this subunit subunit is required for full activity and valine sensitivity 3B also known as acetolactate	28.4	8.20E-18	gene2149
SA2981_RS10745			acetolactate synthase	synthase small	13.8	2.80E-09	gene2150
SA2981_RS10750			ketol-acid reductoisomerase		38.2	5.80E-21	gene2151
SA2981_RS10755			2-isopropylmalate synthase		72.1	4.10E-41	gene2152
SA2981_RS10760			3-isopropylmalate dehydrogenase		60.2	3.20E-31	gene2153
SA2981_RS10765			3-isopropylmalate dehydratase large subunit		24.6	4.20E-15	gene2154
			3-isopropylmalate dehydratase small subunit	catalyzes the isomerization between 2-isopropylmalate and 3-isopropylmalate in leucine biosynthesis 3B forms a heterodimer of LeuC/D			
SA2981_RS10770					39.9	4.80E-21	gene2155
SA2981_RS10775			L-threonine dehydratase biosynthetic IlvA	catalyzes the formation of 2-oxobutanoate from L-threonine 3B biosynthetic	38.5	3.50E-22	gene2156
SA2981_RS10800	**operon_1014**		16S ribosomal RNA		6.3	2.00E-11	gene2161
SA2981_RS10805			tRNA-Gly		42.5	3.30E-08	gene2162
SA2981_RS10810			tRNA-Leu		22.3	3.50E-14	gene2163
SA2981_RS11330	**operon_1064**		arginase				gene2266
SA2981_RS11335			tRNA-Lys				gene2267
SA2981_RS11340			tRNA-Gln				gene2268
SA2981_RS11345			tRNA-Tyr				gene2269
SA2981_RS11350			tRNA-Val				gene2270
SA2981_RS11355			tRNA-Glu				gene2271
SA2981_RS11360			tRNA-Asn				gene2272
SA2981_RS11365			5S ribosomal RNA				gene2273
SA2981_RS11370			23S ribosomal RNA				gene2274

		iron-dicitrate ABC transporter permease	Ferric citrate binds FecA and is transported across the outer membrane while transmits a signal across the cytoplasmic membrane protein FecR. FecR transmits a signal across the membrane and activates the cytoplasmic FecI that directs the RNA polymerase to express the fecABCDE operon (which encodes the ferric citrate outer membrane receptor and the ferric citrate ABC transporter) 2C as well as fecIR. FecD is one of two (along with FecC) integral membrane protein components of the iron dicitrate ABC transporter.			
SA2981_RS11430	**operon_1071**			51.4	3.10E-24	gene2286
SA2981_RS11435		siderophore ABC transporter permease		13.7	4.40E-18	gene2287
SA2981_RS11440		ABC transporter substrate-binding protein		3.9	1.00E-06	gene2288
SA2981_RS12730		6-carboxyhexanoate-CoA ligase	catalyzes the formation of pimeloyl-CoA from pimelate and coenzyme A			
	operon_1213					gene2546
SA2981_RS12735		8-amino-7-oxononanoate synthase				gene2547
SA2981_RS12740		biotin synthase				gene2548
SA2981_RS12745		adenosylmethionine-8-amino-7-oxononanoate aminotransferase BioA	catalyzes the formation of S-adenosyl-4-methylthionine-2-oxobutanoate and 7 2C8-diaminononanoate from S-adenosyl-L-methionine and 8-amino-7-oxononanoate			gene2549
SA2981_RS12750		ATP-dependent dethiobiotin synthetase		-79.8	2.70E-02	gene2550
SA2981_RS12765		multidrug ABC transporter ATP-binding protein		-4.4	6.10E-03	gene2553
SA2981_RS12770		multidrug ABC transporter permease				gene2554
SA2981_RS12965	**operon_1238**	antibiotic MFS transporter		4	4.90E-04	gene2593
SA2981_RS12970		peptide ABC transporter ATP-binding protein				gene2594
SA2981_RS12975		peptide ABC transporter ATP-binding protein				gene2595
SA2981_RS12980		peptide ABC transporter permease		3.9	4.50E-02	gene2596
SA2981_RS12985		nickel ABC transporter permease	with NikACDE is involved in nickel transport into the cell	5.4	1.50E-06	gene2597
SA2981_RS12990		nickel ABC transporter 2C nickel/metallophore periplasmic binding protein		4.4	3.80E-05	gene2598
SA2981_RS13005		diaminopimelate epimerase		173.4	1.60E-25	gene2601
SA2981_RS13585	**operon_1312**	TetR family transcriptional regulator		202.2	1.40E-47	gene2717
SA2981_RS13590		short-chain dehydrogenase		18.5	1.60E-13	gene2718
SA2981_RS13595		amidohydrolase		4	3.00E-06	gene2719
SA2981_RS13600		hydrolase				gene2720
SA2981_RS14115	**operon_1372**	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase	catalyzes the formation of 1-(5-phosphoribosyl)-AMP from 1-(5-phosphoribosyl)-ATP and the subsequent formation of 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-AMP in histidine biosynthesis	13.8	1.90E-17	gene2823
SA2981_RS14120		imidazole glycerol phosphate synthase cyclase subunit	catalyzes the conversion of 5-[(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino]-1-(5-phosphoribosyl)imidazole-4-carboxamideand glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamideribonucleotide and glutamate 3B the HisF subunit acts as a cyclase			gene2824
SA2981_RS14125		1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide isomerase	catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-l-(5-hosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide			gene2825

	imidazole glycerol phosphate synthase subunit HisH	with HisF IGPS catalyzes the conversion of phosphoribulosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide phosphate and glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamide ribonucleotide 2C and glutamate in histidine biosynthesis 3B the HisH subunit provides the glutamine amidotransferase activity that produces the ammonia necessary to HisF for the synthesis of imidazole-glycerol phosphate and 5-aminoimidazol-4-carboxamide ribonucleotide			
SA2981_RS14130					gene2826
SA2981_RS14135	imidazole glycerol-phosphate dehydratase				gene2827
SA2981_RS14140	histidinol-phosphate aminotransferase				gene2828
	histidinol dehydrogenase	catalyzes the oxidation of L-histidinol to L-histidinaldehyde and then to L-histidine in histidine biosynthesis 3B functions as a dimer			
SA2981_RS14145			5.8	3.20E-02	gene2829
	ATP phosphoribosyltransferase (HisG)	short form of enzyme 3B requires HisZ for function 3B catalyzes the formation of N'-5'-phosphoribosyl-ATP from phosphoribosyl pyrophosphate 3B crucial role in histidine biosynthesis 3B forms heteromultimer of HisG and HisZ			
SA2981_RS14150			19	2.10E-04	gene2830
	ATP phosphoribosyltransferase regulatory subunit (HisZ)	May allow the feedback regulation of ATP phosphoribosyltransferase activity by histidine			
SA2981_RS14155					gene2831

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 80 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
SA2981_RS00310	**operon_0038**	**	transposase		-20.4	9.80E-05	gene61
SA2981_RS00315			DNA-binding protein				gene62
SA2981_RS00320			transposase		207.7	2.00E-03	gene63
SA2981_RS00420	**operon_0049**	**	dihydroneopterin aldolase		20.6	4.50E-22	gene83
SA2981_RS00425			Zn-dependent hydrolase		35.2	1.30E-31	gene84
SA2981_RS00430			pyridine nucleotide-disulfide oxidoreductase		2.1	1.10E-02	gene85
SA2981_RS00445	**operon_0052**	**	membrane protein		-1629.8	1.80E-17	gene88
SA2981_RS00450			transcriptional regulator		-2453.2	4.00E-22	gene89
SA2981_RS00500	**operon_0056**	*	AraC family transcriptional regulator				gene99
SA2981_RS00570	**operon_0067**	**	siderophore biosynthesis protein SbnA		173.3	4.10E-07	gene113
SA2981_RS00575			2 2C3-diaminopropionate biosynthesis protein SbnB		377.4	1.20E-04	gene114
SA2981_RS00580			siderophore biosynthesis protein SbnC		5.7	1.20E-02	gene115
SA2981_RS00585			siderophore biosynthesis protein SbnD				gene116
SA2981_RS00590			siderophore biosynthesis protein SbnE		22.3	1.00E-06	gene117
SA2981_RS00595			siderophore biosynthesis protein SbnF		42.8	2.60E-07	gene118
SA2981_RS00600			siderophore biosynthesis protein SbnG		37.2	5.60E-11	gene119
SA2981_RS00605			diaminopimelate decarboxylase		5.3	1.80E-03	gene120
SA2981_RS00610			siderophore biosynthesis protein SbnI		5.4	6.30E-06	gene121
SA2981_RS00625	**operon_0070**	*	diacetyl reductase ((S)-acetoin forming)		-2.3	2.30E-02	gene124
SA2981_RS00630			hypothetical protein		-2163.1	8.00E-06	gene125
SA2981_RS00635			NAD-dependent dehydratase		-79.3	7.00E-03	gene126
SA2981_RS00640			UDP-phosphate N-acetylgalactosaminyl-1-phosphate transferase				gene127
SA2981_RS00745	**operon_0081**	*	capsular polysaccharide type 5 biosynthesis protein Cap5A		-76.9	3.80E-31	gene148
SA2981_RS00750			capsular polysaccharide biosynthesis protein Cap5B		-29	4.80E-28	gene149
SA2981_RS00755			capsular polysaccharide biosynthesis protein Cap8C		-20204.6	7.70E-53	gene150
SA2981_RS00760			polysaccharide biosynthesis protein EpsC		-13.2	4.80E-19	gene151
SA2981_RS00765			UDP-glucose 4-epimerase		-5.5	1.90E-07	gene152
SA2981_RS00770			capsular polysaccharide biosynthesis protein Cap8F		-3.1	9.20E-04	gene153
SA2981_RS00775			UDP-N-acetyl glucosamine 2-epimerase				gene154
SA2981_RS00780			O-acetyltransferase				gene155
SA2981_RS00785			capsular polysaccharide biosynthesis protein				gene156
SA2981_RS00790			capsular polysaccharide biosynthesis protein				gene157
SA2981_RS00795			capsular polysaccharide biosynthesis protein				gene158
SA2981_RS00800			glycosyltransferase WbuB				gene159
SA2981_RS00805			capsular polysaccharide biosynthesis protein Cap8M		19.2	1.70E-18	gene160
SA2981_RS00810			UDP-glucose 4-epimerase		2.1	2.50E-02	gene161
SA2981_RS00815			UDP-N-acetyl-D-mannosamine dehydrogenase				gene162
SA2981_RS00815			UDP-N-acetyl glucosamine 2-epimerase				gene163
SA2981_RS01445	**operon_0146**	*	virulence factor EsxA		5.8	3.60E-04	gene288
SA2981_RS01450			protein EsaA		-4.4	2.70E-03	gene289
SA2981_RS01455			protein EssA				gene290
SA2981_RS01460			protein EsaB		-313.4	4.20E-05	gene291
SA2981_RS01465			protein EssB				gene292
SA2981_RS01470			protein EssC				gene293
SA2981_RS01475			protein EsaC		-7.7	5.30E-03	gene294
SA2981_RS01480			virulence factor EsxB		-1232.5	5.60E-07	gene295
SA2981_RS01510	**operon_0147**	*	membrane protein		-4.6	1.50E-04	gene301
SA2981_RS01515			membrane protein				gene302
SA2981_RS01725	**operon_0168**	**	glyoxalase		4.8	5.30E-09	gene344
SA2981_RS01730			luciferase		10.8	4.90E-18	gene345
SA2981_RS01735			FMN reductase		11.3	3.30E-18	gene346
SA2981_RS02675	**operon_0261**	*****	transcriptional regulator CtsR		22.4	1.90E-19	gene535
SA2981_RS02680			excinuclease ABC subunit B		11.1	1.50E-14	gene536
SA2981_RS02685			protein arginine kinase		10.4	1.10E-14	gene537
SA2981_RS02690			ATP-dependent Clp protease ATP-binding subunit ClpC		21.9	8.50E-20	gene538
SA2981_RS02940	**operon_0289**	*	long-chain-fatty-acid-CoA ligase		-26.4	4.00E-06	gene588

			acetyl-CoA acetyltransferase	Catalyzes the synthesis of acetoacetyl coenzyme A from two molecules of acetyl coenzyme A. It can also act as a thiolase 2C catalyzing the reverse reaction and generating two-carbon units from the four-carbon product of fatty acid oxidation			
SA2981_RS02945							gene589
SA2981_RS02950			protein VraC				gene590
SA2981_RS03035	**operon_0298**	**	dihydrolipoamide dehydrogenase		138.9	1.40E-38	gene607
SA2981_RS03040			transcriptional regulator		118.3	7.10E-47	gene608
SA2981_RS03310	**operon_0326**	**	iron-dicitrate ABC transporter ATP-binding protein	with FepBDE is involved in the transport of ferric enterobactin			gene661
SA2981_RS03315			ferrichrome ABC transporter permease		14.6	7.50E-15	gene662
SA2981_RS03320			iron ABC transporter permease		10.9	2.90E-13	gene663
SA2981_RS03775	**operon_0373**	*	iron ABC transporter permease				gene754
SA2981_RS03780			iron ABC transporter permease				gene755
SA2981_RS03785			iron ABC transporter ATP-binding protein				gene756
SA2981_RS03860	**operon_0383**	**	comf operon protein 1		10.1	4.70E-02	gene771
SA2981_RS03865			competence protein ComF		120.8	4.30E-06	gene772
SA2981_RS03895	**operon_0386**	**	peptidase M23		14.7	4.60E-13	gene778
SA2981_RS04455	**operon_0433**	*	terminase		3.6	7.60E-04	gene891
SA2981_RS04460			terminase		3.3	5.30E-03	gene892
SA2981_RS04465			phage portal protein		3.3	5.00E-02	gene893
SA2981_RS04470			phage head morphogenesis protein		-10.8	9.40E-04	gene894
SA2981_RS04480			phage capsid protein		3.8	5.10E-04	gene896
SA2981_RS04485			major capsid protein		3.3	2.20E-03	gene897
SA2981_RS04495			phage head-tail adapter protein		3.1	4.50E-03	gene899
SA2981_RS04515			tail protein				gene903
SA2981_RS04535			phage tail protein				gene907
SA2981_RS04540			peptidase				gene908
SA2981_RS04545			minor structural protein				gene909
SA2981_RS04570			cell wall hydrolase				gene914
SA2981_RS04575			tail protein				gene915
SA2981_RS04585			phage holin		9.1	2.20E-03	gene917
SA2981_RS04590			amidase				gene918
SA2981_RS04990	**operon_0474**	*	ABC transporter substrate-binding protein		4	2.10E-02	gene998
SA2981_RS04995			peptide ABC transporter ATP-binding protein		6	1.10E-03	gene999
SA2981_RS05000			peptide ABC transporter substrate-binding protein		20.5	1.10E-05	gene1000
SA2981_RS05005			peptide ABC transporter permease		25.4	4.00E-08	gene1001
SA2981_RS05010			peptide ABC transporter permease		2.8	3.50E-02	gene1002
SA2981_RS05715	**operon_0551**	*	iron-regulated surface determinant protein C		-5	2.90E-02	gene1143
SA2981 RS05720			hypothetical protein				gene1144
SA2981_RS05725			heme uptake system protein IsdE		-51.4	9.50E-07	gene1145
SA2981_RS05730			hemin ABC transporter permease		-3	4.60E-02	gene1146
SA2981_RS05735			SrtB family sortase		-8.3	2.70E-02	gene1147
SA2981_RS05740			monooxygenase IsdI	iron regulated 3B catalyzes the release of heme from hemoglobin allowing bacterial pathogens to use the host heme as an iron source	-42.1	4.40E-12	gene1148
SA2981_RS06995	**operon_0676**	*	anthranilate synthase subunit I	with component II 2C the glutamine amidotransferase 2C catalyzes the formation of anthranilate from chorismate and glutamine			gene1399
SA2981_RS07000			glutamine amidotransferase				gene1400
SA2981_RS07005			anthranilate phosphoribosyltransferase				gene1401
SA2981_RS07010			indole-3-glycerol-phosphate synthase		-559.9	1.10E-05	gene1402
SA2981_RS07015			phosphoribosylanthranilate isomerase	catalyzes the formation of 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate from N-(5-phospho-beta-D-ribosyl)-anthranilate in tryptophan biosynthesis	-463.4	1.50E-04	gene1403
SA2981_RS07020			tryptophan synthase subunit beta	catalyzes the formation of L-tryptophan from L-serine and 1-(indol-3-yl)glycerol 3-phosphate			gene1404
SA2981_RS07025			tryptophan synthase subunit alpha		2.1	2.80E-02	gene1405

SA2981_RS07265	**operon_0700**	*	phosphatidic acid phosphatase		-2.4	9.50E-04	gene1453
SA2981_RS07270			UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase 3B involved in cell wall formation 3B inner membrane-associated 3B last step of peptidoglycan synthesis			gene1454
SA2981_RS07275			acetyltransferase		2.1	3.50E-02	gene1455
SA2981_RS07915	**operon_0760**	*	shikimate kinase				gene1585
SA2981_RS07925			competence protein ComGF		-789.8	2.60E-08	gene1587
SA2981_RS07930			competence protein ComGE				gene1588
SA2981_RS07940			competence protein ComGC				gene1590
SA2981 RS07945			competence protein ComGB		-363.7	4.20E-04	gene1591
SA2981_RS07955			hydroxyacylglutathione hydrolase		-3.3	1.10E-05	gene1593
SA2981_RS07965			glucokinase		-4.7	1.40E-08	gene1595
SA2981_RS07975			rhomboid family intramembrane serine protease		-9.1	1.40E-15	gene1597
SA2981_RS07980			5-formyltetrahydrofolate cyclo-ligase		-96.4	6.00E-42	gene1598
SA2981 RS08000	**operon_0762**	**	transcriptional repressor		120.8	9.00E-39	gene1602
SA2981_RS08005			iron ABC transporter permease		12.3	5.90E-19	gene1603
SA2981_RS08010			zinc ABC transporter ATP-binding protein		9.2	2.50E-14	gene1604
SA2981_RS08015			endonuclease		11.4	7.50E-19	gene1605
SA2981_RS08020			DEAD/DEAH box family ATP-dependent RNA helicase		9.6	6.10E-13	gene1606
SA2981_RS08025			Nif3-like dinuclear metal center hexameric protein		4.2	2.20E-07	gene1607
SA2981_RS08030			tRNA methyltransferase		8.6	1.70E-11	gene1608
			RNA polymerase sigma factor SigA	sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released 3B primary sigma factor of bacterium			
SA2981_RS08035							gene1609
SA2981_RS08110	**operon_0768**	**	tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase MtaB		3.9	6.00E-07	gene1624
SA2981_RS08115			16S rRNA (uracil(1498)-N(3))-methyltransferase		7.3	6.30E-13	gene1625
SA2981_RS08120			ribosomal protein L11 methyltransferase		19.2	2.00E-25	gene1626
			molecular chaperone DnaJ	chaperone Hsp40 3B co-chaperone with DnaK 3B Participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins and by disaggregating proteins 2C also in an autonomous 2C dnaK-independent fashion			
SA2981_RS08125			molecular chaperone DnaK	heat shock protein 70 3B assists in folding of nascent polypeptide chains 3B refolding of misfolded proteins 3B utilizes ATPase activity to help fold 3B co-chaperones are DnaJ and GrpE 3B multiple copies in some bacteria	16.8	2.50E-20	gene1627
SA2981_RS08130			nucleotide exchange factor GrpE	with DnaK and DnaJ acts in response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins 3B may act as a thermosensor	9.7	7.70E-11	gene1628
SA2981_RS08135			HrcA family transcriptional regulator		10.1	3.20E-12	gene1629
SA2981_RS08140			coproporphyrinogen III oxidase		6.4	1.50E-08	gene1630
SA2981_RS08145							gene1631
SA2981 RS09290	**operon_0878**	**	integrase		-1385.8	3.10E-10	gene1860
SA2981_RS09295			transposase		-2360.1	6.60E-15	gene1861
SA2981_RS09300			transposase				gene1862
SA2981_RS09335	**operon_0883**	*	serine protease SplD		6.8	4.50E-02	gene1869
SA2981_RS09340			serine protease SplC		-15	3.60E-03	gene1870
SA2981_RS09345			serine protease SplB		-70.2	3.30E-02	gene1871
SA2981_RS09350			serine protease SplA		-135.4	6.90E-03	gene1872
SA2981_RS10410	**operon_0982**	**	transcriptional activator RinB				gene2083
SA2981_RS10420			membrane protein		20.5	4.80E-06	gene2085
SA2981_RS10450			Holliday junction DNA helicase				gene2091
SA2981_RS10460			replication protein DnaD		4.6	1.30E-02	gene2093
SA2981_RS10465			single-stranded DNA-binding protein		-456.8	5.70E-06	gene2094
SA2981_RS10470			MBL fold metallo-hydrolase		14	3.30E-04	gene2095

SA2981_RS10735			dihydroxy-acid dehydratase	catalyzes the dehydration of 2 2C3-dihydroxy-3-methylbutanoate to 3-methyl-2-oxobutanoate in valine and			
	operon_1010	**		isoleucine biosynthesis	7.2	9.90E-08	gene2148
			acetolactate synthase 2C large subunit 2C biosynthetic type	catalyzes the formation of 2-acetolactate from pyruvate 3B also known as acetolactate			
SA2981_RS10740				synthase large subunit	2.4	1.60E-02	gene2149
			acetolactate synthase	with IlvI catalyzes the formation of 2-acetolactate from pyruvate 2C this subunit subunit is required for full activity and valine sensitivity 3B also			
SA2981_RS10745				known as acetolactate synthase	10.3	5.50E-08	gene2150
SA2981_RS10750			ketol-acid reductoisomerase		8.8	1.00E-09	gene2151
SA2981_RS10755			2-isopropylmalate synthase		70.1	9.00E-42	gene2152
SA2981_RS10760			3-isopropylmalate dehydrogenase		17.9	2.10E-19	gene2153
			3-isopropylmalate dehydratase large subunit	dehydratase component 2C catalyzes the isomerization between 2-isopropylmalate and			
SA2981_RS10765				3-isopropylmalate	5.1	1.40E-05	gene2154
			3-isopropylmalate dehydratase small subunit	catalyzes the isomerization between 2-isopropylmalate and 3-isopropylmalate in leucine biosynthesis 3B forms a			
SA2981_RS10770				heterodimer of LeuC/D	8.3	1.50E-09	gene2155
			L-threonine dehydratase biosynthetic IlvA	catalyzes the formation of 2-oxobutanoate from L-threonine			
SA2981_RS10775				3B biosynthetic	18.7	3.20E-16	gene2156
SA2981_RS11255	**operon_1056**	**	transcriptional regulator		59.4	2.30E-37	gene2251
SA2981_RS11260			cation transporter		51.3	1.10E-19	gene2252
			6-carboxyhexanoate-CoA ligase	catalyzes the formation of pimeloyl-CoA from pimelate			
SA2981_RS12730	**operon_1213**	*		and coenzyme A			gene2546
SA2981_RS12735			8-amino-7-oxononanoate synthase				gene2547
SA2981_RS12740			biotin synthase				gene2548
			adenosylmethionine-8-amino-7-oxononanoate aminotransferase BioA	catalyzes the formation of S-adenosyl-4-methylthionine-2-oxobutanoate and 7-2C8-diaminononanoate from S-adenosyl-L-methionine and 8-amino-7-oxononanoate			
SA2981_RS12745							gene2549
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		-61.2	4.20E-02	gene2550
SA2981_RS12765			multidrug ABC transporter ATP-binding protein		-5.5	1.20E-03	gene2553
SA2981_RS12770			multidrug ABC transporter permease				gene2554
SA2981_RS12965	**operon_1238**	**	antibiotic MFS transporter		4.1	4.70E-04	gene2593
SA2981_RS12970			peptide ABC transporter ATP-binding protein		8.4	1.50E-03	gene2594
SA2981_RS12975			peptide ABC transporter ATP-binding protein		10.2	3.00E-03	gene2595
SA2981_RS12980			peptide ABC transporter permease		-558.2	7.90E-07	gene2596
SA2981_RS12985			nickel ABC transporter permease	with NikACDE is involved in nickel transport into the cell			gene2597
SA2981_RS12990			nickel ABC transporter 2C nickel/metallophore periplasmic binding protein				gene2598
SA2981_RS12995			hypothetical protein		14.9	2.30E-03	gene2599
SA2981_RS13000			hypothetical protein		7	2.20E-05	gene2600
SA2981_RS13005			diaminopimelate epimerase		83.2	1.00E-15	gene2601
SA2981_RS13570	**operon_1311**	**	glyoxalase		9	8.00E-16	gene2714
SA2981_RS13905	**operon_1352**	*	transcriptional regulator		-2.1	6.10E-03	gene2781
			carbamate kinase 2	catalyzes the reversible synthesis of carbamate and ATP from carbamoyl phosphate and			
SA2981_RS13910				ADP	-5.5	5.80E-07	gene2782
SA2981_RS13915			arginine-ornithine antiporter		-6.6	1.40E-05	gene2783
SA2981_RS13920			ornithine carbamoyltransferase		-26.1	6.00E-11	gene2784
SA2981_RS13925			arginine deiminase		-20	3.20E-19	gene2785
SA2981_RS14085			poly-beta-1-2C6 N-acetyl-D-glucosamine synthase				
	operon_1369	*			29.9	4.90E-07	gene2817
SA2981_RS14090			poly-beta-1- 2C6-N-acetyl-D-glucosamine synthesis protein IcaD				gene2818
SA2981_RS14095			intercellular adhesin biosynthesis polysaccharide N-deacetylase		-102.4	7.60E-03	gene2819
SA2981_RS14100			poly-beta-1-2C6-N-acetyl-D-glucosamine export protein				gene2820

			bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase	catalyzes the formation of 1-(5-phosphoribosyl)-AMP from 1-(5-phosphoribosyl)-ATP and the subsequent formation of 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-AMP in			
SA2981_RS14115	**operon_1372**	**		histidine biosynthesis	6.2	9.50E-10	gene2823
			imidazole glycerol phosphate synthase cyclase subunit	catalyzes the conversion of 5-[(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino]-1-(5-phosphoribosyl)imidazole-4-carboxamideand glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamideribonucleotide and glutamate 3B the HisF subunit acts as a cyclase			
SA2981_RS14120							gene2824
			1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide isomerase	catalyzes the formation of 5-(5-phospho-1-deoxyribulose-1-ylamino)methylideneamino-l-(5-hosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide			
SA2981_RS14125							gene2825
			imidazole glycerol phosphate synthase subunit HisH	with HisF IGPS catalyzes the conversion of phosphoribulosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide phosphate and glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamide ribonucleotide 2C and glutamate in histidine biosynthesis 3B the HisH subunit provides the glutamine amidotransferase activity that produces the ammonia necessary to HisF for the synthesis of imidazole-glycerol phosphate and 5-aminoimidazol-4-carboxamide ribonucleotide			
SA2981_RS14130					-1463.2	1.10E-11	gene2826
			imidazole glycerol-phosphate dehydratase				
SA2981_RS14135					9.4	2.50E-02	gene2827
			histidinol-phosphate aminotransferase				
SA2981_RS14140							gene2828
			histidinol dehydrogenase	catalyzes the oxidation of L-histidinol to L-histidinaldehyde and then to L-histidine in histidine biosynthesis 3B			
SA2981_RS14145				functions as a dimer	-70.2	1.20E-02	gene2829
			ATP phosphoribosyltransferase	short form of enzyme 3B requires HisZ for function 3B catalyzes the formation of N'-5'-phosphoribosyl-ATP from phosphoribosyl pyrophosphate 3B crucial role in histidine biosynthesis 3B forms heteromultimer of HisG and			
SA2981_RS14150				HisZ	-156.8	8.10E-04	gene2830
			ATP phosphoribosyltransferase regulatory subunit	May allow the feedback regulation of ATP phosphoribosyltransferase			
SA2981_RS14155				activity by histidine			gene2831

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Expression of operons in *S. aureus* 04-02981 on exposure to AGXX® for 120 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
SA2981_RS00210	**operon_0029**	*	rhodanese				gene41
SA2981_RS00215			MBL fold hydrolase		96.5	1.30E-04	gene42
SA2981_RS00220			dihydroneopterin aldolase		14.9	2.30E-02	gene43
SA2981_RS00340	**operon_0041**	*	ATPase				gene67
SA2981_RS00345			potassium-transporting ATPase subunit A		43.6	1.30E-02	gene68
SA2981_RS00350			potassium-transporting ATPase subunit B		10.9	5.00E-02	gene69
SA2981_RS00355			potassium-transporting ATPase subunit C	one of the components of the high-affinity ATP-driven potassium transport (or KDP)system 2C which catalyzes the hydrolysis of ATP coupled with the exchange of hydrogen and potassium ions 3B the C subunit may be involved in assembly of the KDP complex 3B frameshifted			gene70
SA2981_RS00420	**operon_0049**	*	dihydroneopterin aldolase		33.4	3.40E-28	gene83
SA2981_RS00425			Zn-dependent hydrolase		37.9	3.60E-33	gene84
SA2981_RS00430			pyridine nucleotide-disulfide oxidoreductase		5.3	1.10E-08	gene85
SA2981_RS00570	**operon_0067**	*	siderophore biosynthesis protein SbnA				gene113
SA2981_RS00575			2 2C3-diaminopropionate biosynthesis protein SbnB				gene114
SA2981_RS00580			siderophore biosynthesis protein SbnC				gene115
SA2981_RS00585			siderophore biosynthesis protein SbnD				gene116
SA2981_RS00590			siderophore biosynthesis protein SbnE		8.3	8.40E-04	gene117
SA2981_RS00595			siderophore biosynthesis protein SbnF		4.7	1.70E-02	gene118
SA2981_RS00600			siderophore biosynthesis protein SbnG		2.9	4.00E-02	gene119
SA2981_RS00605			diaminopimelate decarboxylase		15.7	2.60E-06	gene120
SA2981_RS00610			siderophore biosynthesis protein SbnI		12.1	1.90E-10	gene121
SA2981_RS00745			capsular polysaccharide type 5				gene148
	operon_0081	*	biosynthesis protein Cap5A		-61.9	4.60E-30	
			capsular polysaccharide				gene149
SA2981_RS00750			biosynthesis protein Cap5B		-38.1	8.00E-32	
			capsular polysaccharide				gene150
SA2981_RS00755			biosynthesis protein Cap8C		-52.9	2.10E-32	
			polysaccharide biosynthesis				gene151
SA2981_RS00760			protein EpsC		-34.5	9.90E-31	
SA2981_RS00765			UDP-glucose 4-epimerase		-4.5	2.00E-06	gene152
SA2981_RS00770			capsular polysaccharide				gene153
			biosynthesis protein Cap8F		-2	3.80E-02	
			UDP-N-acetyl glucosamine 2-				gene154
SA2981_RS00775			epimerase				
SA2981_RS00780			O-acetyltransferase		4.9	7.60E-07	gene155
			capsular polysaccharide				gene156
SA2981_RS00785			biosynthesis protein		3.8	3.60E-05	
			capsular polysaccharide				gene157
SA2981_RS00790			biosynthesis protein		2.9	6.30E-04	
			capsular polysaccharide				gene158
SA2981_RS00795			biosynthesis protein		3.2	5.10E-04	
SA2981_RS00800			glycosyltransferase WbuB		4.4	9.90E-07	gene159
			capsular polysaccharide				gene160
SA2981_RS00805			biosynthesis protein Cap8M		13.2	1.80E-15	
SA2981_RS00810			UDP-glucose 4-epimerase		6.9	1.40E-08	gene161
			UDP-N-acetyl-D-				gene162
SA2981_RS00815			mannosamine dehydrogenase				
			UDP-N-acetyl glucosamine 2-				gene163
SA2981_RS00820			epimerase		2.1	4.50E-03	
SA2981_RS00960	**operon_0098**	*	N-acetylmuramic acid 6-phosphate etherase	catalyzes the cleavage of the lactyl ether moiety of N-acetylmuramic acid-6-phosphate (MurNAc-6-P) to form N-acetylglucosamine-6-phosphate (GlcNAc-6-P) and lactate 3B involved in MurNAc dissimilation pathway	-34.8	9.50E-14	gene191
SA2981_RS00965			permease		-14.8	2.80E-14	gene192
SA2981_RS00970			RpiR family transcriptional regulator		-6.2	6.20E-11	gene193
SA2981_RS01005	**operon_0103**	*	membrane protein		-14.7	3.80E-15	gene200
SA2981_RS01010			membrane protein		-16.5	3.00E-11	gene201
SA2981_RS01015			membrane protein		-3.2	1.90E-02	gene202
SA2981_RS01020			ABC transporter ATP-binding protein		-8	4.80E-06	gene203
SA2981_RS01175	**operon_0119**	*	glutaryl-CoA dehydrogenase		-23.2	1.60E-06	gene234
SA2981_RS01180			long-chain-fatty-acid-CoA ligase		-7.2	1.40E-03	gene235
SA2981_RS01185			acyl CoA:acetate/3-ketoacid CoA transferase		-23.2	2.40E-18	gene236
SA2981_RS01245	**operon_0128**	*	transcriptional antiterminator				gene248

SA2981_RS01250			PTS sugar transporter subunit IIA		276.3	2.90E-03	gene249
SA2981_RS01255			PTS galactitol transporter subunit IIB		57.8	3.60E-02	gene250
SA2981_RS01260	**operon_0129**	*	PTS galactitol transporter subunit IIC (gatc)	with GatAB forms a phosphoenolpyruvate-dependent sugar phosphotransferase transporter for galactitol 3B subunit IIC forms the translocation channel and contains the substrate binding site			gene251
SA2981_RS01265			sorbitol dehydrogenase				gene252
SA2981_RS01270			hypothetical protein		778.1	6.90E-04	gene253
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase		8.5	1.00E-07	gene254
SA2981_RS01510	**operon_0147**	*	membrane protein		-3.7	1.00E-03	gene301
SA2981_RS01515			membrane protein		2.1	2.30E-02	gene302
SA2981_RS01785	**operon_0173**	*	transcriptional regulator		-1724.5	3.30E-12	gene356
SA2981_RS01795			multidrug ABC transporter ATP-binding protein		3.7	6.40E-04	gene358
SA2981_RS01800			membrane protein		-2.6	6.80E-04	gene359
SA2981_RS02295	**operon_0232**	**	glutamate synthase		9.7	2.00E-12	gene458
SA2981_RS02300			glutamate synthase subunit beta		47.4	4.30E-38	gene459
SA2981_RS02675	**operon_0261**	*	transcriptional regulator CtsR		10.4	1.00E-12	gene535
SA2981_RS02680			excinuclease ABC subunit B		5	7.40E-08	gene536
SA2981_RS02685			protein arginine kinase		5.5	7.00E-09	gene537
SA2981_RS02690			ATP-dependent Clp protease ATP-binding subunit ClpC		10.7	2.50E-13	gene538
SA2981_RS03035	**operon_0298**	**	dihydrolipoamide dehydrogenase		183.1	2.50E-41	gene607
SA2981_RS03040			transcriptional regulator		260.8	2.40E-56	gene608
SA2981_RS03485	**operon_0341**	*	Rossman fold protein 2C TIGR00730 family		-3.1	1.80E-04	gene696
SA2981_RS03490			hypothetical protein		-20.4	2.30E-13	gene697
SA2981_RS03495			hypothetical protein		-34.4	9.50E-14	gene698
SA2981_RS03605	**operon_0356**	**	glyoxal reductase		-19	4.40E-19	gene720
SA2981_RS03610			glycosyl transferase		-19.6	1.60E-18	gene721
SA2981_RS03775	**operon_0373**	*	iron ABC transporter permease				gene754
SA2981_RS03780			iron ABC transporter permease		8.4	4.40E-04	gene755
SA2981_RS03785			iron ABC transporter ATP-binding protein		14.8	6.90E-09	gene756
SA2981_RS04645	**operon_0439**	*	membrane protein		-3.6	8.50E-06	gene755
SA2981_RS04650			multifunctional 2' 2C3'-cyclic-nucleotide 2'-phosphodiesterase/5'-nucleotidase/3'-nucleotidase				gene756
SA2981_RS04655			lipoyl synthase				gene757
SA2981_RS04990	**operon_0474**	*	ABC transporter substrate-binding protein		3.8	2.10E-02	gene998
SA2981_RS04995			peptide ABC transporter ATP-binding protein		14.3	1.50E-06	gene999
SA2981_RS05000			peptide ABC transporter substrate-binding protein		590	1.50E-11	gene1000
SA2981_RS05005			peptide ABC transporter permease		247.5	9.00E-14	gene1001
SA2981_RS05010			peptide ABC transporter permease				gene1002
SA2981_RS05210	**operon_0494**	*	bacteriocin ABC transporter ATP-binding protein		-12.8	1.40E-07	gene1042
SA2981_RS05330	**operon_0510**	*	quinol oxidase subunit 4		9.7	4.60E-08	gene1066
SA2981_RS05335			quinol oxidase subunit 3		9.6	5.50E-10	gene1067
SA2981_RS05340			cytochrome ubiquinol oxidase subunit I		2.9	1.10E-03	gene1068
SA2981_RS05345			quinol oxidase subunit 2		2.1	3.10E-02	gene1069
SA2981_RS05715	**operon_0551**	*	iron-regulated surface determinant protein C		-9.1	4.40E-04	gene1143
SA2981_RS05725			heme uptake system protein IsdE				gene1145
SA2981_RS05730			hemin ABC transporter permease				gene1146
SA2981_RS05735			SrtB family sortase		-22.4	3.50E-03	gene1147
SA2981_RS05740			monooxygenase IsdI	iron regulated 3B catalyzes the release of heme from hemoglobin allowing bacterial pathogens to use the host heme as an iron source	-1312.9	1.10E-14	gene1148
SA2981_RS05975	**operon_0579**	*	noncanonical pyrimidine nucleotidase 2C YjjG family				gene1195
SA2981_RS06710	**operon_0640**	*	phage head morphogenesis protein		216.6	1.40E-03	gene1342

SA2981_RS06945	**operon_0668**	*	protein GlcT		-59.7	1.80E-24	gene1389
SA2981_RS06955			AI-2E family transporter		-2.6	4.10E-04	gene1391
SA2981_RS06995	**operon_0676**	*	anthranilate synthase subunit I	with component II 2C the glutamine amidotransferase 2C catalyzes the formation of anthranilate from chorismate and glutamine			gene1399
SA2981_RS07000			glutamine amidotransferase		1600.4	2.90E-08	gene1400
SA2981_RS07005			anthranilate phosphoribosyltransferase				gene1401
SA2981_RS07010			indole-3-glycerol-phosphate synthase				gene1402
SA2981_RS07015			phosphoribosylanthranilate isomerase	catalyzes the formation of 1-(2-carboxyphenylamino)-1-deoxy-D-ribulose 5-phosphate from N-(5-phospho-beta-D-ribosyl)-anthranilate in tryptophan biosynthesis	-179.5	1.60E-03	gene1403
SA2981_RS07020			tryptophan synthase subunit beta	catalyzes the formation of L-tryptophan from L-serine and 1-(indol-3-yl)glycerol 3-phosphate			gene1404
SA2981_RS07025			tryptophan synthase subunit alpha		2.1	1.70E-02	gene1405
SA2981_RS07265			phosphatidic acid phosphatase				
	operon_0700				2.3	1.20E-03	gene1453
SA2981_RS07270			UDP-N-acetylglucosamine-N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	UDP-N-acetylglucosamine--N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase 3B involved in cell wall formation 3B inner membrane-associated 3B last step of peptidoglycan synthesis	2	3.80E-02	gene1454
SA2981_RS07275			acetyltransferase				gene1455
SA2981_RS07535	**operon_0723**	*	nucleoside-diphosphate kinase	catalyzes the formation of nucleoside triphosphate from ATP and nucleoside diphosphate	-18.9	2.10E-22	gene1509
SA2981_RS07540			heptaprenyl diphosphate synthase subunit II				gene1510
SA2981_RS07545			demethylmenaquinone methyltransferase				gene1511
SA2981_RS07550			heptaprenyl pyrophosphate synthase subunit A		-13.6	1.80E-20	gene1512
SA2981_RS07915	**operon_0760**	**	shikimate kinase		-3.6	5.70E-03	gene1585
SA2981_RS07925			competence protein ComGF		-34.7	5.50E-07	gene1587
SA2981_RS07930			competence protein ComGE				gene1588
SA2981_RS07940			competence protein ComGC				gene1590
SA2981_RS07945			competence protein ComGB		31.6	2.40E-03	gene1591
SA2981_RS07955			hydroxyacylglutathione hydrolase				gene1593
SA2981_RS07965			glucokinase		-2	1.00E-02	gene1595
SA2981_RS07975			rhomboid family intramembrane serine protease		-5.6	1.50E-10	gene1597
SA2981_RS07980			5-formyltetrahydrofolate cyclo-ligase		-25.2	3.40E-26	gene1598
SA2981_RS08000	**operon_0762**	**	transcriptional repressor		131.9	1.10E-39	gene1602
SA2981_RS08005			iron ABC transporter permease		26.8	2.00E-28	gene1603
SA2981_RS08010			zinc ABC transporter ATP-binding protein		14	6.50E-19	gene1604
SA2981_RS08015			endonuclease		7.5	4.10E-14	gene1605
SA2981_RS08020			DEAD/DEAH box family ATP-dependent RNA helicase		13.3	5.00E-16	gene1606
SA2981_RS08025			Nif3-like dinuclear metal center hexameric protein		3	5.50E-05	gene1607
SA2981_RS08030			tRNA methyltransferase		8.7	1.10E-11	gene1608
SA2981_RS08035			RNA polymerase sigma factor SigA	sigma factors are initiation factors that promote the attachment of RNA polymerase to specific initiation sites and are then released 3B primary sigma factor of bacterium			gene1609
SA2981_RS08110	**operon_0768**	*	tRNA (N(6)-L-threonylcarbamoyladenosine(37)-C(2))-methylthiotransferase MtaB		5.1	1.30E-09	gene1624
SA2981_RS08115			16S rRNA (uracil(1498)-N(3))-methyltransferase		5	3.10E-09	gene1625
SA2981_RS08120			ribosomal protein L11 methyltransferase		12.2	1.10E-19	gene1626
SA2981_RS08125			molecular chaperone DnaJ	chaperone Hsp40 3B co-chaperone with DnaK 3B Participates actively in the response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins and by disaggregating proteins 2C also in an autonomous 2C dnaK-independent fashion	10.4	2.30E-15	gene1627

SA2981_RS08130			molecular chaperone DnaK	heat shock protein 70 3B assists in folding of nascent polypeptide chains 3B refolding of misfolded proteins 3B utilizes ATPase activity to help fold 3B co-chaperones are DnaJ and GrpE 3B multiple copies in some bacteria	4.7	3.90E-06	gene1628
SA2981_RS08135			nucleotide exchange factor GrpE	with DnaK and DnaJ acts in response to hyperosmotic and heat shock by preventing the aggregation of stress-denatured proteins 3B may act as a thermosensor	3.2	2.20E-04	gene1629
SA2981_RS08140			HrcA family transcriptional regulator		2.4	5.50E-03	gene1630
SA2981_RS08145			coproporphyrinogen III oxidase				gene1631
SA2981_RS08360	**operon_0781**	*	CsbD family protein		-16.7	1.80E-10	gene1674
SA2981_RS08365			Rrf2 family transcriptional regulator				gene1675
SA2981_RS08745			acetyl-CoA carboxylase carboxyltransferase subunit alpha	catalyzes the carboxylation of acetyl-CoA to malonyl-CoA 3B forms a tetramer composed of two alpha (AccA) and two beta (AccD) subunits 3B one of the two catalytic subunits that can form the acetyl CoA carboxylase enzyme together with a carrier protein			
	operon_0816	**			32	1.40E-33	gene1752
SA2981_RS08750			acetyl-CoA carboxylase carboxyltransferase subunit beta		10.2	4.80E-13	gene1753
SA2981_RS08940	**operon_0840**	**	acetoin dehydrogenase acetoin utilization protein		-14	5.60E-13	gene1791
SA2981_RS08945			AcuC		-19.7	2.40E-13	gene1792
SA2981_RS08980	**operon_0844**	*	UDP-N-acetylmuramate-L-alanine ligase				gene1799
SA2981_RS08985			cell division protein FtsK				gene1800
SA2981_RS08990			tRNA-binding protein		-2.5	6.60E-04	gene1801
SA2981_RS08995			hypothetical protein		-15.4	1.10E-19	gene1802
SA2981_RS09000			thiol reductase thioredoxin				gene1803
SA2981_RS09005			glutamyl aminopeptidase		-3.7	1.20E-06	gene1804
SA2981_RS09155	**operon_0863**	**	RNA polymerase sigma factor SigS		-2127.5	1.20E-15	gene1797
SA2981_RS09160			competence protein ComK		-1764.2	2.00E-09	gene1798
SA2981_RS09290	**operon_0878**	*	integrase				gene1860
SA2981_RS09295			transposase		-754.5	2.20E-10	gene1861
SA2981_RS09300			transposase		-1331.1	1.10E-11	gene1862
SA2981_RS09830	**operon_0923**	**	multidrug ABC transporter ATP-binding protein		20.6	1.20E-18	gene1968
SA2981_RS10635	**operon_1000**	*	accessory gene regulator protein B		-40.8	1.90E-18	gene2128
SA2981_RS10640			autoinducing peptide AgrD		-17.5	3.80E-13	gene2129
SA2981_RS10645			histidine kinase		-7	1.50E-08	gene2130
SA2981_RS10650			DNA-binding response regulator				gene2131
SA2981_RS10680	**operon_1003**	**	oxidoreductase		17	6.20E-21	gene2137
SA2981_RS10685			membrane protein		12.3	1.50E-16	gene2138
SA2981_RS10735	**operon_1010**	*****	dihydroxy-acid dehydratase	catalyzes the dehydration of 2 2C3-dihydroxy-3-methylbutanoate to 3-methyl-2-oxobutanoate in valine and isoleucine biosynthesis	11.4	1.00E-10	gene2148
SA2981_RS10740			acetolactate synthase 2C large subunit 2C biosynthetic type acetolactate synthase	catalyzes the formation of 2-acetolactate from pyruvate 3B also known as acetolactate synthase large subunit with IlvI catalyzes the formation of 2-acetolactate from pyruvate 2C this subunit is required for full activity and valine sensitivity 3B also known as acetolactate synthase small	2.9	2.00E-03	gene2149
SA2981_RS10745					10.9	2.40E-08	gene2150
SA2981_RS10750			ketol-acid reductoisomerase		9.1	2.70E-10	gene2151
SA2981_RS10755			2-isopropylmalate synthase				gene2152
SA2981_RS10760			3-isopropylmalate dehydrogenase		22.3	5.80E-22	gene2153
SA2981_RS10765			3-isopropylmalate dehydratase large subunit 3-isopropylmalate dehydratase small subunit	dehydratase component 2C catalyzes the isomerization between 2-isopropylmalate and 3-isopropylmalate catalyzes the isomerization between 2-isopropylmalate and 3-isopropylmalate in leucine biosynthesis 3B forms a heterodimer of LeuC/D	8.8	1.00E-08	gene2154
SA2981_RS10770			L-threonine dehydratase biosynthetic IlvA	catalyzes the formation of 2-oxobutanoate from L-threonine 3B biosynthetic	29.8	2.10E-19	gene2155
SA2981_RS10775					33	1.50E-21	gene2156
SA2981_RS11255	**operon_1056**	**	transcriptional regulator		17.2	5.80E-22	gene2158
SA2981_RS11260			cation transporter		76.1	1.50E-22	gene2159
SA2981_RS11670	**operon_1098**	*	50S ribosomal protein L17		12.5	8.30E-14	gene2161

SA2981_RS11675			DNA-directed RNA polymerase subunit alpha		8.6	2.00E-11	gene2162
SA2981_RS11680			30S ribosomal protein S11		3.7	9.60E-05	gene2163
SA2981_RS11685			30S ribosomal protein S13		2.6	7.40E-04	gene2164
SA2981_RS11690			50S ribosomal protein L36	smallest protein in the large subunit 3B similar to what is found with protein L31 and L33 several bacterial genomes contain paralogs which may be regulated by zinc 3B the protein from Thermus thermophilus has a zinc-binding motif and contains a bound zinc ion 3B the proteins in this group have the motif	2.6	6.70E-04	gene2165
SA2981_RS11695			translation initiation factor IF-1	stimulates the activities of the other two initiation factors 2C IF-2 and IF-3	3.5	1.30E-04	gene2166
SA2981_RS11700	**operon_1099**	*	adenylate kinase		3.8	1.50E-06	gene2340
SA2981_RS11705			protein translocase subunit SecY				gene2341
SA2981_RS11710			50S ribosomal protein L15				gene2342
SA2981_RS11715			50S ribosomal protein L30		7.2	1.60E-11	gene2343
SA2981_RS11720			30S ribosomal protein S5		4.9	2.10E-08	gene2344
SA2981_RS11725			50S ribosomal protein L18		8.7	4.40E-12	gene2345
SA2981_RS11730			50S ribosomal protein L6		6.4	3.10E-10	gene2346
SA2981_RS11735			30S ribosomal protein S8		10.9	5.90E-13	gene2347
SA2981_RS11740			30S ribosomal protein S14 type Z	located in the peptidyl transferase center and involved in assembly of 30S ribosome subunit 3B similar to what is observed with proteins L31 and L33 2C some proteins in this family contain CXXC motifs that are involved in zinc binding 3B if two copies are present in a genome 2C then the duplicated copy appears to have lost the zinc-binding motif and is instead regulated by zinc 3B the proteins in this group appear to contain the zinc-binding motif	18.2	1.50E-16	gene2348
SA2981_RS11745			50S ribosomal protein L5		3.6	3.90E-05	gene2349
SA2981_RS11750			50S ribosomal protein L24		3.2	3.10E-05	gene2350
SA2981_RS11755			50S ribosomal protein L14		2.6	1.10E-03	gene2351
SA2981_RS11760			30S ribosomal protein S17		2.1	1.90E-02	gene2352
SA2981_RS11765			50S ribosomal protein L29		2.9	7.10E-04	gene2353
SA2981_RS11770			50S ribosomal protein L16		3.3	5.70E-05	gene2354
SA2981_RS11775			30S ribosomal protein S3		2.7	5.20E-04	gene2355
SA2981_RS11780			50S ribosomal protein L22		2.5	1.50E-03	gene2356
SA2981_RS11785			30S ribosomal protein S19		2.1	8.10E-03	gene2357
SA2981_RS11790			50S ribosomal protein L2	one of the primary rRNA-binding proteins 3B required for association of the 30S and 50S subunits to form the 70S ribosome 2C for tRNA binding and peptide bond formation	2.1	1.50E-02	gene2358
SA2981_RS11795			50S ribosomal protein L23		2.4	4.90E-03	gene2359
SA2981_RS11800			50S ribosomal protein L4		2	1.30E-02	gene2360
SA2981_RS11805			50S ribosomal protein L3		2.7	1.40E-03	gene2361
SA2981_RS11810			30S ribosomal protein S10		2.8	7.70E-04	gene2362
SA2981_RS12230	**operon_1150**	**	imidazolone propionase		-28	2.40E-30	gene2446
SA2981_RS12235			urocanate hydratase	catalyzes the formation of 4-imidazolone-5-propanoate from urocanate during histidine metabolism	-22.5	3.30E-17	gene2447
SA2981_RS12445	**operon_1181**	*	GNAT family acetyltransferase		-21.2	4.00E-25	gene2489
SA2981_RS12450			ferredoxin-NADP(+) reductase				gene2490
SA2981_RS12460			membrane protein				gene2492
SA2981_RS12730	**operon_1213**	*	6-carboxyhexanoate-CoA ligase	catalyzes the formation of pimeloyl-CoA from pimelate and coenzyme A			gene2546
SA2981_RS12735			8-amino-7-oxononanoate synthase				gene2547
SA2981_RS12740			biotin synthase				gene2548
SA2981_RS12745			adenosylmethionine-8-amino-7-oxononanoate aminotransferase BioA	catalyzes the formation of S-adenosyl-4-methylthionine-2-oxobutanoate and 7 2C8-diaminononanoate from S-adenosyl-L-methionine and 8-amino-7-oxononanoate			gene2549
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		-99.4	1.80E-02	gene2550
SA2981_RS12765			multidrug ABC transporter ATP-binding protein		-4.5	3.20E-03	gene2553
SA2981_RS12770			multidrug ABC transporter permease		-15.4	1.30E-05	gene2554
SA2981_RS12920	**operon_1232**	**	iron export ABC transporter permease subunit FetB		-12.8	6.60E-15	gene2584
SA2981_RS12925			methionine ABC transporter ATP-binding protein		-19.9	5.80E-22	gene2585
SA2981_RS13305	**operon_1277**	**	peptide ABC transporter ATP-binding protein		-1796.7	5.60E-19	gene2661
SA2981_RS13310			peptide ABC transporter permease		-20.8	3.20E-14	gene2662
SA2981_RS13495	**operon_1302**	*	dehydrosqualene desaturase		-7.2	4.90E-13	gene2699
SA2981_RS13500			dehydrosqualene synthase		-9.2	3.90E-16	gene2700

			4 2C4'-diaponeurosporenoate glycosyltransferase				
SA2981_RS13505					-4.2	4.00E-08	gene2701
SA2981_RS13510			diapolycopene oxygenase		-14.8	8.40E-22	gene2702
SA2981_RS13515			glycosyl-4 2C4'-diaponeurosporenoate acyltransferase		-85.2	2.80E-36	gene2703
SA2981_RS13585	**operon_1312**	*	TetR family transcriptional regulator		47.5	5.50E-29	gene2717
SA2981_RS13590			short-chain dehydrogenase		9.6	2.70E-09	gene2718
SA2981_RS13595			amidohydrolase		2.8	5.30E-04	gene2719
SA2981_RS13600			hydrolase				gene2720
SA2981_RS13675	**operon_1322**	**	peptidase		-60.3	9.90E-15	gene2735
SA2981_RS14115	**operon_1372**	*	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase	catalyzes the formation of 1-(5-phosphoribosyl)-AMP from 1-(5-phosphoribosyl)-ATP and the subsequent formation of 1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-AMP in histidine biosynthesis	13.6	5.30E-17	gene2823
SA2981_RS14120			imidazole glycerol phosphate synthase cyclase subunit	catalyzes the conversion of 5-[(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino]-1-(5-phosphoribosyl)imidazole-4-carboxamideand glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamideribonucleotide and glutamate 3B the HisF subunit acts as a cyclase	-3.2	3.40E-02	gene2824
SA2981_RS14125			1-(5-phosphoribosyl)-5-((5-phosphoribosylamino)methylideneamino)imidazole-4-carboxamide isomerase	catalyzes the formation of 5-(5-phospho-1-deoxyribulos-1-ylamino)methylideneamino-l-(5-hosphoribosyl)imidazole-4-carboxamide from 1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide	-4.7	5.50E-04	gene2825
SA2981_RS14130			imidazole glycerol phosphate synthase subunit HisH	with HisF lGPS catalyzes the conversion of phosphoribulosyl-formimino-5-aminoimidazole-4-carboxamide ribonucleotide phosphate and glutamine to imidazole-glycerol phosphate 2C 5-aminoimidazol-4-carboxamide ribonucleotide 2C and glutamate in histidine biosynthesis 3B the HisH subunit provides the glutamine amidotransferase activity that produces the ammonia necessary to HisF for the synthesis of imidazole-glycerol phosphate and 5-aminoimidazol-4-carboxamide ribonucleotide	-5.3	3.30E-03	gene2826
SA2981_RS14135			imidazoleglycerol-phosphate dehydratase				gene2827
SA2981_RS14140			histidinol-phosphate aminotransferase		196.1	5.90E-07	gene2828
SA2981_RS14145			histidinol dehydrogenase	catalyzes the oxidation of L-histidinol to L-histidinaldehyde and then to L-histidine in histidine biosynthesis 3B functions as a dimer			gene2829
SA2981_RS14150			ATP phosphoribosyltransferase	short form of enzyme 3B requires HisZ for function 3B catalyzes the formation of N'-5'-phosphoribosyl-ATP from phosphoribosyl pyrophosphate 3B crucial role in histidine biosynthesis 3B forms heteromultimer of HisG and HisZ	29.4	1.60E-04	gene2830
SA2981_RS14155			ATP phosphoribosyltransferase regulatory subunit	May allow the feedback regulation of ATP phosphoribosyltransferase activity by histidine	-246.9	8.10E-04	gene2831

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 12 minutes

Locus Tag	GO	Rate	Gene product	Description	gene number	Fold change	p- value
SA2981_RS07375	GO:0003333	*	amino acid permease	Amino acid transmembrane transport	gene1476	-2.8	3.00E-03
SA2981_RS12905			amino acid:proton symporter	amino acid membrane transport	gene2581	4.4	5.30E-06
SA2981_RS13720			amino acid permease		gene2744	4.7	4.30E-08
SA2981_RS00200	GO:0003677	*	mecA-type methicillin resistance repressor MecI	DNA binding	gene39	-3.3	3.40E-05
SA2981_RS00225			hypothetical protein		gene44	58.8	2.40E-29
SA2981_RS00415			hypothetical protein		gene82	19.1	5.80E-12
SA2981_RS01025			transcriptional regulator		gene204	-3.6	1.90E-03
SA2981_RS01115			DNA-binding response regulator		gene222	-2.9	7.50E-04
SA2981_RS01330			DNA-binding response regulator		gene265	-3	2.50E-03
SA2981_RS02400			DNA polymerase III subunit delta'		gene480	-3.1	3.20E-04
SA2981_RS02675			transcriptional regulator CtsR		gene535	6.6	3.70E-08
SA2981_RS03180			recombinase		gene635	8.3	3.50E-12
SA2981_RS07180			cold-shock protein CspA		gene1436	-4	2.80E-02
SA2981_RS07680			DNA-binding response regulator		gene1538	-4.6	4.00E-06
SA2981_RS09580			transcriptional regulator		gene1919	-2.5	2.20E-03
SA2981_RS13555			TetR family transcriptional regulator		gene2711	8.6	8.80E-09
SA2981_RS13585			TetR family transcriptional regulator		gene2717	10.9	5.00E-13
SA2981_RS05370	GO:0006189	*	N5-carboxyaminoimidazole ribonucleotide synthase	de novo' IMP biosynthetic prc	gene1074	-2.7	3.20E-03
SA2981_RS05405			phosphoribosylglycinamide formyltransferase	'de novo' purine biosynthesis	gene1081	-2.3	2.80E-02
SA2981_RS00225	GO:0006355	*	hypothetical protein	regulation of gene-specific transcription	gene44	58.8	2.40E-29
SA2981_RS00415			hypothetical protein	regulation of cellular transcription, DNA-dependent	gene82	19.1	5.80E-12
SA2981_RS01115			DNA-binding response regulator	transcriptional control	gene222	-2.9	7.50E-04
SA2981_RS01700			hypothetical protein		gene339	-2.3	4.90E-02
SA2981_RS02675			transcriptional regulator CtsR		gene535	6.6	3.70E-08
SA2981_RS03420			transcriptional regulator		gene683	-5.6	4.10E-09
SA2981_RS05990			division/cell wall cluster transcriptional repressor MraZ		gene1198	-3.3	1.10E-02
SA2981_RS06640			RNA-binding protein Hfq		gene1328	-2.6	1.50E-03
SA2981_RS07180			cold-shock protein CspA		gene1436	-4	2.80E-02
SA2981_RS07680			DNA-binding response regulator		gene1538	-4.6	4.00E-06
SA2981_RS07830			arginine repressor		gene1568	-4.7	1.30E-07
SA2981_RS10060			hypothetical protein		gene2015	-2.7	1.50E-03
SA2981_RS05935	GO:0006525	*	carbamate kinase 1	arginine metabolism	gene1187	-3.6	6.70E-05
SA2981_RS07830			arginine repressor		gene1568	-4.7	1.30E-07
SA2981_RS02675	GO:0006950	*	transcriptional regulator CtsR	response to stress	gene535	6.6	3.70E-08
SA2981_RS11225			DNA starvation/stationary phase protection protein		gene2254	16.5	9.80E-09
SA2981_RS00740	GO:0008152	*	bifunctional acetaldehyde-CoA/alcohol dehydrogena	metabolism	gene147	-2.6	1.70E-02
SA2981_RS01130			formate acetyltransferase	metabolic process	gene225	-3.7	4.40E-04
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234	141.5	1.40E-05
SA2981_RS01180			long-chain-fatty-acid-CoA ligase		gene235	5.1	3.30E-02
SA2981_RS01185			acyl CoA:acetate/3-ketoacid CoA transferase		gene236	17.8	1.10E-15
SA2981_RS01925			phosphoglycerate mutase		gene384	-3.1	3.10E-03
SA2981_RS07805			2-oxoglutarate dehydrogenase E2		gene1536	-3.3	1.10E-02
SA2981_RS08895			1-acyl-sn-glycerol-3-phosphate acyltransferase		gene1782	-3.3	2.50E-05
SA2981_RS10105			prephenate dehydratase		gene2024	-3	1.90E-04
SA2981_RS12225			N-acetyl-L2CL-diaminopimelate deacetylase		gene2445	6.6	1.60E-06
SA2981_RS13295			lactate dehydrogenase		gene2659	-4.4	2.50E-05
SA2981_RS07375	GO:0015171	*	amino acid permease	amino acid permease activity	gene1476	-2.8	3.00E-03
SA2981_RS12905			amino acid:proton symporter	amino acid transporter activity	gene2581	4.4	5.30E-06
SA2981_RS13720			amino acid permease		gene2744	4.7	4.30E-08
SA2981_RS00525	GO:0016020	*	membrane protein	membrane	gene104	-202.9	2.90E-02
SA2981_RS00950			PTS glucose EIICBA component		gene189	-2.8	4.50E-04
SA2981_RS01085			maltose ABC transporter permease		gene216	-2.7	2.00E-02
SA2981_RS01555			formate/nitrite transporter		gene310	-6.3	7.30E-11
SA2981_RS05725			heme uptake system protein IsdE		gene1145	6.1	4.00E-02
SA2981_RS06430			phosphatidate cytidylyltransferase		gene1286	-2.7	1.70E-03
SA2981_RS06745			multidrug ABC transporter permease		gene1349	5.1	8.40E-03
SA2981_RS07375			amino acid permease		gene1476	-2.8	3.00E-03
SA2981_RS10610			CAAX amino protease		gene2123	-2.7	8.10E-04
SA2981_RS12905			amino acid:proton symporter		gene2581	4.4	5.30E-06
SA2981_RS12985			nickel ABC transporter permease		gene2597	5.9	4.30E-06
SA2981_RS13720			amino acid permease		gene2744	4.7	4.30E-08
SA2981_RS14180			membrane protein		gene2836	8.1	5.50E-07
SA2981_RS00230	GO:0016021	*	membrane protein	transmembrane	gene45	26.7	3.40E-17
SA2981_RS00410			membrane protein		gene81	15.9	6.20E-13
SA2981_RS00525			membrane protein		gene104	-202.9	2.90E-02
SA2981_RS00950			PTS glucose EIICBA component	integral to membrane	gene189	-2.8	4.50E-04
SA2981_RS01335			antiholin-like protein LrgA		gene266	-2.6	2.50E-03
SA2981_RS01740			membrane protein		gene347	-2.5	1.40E-02
SA2981_RS05950			permease		gene1190	-4	3.70E-04
SA2981_RS06000			cell division protein FtsL		gene1200	-2.6	1.10E-02
SA2981_RS09520			multidrug ABC transporter ATP-binding protein		gene1907	-2.3	1.70E-02
SA2981_RS10635			accessory gene regulator protein B		gene2128	-5.7	3.30E-05
SA2981_RS12340			multidrug MFS transporter		gene2468	-2.4	1.80E-02
SA2981_RS12530			nitrate transporter NarT		gene2506	-2.3	8.50E-03
SA2981_RS13435			ferrous iron transporter B		gene2687	7.1	3.30E-02
SA2981_RS13475			copper-exporting P-type ATPase A		gene2695	13.8	7.50E-17
SA2981_RS00400	GO:0016491	*	tRNA-dihydrouridine synthase	oxidoreductase activity	gene79	26.3	7.00E-21
SA2981_RS00740			bifunctional acetaldehyde-CoA/alcohol dehydrogena	redox activity	gene147	-2.6	1.70E-02

SA2981_RS01135			pyruvate formate-lyase-activating enzyme		gene226	-4.2	2.20E-04
SA2981_RS01220			L-lactate dehydrogenase		gene243	-2.6	4.00E-02
SA2981_RS02870			FMN-dependent NADPH-azoreductase		gene574	-2.5	3.20E-03
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	55.1	3.00E-28
SA2981_RS03100			zinc-dependent alcohol dehydrogenase		gene619	-3.9	4.30E-05
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	36.6	8.80E-19
SA2981_RS07820			dihydrolipoyl dehydrogenase		gene1566	-2.4	6.30E-03
SA2981_RS10620			nitroreductase		gene2125	10.4	2.10E-13
SA2981_RS12575			nitrate reductase subunit alpha (NarZ)		gene2515	-2.5	3.30E-02
SA2981_RS12995			hypothetical protein		gene2599	8.2	1.70E-02
SA2981_RS13290			NAD(P)H-dependent oxidoreductase		gene2658	4.6	3.10E-06
SA2981_RS05930	GO:0016597	*	ornithine carbamoyltransferase	amino acid binding	gene1186	-4.9	3.00E-07
SA2981_RS10105			prephenate dehydratase		gene2024	-3	1.90E-04
SA2981_RS00740	GO:0016620	*	bifunctional acetaldehyde-CoA/alcohol dehydrogena	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	gene147	-2.6	1.70E-02
SA2981_RS00920			N-acetyl-gamma-glutamyl-phosphate reductase		gene183	-6.2	9.90E-03
SA2981_RS08680			glyceraldehyde-3-phosphate dehydrogenase	NAD-dependent, catalyzes th	gene1739	-3.5	2.40E-04
SA2981_RS03035	GO:0016668	*	dihydrolipoamide dehydrogenase	oxidoreductase activity, acting on a sulfur group of donors, NAD(P) as acceptor	gene607	55.1	3.00E-28
SA2981_RS07820			dihydrolipoyl dehydrogenase		gene1566	-2.4	6.30E-03
SA2981_RS01730	GO:0016705	*	luciferase	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	gene345	4.6	6.40E-08
SA2981_RS08350			hypothetical protein		gene1672	4.6	1.20E-07
SA2981_RS07805	GO:0016746	*	2-oxoglutarate dehydrogenase E2	transferase activity, transferri	gene1563	-3.3	4.20E-05
SA2981_RS08895			1-acyl-sn-glycerol-3-phosphate acyltransferase	acyl transferase activity	gene1782	-3.3	2.50E-05
SA2981_RS02690	GO:0019538	*	ATP-dependent Clp protease ATP-binding subunit	protein metabolism	gene538	4.9	3.20E-06
SA2981_RS04910			chaperone protein ClpB	protein metabolism and modif	gene982	12.7	5.50E-14
SA2981_RS05725	GO:0020037	*	heme uptake system protein IsdE	Heme binding	gene1145	6.1	4.00E-02
SA2981_RS10100			nitric oxide synthase oxygenase		gene2023	-2.9	3.70E-04
SA2981_RS12990			nickel ABC transporter2C nickel/metallophore peripl	asmic binding protein	gene2598	7.5	2.60E-07
SA2981_RS01025	GO:0043565	*	transcriptional regulator	sequence specific DNA bindi	gene204	-3.6	1.90E-03
SA2981_RS01115			DNA-binding response regulator		gene222	-2.9	7.50E-04
SA2981_RS09580			transcriptional regulator		gene1919	-2.5	2.20E-03
SA2981_RS10060			hypothetical protein		gene2015	-2.7	1.50E-03
SA2981_RS00225	GO:0046872	*	hypothetical protein	metal ion binding	gene44	58.8	2.40E-29
SA2981_RS00415			hypothetical protein	heavy metal binding	gene82	19.1	5.80E-12
SA2981_RS00740			bifunctional acetaldehyde-CoA/alcohol dehydrogenase		gene147	-2.6	1.70E-02
SA2981_RS03280			glycerol-3-phosphate cytidylyltransferase		gene655	-2.6	2.90E-03
SA2981_RS13475			copper-exporting P-type ATPase A		gene2695	13.8	7.50E-17
SA2981_RS00400	GO:0050660	*	tRNA-dihydrouridine synthase	flavine-adenine dinucleotide b	gene79	26.3	7.00E-21
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234	141.5	1.40E-05
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	55.1	3.00E-28
SA2981_RS07820			dihydrolipoyl dehydrogenase		gene1566	-2.4	6.30E-03
SA2981_RS00920	GO:0051287	*	N-acetyl-gamma-glutamyl-phosphate reductase	NAD or NADH binding	gene183	-6.2	9.90E-03
SA2981_RS05370			N5-carboxyaminoimidazole ribonucleotide synthase		gene1074	-2.7	3.20E-03
SA2981_RS08680			glyceraldehyde-3-phosphate dehydrogenase		gene1739	-3.5	2.40E-04
SA2981_RS13295			lactate dehydrogenase		gene2659	-4.4	2.50E-05
SA2981_RS00400	GO:0055114	*	tRNA-dihydrouridine synthase	oxidation- reduction process	gene79	26.3	7.00E-21
SA2981_RS00740			bifunctional acetaldehyde-CoA/alcohol dehydrogena	oxidoreductase process	gene147	-2.6	1.70E-02
SA2981_RS00920			N-acetyl-gamma-glutamyl-phosphate reductase		gene183	-6.2	9.90E-03
SA2981_RS01060			FMN-dependent NADH-azoreductase		gene211	15	4.00E-21
SA2981_RS01135			pyruvate formate-lyase-activating enzyme		gene226	-4.2	2.20E-04
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234	141.5	1.40E-05
SA2981_RS01220			L-lactate dehydrogenase		gene243	-2.6	4.00E-02
SA2981_RS01730			luciferase		gene345	4.6	6.40E-08
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	55.1	3.00E-28
SA2981_RS03100			zinc-dependent alcohol dehydrogenase		gene619	-3.9	4.30E-05
SA2981_RS03195			cation:proton antiporter		gene638	6.8	1.00E-06
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	36.6	8.80E-19
SA2981_RS05370			N5-carboxyaminoimidazole ribonucleotide synthase		gene1074	-2.7	3.20E-03
SA2981_RS05740			monooxygenase IsdI		gene1148	-2.8	4.20E-04
SA2981_RS07820			dihydrolipoyl dehydrogenase		gene1566	-2.4	6.30E-03
SA2981_RS08350			hypothetical protein		gene1672	4.6	1.20E-07
SA2981_RS08680			glyceraldehyde-3-phosphate dehydrogenase		gene1739	-3.5	2.40E-04
SA2981_RS10100			nitric oxide synthase oxygenase		gene2023	-2.9	3.70E-04
SA2981_RS11225			DNA starvation/stationary phase protection protein		gene2245	16.5	9.80E-09
SA2981_RS12575			nitrate reductase subunit alpha		gene2515	-2.5	3.30E-02
SA2981_RS12995			hypothetical protein		gene2599	8.2	1.70E-02
SA2981_RS13295			lactate dehydrogenase		gene2659	-4.4	3.80E-07

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 24 minutes

Locus Tag	GO	Rate	Gene product	Description	gene number	Fold change	p- value
SA2981_RS14115	GO:0000105	*	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase	Histidine biosynthetic process	gene2823	13.8	1.90E-17
SA2981_RS14150			ATP phosphoribosyltransferase (hisG)		gene2830	19	2.10E-04
SA2981_RS10740	GO:0000287	*	acetolactate synthase2C large subunit2C biosynthetic type	magnesium binding	gene2149	28.4	8.20E-18
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	60.2	3.20E-31
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		gene2550	-79.8	2.70E-02
SA2981_RS10765	GO:0003861	**	3-isopropylmalate dehydratase large subunit	isopropylmalate isomerase activity (IPM)	gene2154	24.6	4.20E-15
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	39.9	4.80E-21
SA2981_RS00585	GO:0005215	*	siderophore biosynthesis protein SbnD	transporter activity	gene116	20.7	9.30E-05
SA2981_RS01695			PTS ascorbate transporter subunit IIA		gene338	-94.8	6.80E-21
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	14.3	5.40E-12
SA2981_RS11430			iron-dicitrate ABC transporter permease (fecD)		gene2286	51.4	3.10E-24
SA2981_RS11435			siderophore ABC transporter permease		gene2287	13.7	4.40E-18
SA2981_RS00860	GO:0005524	*	sulfonate ABC transporter ATP-binding protein	ATP binding	gene171	52.1	6.60E-39
SA2981_RS00995			peptide ABC transporter ATP-binding protein		gene198	-336.8	2.30E-05
SA2981_RS02245			methionine import ATP-binding protein MetN 1		gene448	18.6	2.80E-18
SA2981_RS03785			iron ABC transporter ATP-binding protein		gene756	17.2	7.80E-10
SA2981_RS04910			chaperone protein ClpB		gene982	24.7	3.40E-21
SA2981_RS04995			peptide ABC transporter ATP-binding protein		gene999	33.4	3.00E-09
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	30.1	9.30E-07
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	14.3	5.40E-12
SA2981_RS06740			antibiotic ABC transporter ATP-binding protein		gene1348	-249.2	1.40E-04
SA2981_RS07095			phosphate ABC transporter ATP-binding protein		gene1419	-1073.5	6.20E-10
SA2981_RS08125			molecular chaperone DnaJ		gene1627	25	3.90E+25
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		gene2550	-79.8	2.70E-02
SA2981_RS02075	GO:0005576	*	hypothetical protein	extracellular region	gene414	-252.4	2.60E-04
SA2981_RS09385			gamma-hemolysin subunit A		gene1879	-378.9	3.60E-04
SA2981_RS09420			enterotoxin		gene1887	-82.2	1.00E-02
SA2981_RS11575			hyaluronate lyase		gene2315	-164	2.20E-13
SA2981_RS00875	GO:0008152	*	butyryl-CoA dehydrogenase	metabolism	gene174	15.9	2.10E-22
SA2981_RS00885			formate dehydrogenase	meabolic processes	gene176	33.9	5.30E-18
SA2981_RS07000			glutamine amidotransferase		gene1400	13.2	8.00E-04
SA2981_RS07015			phosphoribosylanthranilate isomerase		gene1403	-130.7	4.00E-03
SA2981_RS10735			dihydroxy-acid dehydratase		gene2148	32.8	5.50E-18
SA2981_RS10745			acetolactate synthase		gene2150	13.8	2.80E-09
SA2981_RS10765			3-isopropylmalate dehydratase large subunit		gene2154	24.6	4.20E-15
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	39.9	4.80E-21
SA2981_RS12225			N-acetyl-L 2CL-diaminopimelate deacetylase		gene2445	31	2.50E-19
SA2981_RS10735	GO:0009082	**	dihydroxy-acid dehydratase	branched chain family amino acid biosynthetic process	gene2148	32.8	5.50E-18
SA2981_RS10740			acetolactate synthase 2C large subunit 2C biosynthetic type		gene2149	28.4	8.20E-18
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	38.2	5.80E-21
SA2981_RS10755	GO:0009098	***	2-isopropylmalate synthase	leucine biosynthesis	gene2152	72.1	4.10E-41
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	60.2	3.20E-31
SA2981_RS10765			3-isopropylmalate dehydratase large subunit		gene2154	24.6	4.20E-15
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	39.9	4.80E-21
SA2981_RS02075	GO:0009405	*	hypothetical protein	pathogenesis	gene414	-252.4	2.60E-04
SA2981_RS09385			gamma-hemolysin subunit A	virulence	gene1879	-378.9	3.60E-04
SA2981_RS09420			enterotoxin		gene1887	-82.2	1.00E-02
SA2981_RS09425			enterotoxin		gene1888	-144.2	2.60E-03
SA2981_RS04910	GO:0009408	**	chaperone protein ClpB	response to heat shock	gene982	24.7	3.40E-21
SA2981_RS08125			molecular chaperone DnaJ		gene1627	25	3.90E-25
SA2981_RS00580	GO:0015343	*	siderophore biosynthesis protein SbnC	iron-siderophore transporter activity	gene115	30.6	4.40E-06
SA2981_RS00590			siderophore biosynthesis protein SbnE	siderophore-iron transmembrane transporter activity	gene117	15.9	9.50E-06
SA2981_RS04995	GO:0015833	*	peptide ABC transporter ATP-binding protein	peptide transport	gene999	33.4	3.0-09
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	30.1	9.30E-07
SA2981_RS00400	GO:0016491	*	tRNA-dihydrouridine synthase	oxidoreductase acivity	gene79	22.5	2.00E-20
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase	redox activity	gene254	20.4	1.00E-12
SA2981_RS02300			glutamate synthase subunit beta		gene459	13.7	6.80E-21

SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	158.1	1.00E-39
SA2981_RS04165			nitroreductase		gene833	35.6	2.20E-31
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	72.8	8.30E-26
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	38.2	5.80E-21
SA2981_RS12995			hypothetical protein		gene2599	31	1.60E-05
SA2981_RS13590			short-chain dehydrogenase		gene2718	18.5	1.60E-13
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	22.5	5.80E-05
SA2981_RS00860	GO:0016887	*	sulfonate ABC transporter ATP-binding protein	ATPase activity	gene171	52.1	6.60E-39
SA2981_RS00995			peptide ABC transporter ATP-binding protein		gene198	-336.8	2.30E-05
SA2981_RS02245			methionine import ATP-binding protein MetN 1		gene448	18.6	2.80E-18
SA2981_RS03785			iron ABC transporter ATP-binding protein		gene756	17.2	7.80E-10
SA2981_RS04995			peptide ABC transporter ATP-binding protein		gene999	33.4	3.00E-09
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	30.1	9.30E-07
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	14.3	5.40E-12
SA2981_RS06740			antibiotic ABC transporter ATP-binding protein		gene1348	-249.2	1.40E-04
SA2981_RS07095			phosphate ABC transporter ATP-binding protein		gene1419	-1073.5	6.20E-10
SA2981_RS00580	GO:0019290	*	siderophore biosynthesis protein SbnC	siderophore formation	gene115	30.6	4.40E-06
SA2981_RS00590			siderophore biosynthesis protein SbnE	siderophore biosynthetic process, peptide formation	gene117	15.9	9.50E-06
SA2981_RS07935	GO:0030420	*	hypothetical protein	establishment of competence for transformation	gene1589	-129.9	5.80E-03
SA2981_RS07940			competence protein ComGC		gene1590	-495.1	4.80E-05
SA2981_RS03035	GO:0045454	*	dihydrolipoamide dehydrogenase	cell redox homeostasis	gene607	158.1	1.00E-39
SA2981_RS05475			NrdH-redoxin	regulation of cell redox homeostasis	gene1095	29.1	7.60E-32
SA2981_RS00400	GO:0050660	*	tRNA-dihydrouridine synthase	flavine-adenine dinucleotide binding	gene79	22.5	2.00E-20
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	158.1	1.00E-39
SA2981_RS10740			acetolactate synthase 2C large subunit 2C biosynthetic type		gene2149	28.4	8.20E-18
SA2981_RS00400	GO:0055114	*	tRNA-dihydrouridine synthase	oxidation- reduction process	gene79	22.5	2.00E-20
SA2981_RS00875			butyryl-CoA dehydrogenase	oxidoreductase process	gene174	15.9	2.10E-22
SA2981_RS00885			formate dehydrogenase		gene176	33.9	5.30E-18
SA2981_RS01060			FMN-dependent NADH-azoreductase		gene211	27.5	8.10E-30
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase		gene254	20.4	1.00E-12
SA2981_RS02300			glutamate synthase subunit beta		gene459	13.7	6.80E-21
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	158.1	1.00E-39
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	72.8	8.30E-26
SA2981_RS05740			monooxygenase IsdI		gene1148	-2207.3	2.20E-17
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	38.2	5.80E-21
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	60.2	3.20E-31
SA2981_RS12995			hypothetical protein		gene2599	31	1.60E-05
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	22.5	5.80E-05
SA2981_RS14055			methionine sulfoxide reductase A		gene2811	-941.4	1.10E-10

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 80 minutes

Locus Tag	GO	Rate	Gene product	Description	gene number	Fold change	p- value
SA2981_RS14130	GO:0000105	*	imidazole glycerol phosphate synthase subunit HisH	Histidine biosynthetic process	gene2826	-1463.2	1.10E-11
SA2981_RS14135			imidazoleglycerol-phosphate dehydratase		gene2827	9.4	2.50E-02
SA2981_RS14145			histidinol dehydrogenase		gene2829	-70.2	1.20E-02
SA2981_RS14150			ATP phosphoribosyltransferase (hisG)		gene2830	-156.8	8.10E-04
SA2981_RS00225	GO:0003677	*	hypothetical protein	DNA binding	gene44	19.2	2.10E-16
SA2981_RS00320			transposase		gene63	207.7	2.00E-03
SA2981_RS02320			trehalose operon repressor		gene463	14.7	3.40E-20
SA2981_RS02675			transcriptional regulator CtsR		gene535	22.4	1.90E-19
SA2981_RS03860			comf operon protein 1		gene771	10.1	4.70E-02
SA2981_RS07705			tyrosine recombinase XerD		gene1543	10.4	6.40E-15
SA2981_RS08015			endonuclease		gene1605	11.4	7.50E-19
SA2981_RS08180			competence protein ComE		gene1638	-51.4	1.70E-07
SA2981_RS08285			transcription elongation factor GreA		gene1659	11	9.10E-12
SA2981_RS10950			hypothetical protein		gene2191	-22.5	2.90E-27
SA2981_RS11565			MerR family transcriptional regulator		gene2313	-1505.3	1.10E-14
SA2981_RS13585			TetR family transcriptional regulator		gene2717	1245.3	1.70E-46
SA2981_RS02320	GO:0003700	*	trehalose operon repressor	transcription factor activity	gene463	14.7	3.40E-20
SA2981_RS04915			LysR family transcriptional regulator	sequence-specific DNA binding transcri	gene983	-131.2	4.50E-03
SA2981_RS08000			transcriptional repressor		gene1602	120.8	9.00E-39
SA2981_RS11255			transcriptional regulator		gene2251	59.4	2.30E-37
SA2981_RS12240			LysR family transcriptional regulator		gene2448	-4762.8	1.20E-27
SA2981_RS13150			transcriptional regulator		gene2630	-454.3	4.70E-06
SA2981_RS09345	GO:0004252	*	serine protease SplB	serine elastase activity	gene1871	-70.2	3.30E-02
SA2981_RS09350			serine protease SplA	blood coagulation factor activity	gene1872	-135.4	6.90E-03
SA2981_RS13680			hypothetical protein		gene2736	-296.1	6.60E-04
SA2981_RS03790	GO:0005488	*	hypothetical protein	binding	gene757	9.6	9.30E-16
SA2981_RS05225			ABC transporter substrate-binding protein		gene1045	-856.6	2.70E-11
SA2981_RS05725			heme uptake system protein IsdE		gene1145	-51.4	9.50E-07
SA2981_RS11990			ferrichrome ABC transporter substrate-binding protein		gene2398	14.4	3.40E-21
SA2981_RS01020	GO:0005524	*	ABC transporter ATP-binding protein	ATP binding	gene203	-32.4	6.70E-12
SA2981_RS02690			ATP-dependent Clp protease ATP-binding subunit ClpC		gene538	21.9	8.50E-20
SA2981_RS03860			comf operon protein 1		gene771	10.1	4.70E-02
SA2981_RS04910			chaperone protein ClpB		gene982	36.7	5.70E-25
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	20.5	1.10E-05
SA2981_RS06065			isoleucine-tRNA ligase (ileS)		gene1213	10.7	1.80E-17
SA2981_RS06740			antibiotic ABC transporter ATP-binding protein		gene1348	-328.2	5.60E-05
SA2981_RS07095			phosphate ABC transporter ATP-binding protein		gene1419	15	6.00E-05
SA2981_RS08005			iron ABC transporter permease		gene1603	12.3	5.90E-19
SA2981_RS08020			DEAD/DEAH box family ATP-dependent RNA helicase		gene1606	9.6	6.10E-13
SA2981_RS08125			molecular chaperone DnaJ		gene1627	16.8	2.50E-20
SA2981_RS08130			molecular chaperone DnaK		gene1628	9.7	7.70E-11
SA2981_RS10605			co-chaperone GroES		gene2122	9.9	2.70E-10
SA2981_RS12655			arginine ABC transporter ATP-binding protein		gene2531	10.3	2.00E-12
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		gene2550	-61.2	4.20E-02
SA2981_RS12975			peptide ABC transporter ATP-binding protein		gene2595	10.2	3.00E-03
SA2981_RS13860			ABC transporter ATP-binding protein		gene2772	-1063.5	8.70E-11
SA2981_RS14255			ABC transporter ATP-binding protein		gene2851	-325.8	1.60E-04
SA2981_RS02070	GO:0005576	*	hypothetical protein	extracellular region	gene413	-25	1.40E-05
SA2981_RS02075			hypothetical protein		gene414	-181.5	9.10E-04
SA2981_RS05920			hypothetical protein		gene1184	-564	3.30E-06
SA2981_RS09385			gamma-hemolysin subunit A		gene1879	-192.2	2.20E-03
SA2981_RS09420			enterotoxin		gene1887	14.5	1.50E-02
SA2981_RS00225	GO:0006355	*	hypothetical protein	regulation of cellular transcription, DNA	gene44	19.2	2.10E-16
SA2981_RS02320			trehalose operon repressor	regulation of gene-specific transcription	gene463	14.7	3.40E-20
SA2981_RS02675			transcriptional regulator CtsR		gene535	22.4	1.90E-19
SA2981_RS04915			LysR family transcriptional regulator		gene983	-131.2	4.50E-03
SA2981_RS08000			transcriptional repressor		gene1602	120.8	9.00E-39
SA2981_RS10950			hypothetical protein		gene2191	-22.5	2.90E-27
SA2981_RS11255			transcriptional regulator		gene2251	59.4	2.30E-37
SA2981_RS11565			MerR family transcriptional regulator		gene2313	-1505.3	1.10E-14
SA2981_RS12240			LysR family transcriptional regulator		gene2448	-4762.8	1.20E-27
SA2981_RS13150			transcriptional regulator		gene2630	-454.3	4.70E-06
SA2981_RS13155			transcriptional regulator		gene2631	-204.6	1.20E-03
SA2981_RS08125	GO:0006457	*	molecular chaperone DnaJ	Protein folding	gene1627	16.8	2.50E-20
SA2981_RS08130			molecular chaperone DnaK		gene1628	9.7	7.70E-11
SA2981_RS08135			nucleotide exchange factor GrpE		gene1629	10.1	3.20E-12

SA2981_RS10605			co-chaperone GroES		gene2122	9.9	2.70E-10
SA2981_RS07010	GO:0006568	*	indole-3-glycerol-phosphate synthase	tryptophan metabolism	gene1402	-559.9	1.10E-05
SA2981_RS07015			phosphoribosylanthranilate isomerase		gene1403	-463.4	1.50E-04
SA2981_RS09345	GO:0008236	*	serine protease SplB	serine protease activity	gene1871	-70.2	3.30E-02
SA2981_RS09350			serine protease SplA		gene1872	-135.4	6.90E-03
SA2981_RS13680			hypothetical protein		gene2736	-296.1	6.60E-04
SA2981_RS05475	GO:0009055	*	NrdH-redoxin	electron carrier activity	gene1095	13.1	5.60E-21
SA2981_RS07630			ferredoxin	thioredoxin-like 2Fe-2S ferredoxin	gene1528	16.8	1.70E-19
SA2981_RS10755	GO:0009098	*	2-isopropylmalate synthase	leucine biosynthesis	gene2152	70.1	9.00E-42
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	17.9	2.10E-19
SA2981_RS00745	GO:0009103	*	capsular polysaccharide type 5 biosynthesis protein Cap5A	lipopolysaccharide synthesis	gene148	-76.1	3.80E-31
SA2981_RS14075			capsular polysaccharide biosynthesis protein CapA	LPS biosynthetic process	gene2815	-329.9	1.70E-04
SA2981_RS02070	GO:0009405	*	hypothetical protein	pathogenesis	gene413	-25	1.40E-05
SA2981_RS02075			hypothetical protein	virulence	gene414	-181.5	9.10E-04
SA2981_RS05920			hypothetical protein		gene1184	-564	3.30E-06
SA2981_RS09385			gamma-hemolysin subunit A		gene1879	-192.2	2.20E-03
SA2981_RS09420			enterotoxin		gene1887	14.5	1.50E-02
SA2981_RS10635			accessory gene regulator protein B		gene2128	-40.7	2.80E-18
SA2981_RS04910	GO:0009408	**	chaperone protein ClpB	response to heat shock	gene982	36.7	5.70E-25
SA2981_RS08125			molecular chaperone DnaJ		gene1627	16.8	2.50E-20
SA2981_RS00310	GO:0015074	*	transposase	DNA integration	gene61	-20.4	9.80E-05
SA2981_RS07705			tyrosine recombinase XerD		gene1543	10.4	6.40E-15
SA2981_RS09290			integrase		gene1860	-1385.8	3.10E-10
SA2981_RS00590	GO:0015343	*	siderophore biosynthesis protein SbnE	iron-siderophore transporter activity	gene117	22.3	1.00E-06
SA2981_RS00595			siderophore biosynthesis protein SbnF		gene118	42.8	2.60E-07
SA2981_RS10715	GO:0016407	*	ribosomal-protein-alanine N-acetyltransferase RimI	acetylase activity	gene2144	-623.3	5.20E-11
SA2981_RS13465			maltose O-acetyltransferase		gene2693	56.2	1.20E-11
SA2981_RS00400	GO:0016491	*	tRNA-dihydrouridine synthase	oxidoreductase acivity	gene79	11.7	3.40E-14
SA2981_RS01265			sorbitol dehydrogenase	redox activity	gene252	36.9	1.80E-03
SA2981_RS01735			FMN reductase		gene346	11.3	3.30E-18
SA2981_RS01950			alkyl hydroperoxide reductase subunit C		gene389	14.6	7.20E-13
SA2981_RS02300			glutamate synthase subunit beta		gene459	10.4	1.60E-17
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	138.9	1.40E-38
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	18.9	1.10E-14
SA2981_RS04820			NADH-dependent flavin oxidoreductase		gene964	12.8	5.30E-20
SA2981_RS10620			nitroreductase		gene2125	20.3	5.40E-21
SA2981_RS12995			hypothetical protein		gene2599	14.9	2.30E-03
SA2981_RS13290			NAD(P)H-dependent oxidoreductase		gene2658	17.5	5.70E-19
SA2981_RS13510			diapolycopene oxygenase		gene2702	-30.8	2.30E-31
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	446.8	1.10E-05
SA2981_RS13805			ribonucleoside-triphosphate reductase		gene2761	-30.4	6.50E-20
SA2981_RS14145			histidinol dehydrogenase		gene2829	-70.2	1.20E-02
SA2981_RS10745	GO:0016597	*	acetolactate synthase	amino acid binding	gene2150	10.3	5.50E-08
SA2981_RS13920			ornithine carbamoyltransferase		gene2784	-26.1	6.00E-11
SA2981_RS01410	GO:0016811	*	choloylglycine hydrolase	hydrolase activity	gene281	18.4	1.30E-10
SA2981_RS14095			intercellular adhesin biosynthesis polysaccharide N-deacetylase		gene2819	-102.4	7.60E-03
SA2981_RS01020	GO:0016887	*	ABC transporter ATP-binding protein	ATP hydrolase activity	gene203	-32.4	6.70E-12
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	20.5	1.10E-05
SA2981_RS06740			antibiotic ABC transporter ATP-binding protein		gene1348	-328.2	6.00E-06
SA2981_RS07095			phosphate transport system regulatory protein PhoU		gene1419	15	6.00E-05
SA2981_RS12655			arginine ABC transporter ATP-binding protein		gene2531	10.3	2.00E-12
SA2981_RS12975			peptide ABC transporter ATP-binding protein		gene2595	10.2	3.00E-03
SA2981_RS13860			ABC transporter ATP-binding protein		gene2772	-1063.5	8.70E-11
SA2981_RS14255			ABC transporter ATP-binding protein		gene2851	-325.8	1.60E-04
SA2981_RS00590	GO:0019290	*	siderophore biosynthesis protein SbnE	siderophore synthesis	gene117	22.3	1.00E-06
SA2981_RS00595			siderophore biosynthesis protein SbnF		gene118	42.8	2.60E-07
SA2981_RS02690	GO:0019538	*	ATP-dependent Clp protease ATP-binding subunit ClpC	protein metabolism and modification	gene538	21.9	8.50E-20

SA2981_RS04910			chaperone protein ClpB		gene982	36.7	5.70E-25
SA2981_RS07935	GO:0030420	*	hypothetical protein	establishment of competence for transfo	gene1589	-221.1	1.90E-03
SA2981_RS09160			competence protein ComK		gene1835	-1255.5	2.00E-08
SA2981_RS08125	GO:0051082	*	molecular chaperone DnaJ	unfolded protein binding	gene1627	16.8	2.50E-20
SA2981_RS08130			molecular chaperone DnaK		gene1628	9.7	7.70E-11
SA2981_RS00400	GO:0055114	*	tRNA-dihydrouridine synthase	oxidation reduction	gene79	11.7	3.40E-14
SA2981_RS00885			formate dehydrogenase	oxidoreductase process	gene176	35.6	1.40E-19
SA2981_RS01060			FMN-dependent NADH-azoreductase		gene211	16.3	9.70E-23
SA2981_RS01265			sorbitol dehydrogenase		gene252	36.9	1.80E-03
SA2981_RS01730			luciferase		gene345	10.8	4.90E-18
SA2981_RS01950			alkyl hydroperoxide reductase subunit C		gene389	14.6	7.20E-13
SA2981_RS02300			glutamate synthase subunit beta		gene459	10.4	1.60E-17
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	138.9	1.40E-38
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	18.9	1.10E-14
SA2981_RS04820			NADH-dependent flavin oxidoreductase		gene964	12.8	5.30E-20
SA2981_RS05740			monooxygenase IsdI		gene1148	-42.1	4.40E-12
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2513	17.9	2.10E-19
SA2981_RS12995			hypothetical protein		gene2599	14.9	2.30E-03
SA2981_RS13510			diapolycopene oxygenase		gene2702	-30.8	2.30E-31
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	446.8	1.10E-05
SA2981_RS13805			ribonucleoside-triphosphate reductase		gene2761	-30.4	6.50E-20
SA2981_RS14145			histidinol dehydrogenase		gene2829	-70.2	1.20E-02

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Gene Ontology assignments on exposing *S. aureus* 04-02981 to AGXX® for 120 minutes

Locus Tag	GO	Rate	Gene product	Description	gene number	Fold change	p- value
SA2981_RS14115	GO:0000105	*	bifunctional phosphoribosyl-AMP cyclohydrolase/phos	histidine biosynthesis	gene2823	13.6	5.30E-17
SA2981_RS14150			ATP phosphoribosyltransferase HisG		gene2830	29.4	1.60E-04
SA2981_RS14155			ATP phosphoribosyltransferase regulatory subunit (hisZ)		gene2831	-246.9	8.10E-04
SA2981_RS00350	GO:0000166	*	potassium-transporting ATPase subunit B	nucleotide binding	gene69	10.9	5.00E-02
SA2981_RS02710			glutamate--tRNA ligase		gene542	10.7	9.00E-17
SA2981_RS04995			peptide ABC transporter ATP-binding protein		gene999	14.3	1.50E-06
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	590	1.50E-11
SA2981_RS06065			isoleucine-tRNA ligase (ileS)		gene1213	16	1.90E-22
SA2981_RS08055			glycine-tRNA ligase		gene1613	8.3	1.10E-09
SA2981_RS00225	GO:0003677	*	hypothetical protein	DNA binding	gene44	8.3	6.20E-11
SA2981_RS00285			recombinase RecB		gene56	11.4	1.30E-02
SA2981_RS00330			DNA-binding response regulator		gene65	-355.5	3.50E-06
SA2981_RS00415			hypothetical protein		gene82	17.2	4.90E-12
SA2981_RS01345			GntR family transcriptional regulator		gene268	-46.5	1.40E-36
SA2981_RS01785			transcriptional regulator		gene356	-1724.5	3.30E-12
SA2981_RS02675			transcriptional regulator CtsR		gene535	10.4	1.00E-12
SA2981_RS04100			cold-shock protein		gene820	-16	8.10E-07
SA2981_RS06365			DNA topoisomerase 1		gene1273	11.9	3.80E-18
SA2981_RS06675			XRE family transcriptional regulator		gene1335	-6825.3	9.50E-25
SA2981_RS06690			XRE family transcriptional regulator		gene1338	-29.8	2.10E-12
SA2981_RS09155			RNA polymerase sigma factor SigS		gene1834	-2127.5	1.20E-15
SA2981_RS09300			transposase		gene1862	-1331.1	1.10E-11
SA2981_RS11675			DNA-directed RNA polymerase subunit alpha		gene2335	8.6	2.00E-11
SA2981_RS13585			TetR family transcriptional regulator		gene2717	47.5	5.50E-29
SA2981_RS01345	GO:0003700	*	GntR family transcriptional regulator	sequence-specific DNA binding transcription factor activity	gene268	-46.5	1.40E-36
SA2981_RS02290			LysR family transcriptional regulator	transcription factor activity	gene457	-92.7	7.40E-13
SA2981_RS08000			transcriptional repressor		gene1602	131.9	1.10E-39
SA2981_RS09155			RNA polymerase sigma factor SigS		gene1834	-2127.5	1.20E-15
SA2981_RS11255			transcriptional regulator		gene2251	17.2	5.80E-22
SA2981_RS12240			LysR family transcriptional regulator		gene2448	-11303.5	1.30E-35
SA2981_RS06830	GO:0003735	*	30S ribosomal protein S14	ribosomal protein	gene1366	18.1	4.50E-24
SA2981_RS08470			50S ribosomal protein L21	ribosomal RNA structural constituent of ribosome	gene1697	8.7	6.10E-11
SA2981_RS11670			50S ribosomal protein L17		gene2334	12.5	8.30E-14
SA2981_RS11725			50S ribosomal protein L18		gene2345	8.7	4.40E-12
SA2981_RS11735			30S ribosomal protein S8		gene2347	10.9	5.90E-13
SA2981_RS11740			30S ribosomal protein S14 type Z (rspN)		gene2348	18.2	1.50E-16
SA2981_RS00400	GO:0003824		tRNA-dihydrouridine synthase	enzyme activity	gene79	23.1	1.00E-20
SA2981_RS00605			diaminopimelate decarboxylase	catalytic activity	gene120	15.7	2.60E-06
SA2981_RS00755			capsular polysaccharide biosynthesis protein Cap8C		gene150	-52.9	2.10E-32
SA2981_RS01170			3-hydroxyacyl-CoA dehydrogenase		gene233	-15.3	9.20E-05
SA2981_RS02295			glutamate synthase		gene458	9.7	2.00E-12
SA2981_RS03975			epimerase		gene794	12.3	4.10E-20
SA2981_RS05415			phosphoribosylamine-glycine ligase		gene1083	16.2	1.20E-21
SA2981_RS06835			guanosine monophosphate reductase		gene1367	15.5	1.20E-15
SA2981_RS07015			phosphoribosylanthranilate isomerase		gene1403	-179.5	1.60E-03
SA2981_RS08935			acetate-CoA ligase		gene1790	-48.4	1.10E-33
SA2981_RS10735			dihydroxy-acid dehydratase		gene2148	11.4	1.00E-10
SA2981_RS10755			2-isopropylmalate synthase		gene2152	50.4	7.50E-38
SA2981_RS12850			epimerase		gene2570	8.6	1.70E-13
SA2981_RS14065			capsular polysaccharide biosynthesis protein Cap8C		gene2813	-12.9	5.40E-06
SA2981_RS14140			histidinol-phosphate aminotransferase		gene2828	196.1	5.90E-07
SA2981_RS10765	GO:0003861	**	3-isopropylmalate dehydratase large subunit	(2R,3S)-3-isopropyl	gene2154	8.8	1.00E-08
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	29.8	2.10E-19
SA2981_RS08745	GO:0003989	*	acetyl-CoA carboxylase carboxyltransferase subunit alp	acetyl coenzyme A c	gene1752	32	1.40E-33
SA2981_RS08750			acetyl-CoA carboxylase carboxyltransferase subunit beta		gene1753	10.2	4.80E-13
SA2981_RS00755	GO:0004725	*	capsular polysaccharide biosynthesis protein Cap8C	phosphoprotein phos	gene150	-52.9	2.10E-32
SA2981_RS14065			capsular polysaccharide biosynthesis protein Cap8C		gene2183	-12.9	5.40E-06
SA2981_RS02710	GO:0004812	*	glutamate-tRNA ligase	aminoacyl-tRNA syn	gene542	10.7	9.00E-17
SA2981_RS06065			isoleucine-tRNA ligase		gene1232	16	1.90E-22
SA2981_RS08055			glycine-tRNA ligase		gene1613	8.3	1.10E-09
SA2981_RS01250	GO:0005215	*	PTS sugar transporter subunit IIA	small-molecule carri	gene249	276.3	2.90E-03
SA2981_RS01605			hypothetical protein	transporter activity	gene320	-35.1	4.60E-24
SA2981_RS03780			iron ABC transporter permease		gene755	8.4	4.40E-04
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	12.5	5.90E-11
SA2981_RS06615			glycerol transporter		gene1323	-12.5	1.70E-19
SA2981_RS11430			iron-dicitrate ABC transporter permease (fecD)		gene2286	9.2	2.00E-10
SA2981_RS00350	GO:0005524	*	potassium-transporting ATPase subunit B	small-molecule carri	gene69	10.9	5.00E-02
SA2981_RS00710			phosphonates import ATP-binding protein PhnC	transporter activity	gene141	-104.9	4.20E-03
SA2981_RS00860			sulfonate ABC transporter ATP-binding protein		gene171	12.3	1.00E-19
SA2981_RS02690			ATP-dependent Clp protease ATP-binding subunit ClpC		gene538	10.7	2.50E-13

SA2981_RS02710			glutamate--tRNA ligase		gene542	10.7	9.00E-17
SA2981_RS03785			iron ABC transporter ATP-binding protein		gene756	14.8	6.90E-09
SA2981_RS04910			chaperone protein ClpB		gene982	24.4	4.50E-21
SA2981_RS04995			peptide ABC transporter ATP-binding protein		gene999	14.3	1.50E-06
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	590	1.50E-11
SA2981_RS05210			bacteriocin ABC transporter ATP-binding protein		gene1042	-12.8	1.40E-07
SA2981_RS05415			phosphoribosylamine-glycine ligase		gene1083	16.2	1.20E-21
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	12.5	5.90E-11
SA2981_RS06065			isoleucine-tRNA ligase (ileS)		gene1213	16	1.90E-22
SA2981_RS07950			hypothetical protein		gene1592	349.1	6.70E-04
SA2981_RS08005			iron ABC transporter permease		gene1603	26.8	2.00E-28
SA2981_RS08010			zinc ABC transporter ATP-binding protein		gene1604	14	6.50E-19
SA2981_RS08020			DEAD/DEAH box family ATP-dependent RNA helicase		gene1606	13.3	5.00E-16
SA2981_RS08055			glycine-tRNA ligase		gene1613	8.3	1.10E-09
SA2981_RS08125			molecular chaperone DnaJ		gene1627	10.4	2.30E-15
SA2981_RS09830			multidrug ABC transporter ATP-binding protein		gene1969	20.6	1.20E-18
SA2981_RS12655			arginine ABC transporter ATP-binding protein		gene2531	8.9	2.50E-11
SA2981_RS12750			ATP-dependent dethiobiotin synthetase		gene2550	-99.4	1.80E-02
SA2981_RS12770			multidrug ABC transporter permease		gene2554	-15.4	1.30E-05
SA2981_RS12925			methionine ABC transporter ATP-binding protein		gene2585	-19.9	5.80E-22
SA2981_RS13240			lantibiotic ABC transporter ATP-binding protein		gene2648	13.6	9.00E-18
SA2981_RS13305			peptide ABC transporter ATP-binding protein		gene2661	-1796.7	5.60E-19
		*					
SA2981_RS02810	GO:0005622		elongation factor Tu	intracellular	gene562	8.2	3.00E-07
SA2981_RS06830			30S ribosomal protein S14		gene1366	18.1	4.50E-24
SA2981_RS08470			50S ribosomal protein L21		gene1697	8.7	6.10E-11
SA2981_RS11670			50S ribosomal protein L17		gene2334	12.5	8.30E-14
SA2981_RS11725			50S ribosomal protein L18		gene2345	8.7	4.40E-12
SA2981_RS11740			30S ribosomal protein S14 type Z (rpsN)		gene2348	18.2	1.50E-16
SA2981_RS02710	GO:0005737	*	glutamate-tRNA ligase	cytoplasm	gene542	10.7	9.00E-17
SA2981_RS03745			7-cyano-7-deazaguanine reductase		gene748	14.6	4.50E-22
SA2981_RS04910			chaperone protein ClpB		gene982	24.4	4.50E-21
SA2981_RS06065			isoleucine-tRNA ligase (ileS)		gene1213	16	1.90E-22
SA2981_RS08055			glycine-tRNA ligase		gene1613	8.3	1.10E-09
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	22.3	5.80E-22
SA2981_RS12230			imidazolonepropionase		gene2446	-28	2.40E-30
SA2981_RS12860			2-dehydropantoate 2-reductase		gene2572	11	2.30E-15
SA2981_RS14150			ATP phosphoribosyltransferase (hisG)		gene2830	29.4	1.60E-04
SA2981_RS14155			ATP phosphoribosyltransferase regulatory subunit (hisZ)		gene2831	-246.9	8.10E-04
SA2981_RS14295			ribosomal RNA small subunit methyltransferase G		gene2859	9.8	2.80E-14
SA2981_RS06830	GO:0005840	*	30S ribosomal protein S14	ribosomal RNA	gene1366	18.1	4.50E-24
SA2981_RS08470			50S ribosomal protein L21		gene1697	8.7	6.10E-11
SA2981_RS11670			50S ribosomal protein L17		gene2334	12.5	8.30E-14
SA2981_RS11725			50S ribosomal protein L18		gene2345	8.7	4.40E-12
SA2981_RS11735			30S ribosomal protein S8		gene2347	10.9	5.90E-13
SA2981_RS11740			30S ribosomal protein S14 type Z (rspN)		gene2348	18.2	1.50E-16
SA2981_RS00225	GO:0006355	*	hypothetical protein	regulation of cellula	gene44	8.3	6.20E-11
SA2981_RS00330			DNA-binding response regulator		gene65	-355.5	3.50E-06
SA2981_RS00415			hypothetical protein		gene82	17.2	4.90E-12
SA2981_RS01345			GntR family transcriptional regulator		gene268	-46.5	1.40E-36
SA2981_RS02290			LysR family transcriptional regulator		gene457	-92.7	7.40E-13
SA2981_RS02675			transcriptional regulator CtsR		gene535	10.4	1.00E-12
SA2981_RS04100			cold-shock protein		gene820	-16	8.10E-07
SA2981_RS06945			protein GlcT		gene1389	-59.7	1.80E-24
SA2981_RS08000			transcriptional repressor		gene1602	131.9	1.10E-39
SA2981_RS09155			RNA polymerase sigma factor SigS		gene1834	-2127.5	1.20E-15
SA2981_RS11255			transcriptional regulator		gene2251	17.2	5.80E-22
SA2981_RS12240			LysR family transcriptional regulator		gene2448	-11303.5	1.30E-35
SA2981_RS06830	GO:0006412	*	30S ribosomal protein S14	protein biosynthesis	gene1366	18.1	4.50E-24
SA2981_RS08470			50S ribosomal protein L21	translation	gene1697	8.7	6.10E-11
SA2981_RS11670			50S ribosomal protein L17		gene2334	12.5	8.30E-14
SA2981_RS11725			50S ribosomal protein L18		gene2345	8.7	4.40E-12
SA2981_RS11735			30S ribosomal protein S8		gene2347	10.9	5.90E-13
SA2981_RS11740			30S ribosomal protein S14 type Z (rspN)		gene2348	18.2	1.50E-16
SA2981_RS02710	GO:0006418	*	glutamate-tRNA ligase	tRNA charging	gene542	10.7	9.00E-17
SA2981_RS06065			isoleucine-tRNA ligase (ileS)		gene1213	16	1.90E-22
SA2981_RS08055			glycine-tRNA ligase		gene1613	8.3	1.10E-09
SA2981_RS02295	GO:0006537	*	glutamate synthase	glutamate synthesis	gene458	9.7	2.00E-12
SA2981_RS02300			glutamate synthase subunit beta		gene459	47.4	4.30E-38
SA2981_RS01080	GO:0006810	*	arabinogalactan ABC transporter permease	transport accessory p	gene215	-12.4	5.90E-09
SA2981_RS01250			PTS sugar transporter subunit IIA	transport	gene249	276.3	2.90E-03
SA2981_RS01605			hypothetical protein	small molecule trans	gene320	-35.1	4.60E-24
SA2981_RS05005			peptide ABC transporter permease		gene1001	247.5	9.00E-14
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	12.5	5.90E-11
SA2981_RS06615			glycerol transporter		gene1323	-12.5	1.70E-19
SA2981_RS07950			hypothetical protein		gene1592	349.1	6.70E-04
SA2981_RS08005			iron ABC transporter permease		gene1603	26.8	2.00E-28
SA2981_RS09830			multidrug ABC transporter ATP-binding protein		gene1969	20.6	1.20E-18
SA2981_RS12770			multidrug ABC transporter permease		gene2554	-15.4	1.30E-05
SA2981_RS12870			amino acid ABC transporter permease		gene2574	9.3	3.00E-17
SA2981_RS00345	GO:0006813	*	potassium-transporting ATPase subunit A	potassium transport	gene68	43.6	1.30E-02
SA2981_RS00350			potassium-transporting ATPase subunit B		gene69	10.9	5.00E-02
SA2981_RS00835	GO:0008152	*	aldehyde dehydrogenase	metabolic process	gene166	-15.7	7.40E-21

SA2981_RS00885			formate dehydrogenase	metabolism	gene177	25	2.50E-17
SA2981_RS01170			3-hydroxyacyl-CoA dehydrogenase		gene233	-15.3	9.20E-05
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234	-23.2	1.60E-06
SA2981_RS01185			acyl CoA:acetate/3-ketoacid CoA transferase		gene236	-23.2	2.40E-18
SA2981_RS02295			glutamate synthase		gene458	9.7	2.00E-12
SA2981_RS07000			glutamine amidotransferase		gene1400	1600.4	2.90E-08
SA2981_RS07015			phosphoribosylanthranilate isomerase		gene1403	-179.5	1.60E-03
SA2981_RS08935			acetate-CoA ligase		gene1790	-48.4	1.10E-33
SA2981_RS10735			dihydroxy-acid dehydratase		gene2148	11.4	1.00E-10
SA2981_RS10745			acetolactate synthase		gene2150	10.9	2.40E-08
SA2981_RS10765			3-isopropylmalate dehydratase large subunit		gene2154	8.8	1.00E-08
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	29.8	2.10E-19
SA2981_RS12225			N-acetyl-L 2CL-diaminopimelate deacetylase		gene2445	11	3.40E-11
SA2981_RS14140			histidinol-phosphate aminotransferase		gene2828	196.1	5.90E-07
SA2981_RS00345	GO:0008556	*	potassium-transporting ATPase subunit A	potassium-uptake-A	gene68	43.6	1.30E-02
SA2981_RS00350			potassium-transporting ATPase subunit B		gene69	10.9	5.00E-02
SA2981_RS10735	GO:0009082	*	dihydroxy-acid dehydratase	branched chain fami	gene2148	11.4	1.00E-10
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	9.1	2.70E-10
SA2981_RS10755	GO:0009098	***	2-isopropylmalate synthase	leucine biosynthesis	gene2152	50.4	7.50E-38
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	22.3	5.80E-22
SA2981_RS10765			3-isopropylmalate dehydratase large subunit		gene2154	8.8	1.00E-08
SA2981_RS10770			3-isopropylmalate dehydratase small subunit (leuD)		gene2155	29.8	2.10E-19
SA2981_RS08745	GO:0009317	*	acetyl-CoA carboxylase carboxyltransferase subunit alp	acetyl coA-carboxylase complex	gene1752	32	1.40E-33
SA2981_RS08750			acetyl-CoA carboxylase carboxyltransferase subunit beta		gene1753	10.2	4.80E-13
SA2981_RS02035	GO:0009405	*	hypothetical protein	pathogenesis	gene406	-12.6	2.00E-07
SA2981_RS05920			hypothetical protein	virulence	gene1184	-15.3	2.30E-03
SA2981_RS05965			hypothetical protein		gene1193	-12.4	6.00E-08
SA2981_RS05970			hypothetical protein		gene1194	-22.7	9.70E-08
SA2981_RS10270			staphylokinase		gene2057	18.5	7.00E-25
SA2981_RS10635			accessory gene regulator protein B		gene2128	-40.8	1.90E-18
SA2981_RS12710			gamma-hemolysin component A		gene2542	-16.8	4.60E-05
SA2981_RS04910	GO:0009408	*	chaperone protein ClpB	response to heat sho	gene982	24.4	4.50E-21
SA2981_RS08125			molecular chaperone DnaJ		gene1627	10.4	2.30E-15
SA2981_RS04995	GO:0015833	*	peptide ABC transporter ATP-binding protein	peptide transport	gene999	14.3	1.50E-06
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000	590	1.50E-11
SA2981_RS00710	GO:0016020	*	phosphonates import ATP-binding protein PhnC	membrane	gene141	-104.9	4.20E-03
SA2981_RS00745			capsular polysaccharide type 5 biosynthesis protein Cap5A		gene148	-61.9	4.60E-30
SA2981_RS00965			permease		gene192	-14.8	2.80E-14
SA2981_RS01080			arabinogalactan ABC transporter permease		gene215	-12.4	5.90E-09
SA2981_RS01575			peptide ABC transporter permease		gene314	-18.9	9.50E-25
SA2981_RS01605			hypothetical protein		gene320	-35.1	4.60E-24
SA2981_RS03780			iron ABC transporter permease		gene755	8.4	4.40E-04
SA2981_RS05005			peptide ABC transporter permease		gene1001	247.5	9.00E-14
SA2981_RS05335			quinol oxidase subunit 3		gene1067	9.6	5.50E-10
SA2981_RS06345			cell wall hydrolase		gene1269	22.8	5.20E-08
SA2981_RS06615			glycerol transporter		gene1323	-12.5	1.70E-19
SA2981_RS08005			iron ABC transporter permease		gene1603	26.8	2.00E-28
SA2981_RS10610			CAAX amino protease		gene2123	-24.7	2.30E-27
SA2981_RS11430			iron-dicitrate ABC transporter permease (fecD)		gene2286	9.2	2.00E-10
SA2981_RS12870			amino acid ABC transporter permease		gene2574	9.3	3.00E-17
SA2981_RS13145			hypothetical protein		gene2629	20.4	1.80E-13
SA2981_RS14105			lipase		gene2821	-13.8	1.00E-19
SA2981_RS00400	GO:0016491	*	tRNA-dihydrouridine synthase	redox activiy	gene79	23.1	1.00E-20
SA2981_RS00835			aldehyde dehydrogenase	oxidoreductase	gene166	-15.7	7.40E-21
SA2981_RS01170			3-hydroxyacyl-CoA dehydrogenase		gene233	-15.3	9.20E-05
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase		gene254	8.5	1.00E-07
SA2981_RS02295			glutamate synthase		gene458	9.7	2.00E-12
SA2981_RS02300			glutamate synthase subunit beta		gene459	47.4	4.30E-38
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	183.1	2.50E-41
SA2981_RS03605			glyoxal reductase		gene720	-19	4.40E-19
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	13.9	5.20E-13
SA2981_RS06835			guanosine monophosphate reductase		gene1367	15.5	1.20E-15
SA2981_RS09130			pyridine nucleotide-disulfide oxidoreductase		gene1829	-30.7	5.80E-30
SA2981_RS10620			nitroreductase		gene2125	9.5	1.30E-13
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	9.1	2.70E-10
SA2981_RS12585			nitrite reductase NAD(P)H small subunit		gene2517	10.1	2.40E-10
SA2981_RS12860			2-dehydropantoate 2-reductase		gene2572	11	2.30E-15
SA2981_RS13510			diapolycopene oxygenase		gene2702	-14.8	8.40E-22
SA2981_RS13590			short-chain dehydrogenase		gene2718	9.6	2.70E-09
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	878.6	3.30E-07
SA2981_RS13805			ribonucleoside-triphosphate reductase		gene2761	-29	1.20E-19
SA2981_RS00710	GO:0016887	*	phosphonates import ATP-binding protein PhnC	ATP hydrolase activ	gene141	-104.9	4.20E-03
SA2981_RS00860			sulfonate ABC transporter ATP-binding protein	ATPase activity	gene171	12.3	1.00E-19
SA2981_RS03785			iron ABC transporter ATP-binding protein		gene756	14.8	6.90E-09
SA2981_RS04995			peptide ABC transporter ATP-binding protein		gene999	14.3	1.50E-06
SA2981_RS05000			peptide ABC transporter substrate-binding protein		gene1000		
SA2981_RS05210			bacteriocin ABC transporter ATP-binding protein		gene1042	-12.8	1.40E-07
SA2981_RS05550			spermidine/putrescine import ATP-binding protein PotA		gene1110	12.5	5.90E-11
SA2981_RS08010			zinc ABC transporter ATP-binding protein		gene1604	14	6.50E-19
SA2981_RS09830			multidrug ABC transporter ATP-binding protein		gene1969	20.6	1.20E-18

SA2981_RS12655			arginine ABC transporter ATP-binding protein		gene2531	8.9	2.50E-11
SA2981_RS12770			multidrug ABC transporter permease		gene2554	-15.4	1.30E-05
SA2981_RS12925			methionine ABC transporter ATP-binding protein		gene2585	-19.9	5.80E-22
SA2981_RS13240			lantibiotic ABC transporter ATP-binding protein		gene2648	13.6	9.00E-18
SA2981_RS13305			peptide ABC transporter ATP-binding protein		gene2661	-1796.7	5.60E-19
SA2981_RS02690	GO:0019538	*	ATP-dependent Clp protease ATP-binding subunit Clp	protein metabolism a	gene538	10.7	2.50E-13
SA2981_RS04910			chaperone protein ClpB		gene982	24.4	4.50E-21
SA2981_RS00755	GO:0030145	*	capsular polysaccharide biosynthesis protein Cap8C	manganese binding	gene150	-52.9	2.10E-32
SA2981_RS14065					gene2813		
SA2981_RS07935	GO:0030420	*	hypothetical protein	establishment of competence for transformation	gene1589	-205.6	1.80E-03
SA2981_RS09160			competence protein ComK		gene1835	-1764.2	2.00E-09
SA2981_RS08005	GO:0042626	*	iron ABC transporter permease	ABC-type efflux por	gene1603		
SA2981_RS09830			multidrug ABC transporter ATP-binding protein	ABC-type efflux per	gene1969		
SA2981_RS12770			multidrug ATP transporter permease		gene2554		
SA2981_RS02455	GO:0042710	**	hypothetical protein	biofilm formation	gene491	-95.4	1.90E-23
SA2981_RS14085			poly-beta-2C6 N-acetyl-D-glucosamine synthase		gene2817	26.5	5.60E-07
SA2981_RS05330	GO:0042773	*	quinol oxidase subunit 4	ATP synthesis coupled electron transport	gene1066	9.7	4.60E-08
SA2981_RS05335			quinol oxidase subunit 3		gene1067	9.6	5.50E-10
SA2981_RS00225	GO:0046872	*	hypothetical protein	metal binding	gene44		
SA2981_RS00350			potassium transporting ATPase-subunit B	heavy metal binding	gene69		
SA2981_RS00415			hypothetical protein		gene82		
SA2981_RS03230			manganese ABC transporter substrate-binding protein		gene645	-16.4	1.60E-16
SA2981_RS00400	GO:0050660	*	tRNA-dihydrouridine synthase	flavine-adenine dinu	gene79		
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234		
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607		
SA2981_RS08125	GO:0051082	*	molecular chaperone DnaJ	chaperone activity	gene1627		
SA2981_RS12565			nitrate reductase subunit delta	binding unfolded EF	gene2513	10.9	9.20E-09
SA2981_RS00400	GO:0055114	*	tRNA-dihydrouridine synthase	oxidoreductase proc	gene79	23.1	1.00E-20
SA2981_RS00835			aldehyde dehydrogenase	metabolic process	gene166	-15.7	7.40E-21
SA2981_RS00885			formate dehydrogenase	metabolism	gene177	25	2.50E-17
SA2981_RS01060			FMN-dependent NADH-azoreductase			8.5	2.70E-15
SA2981_RS01170			3-hydroxyacyl-CoA dehydrogenase		gene233	-15.3	9.20E-05
SA2981_RS01175			glutaryl-CoA dehydrogenase		gene234	-23.2	1.60E-06
SA2981_RS01275			galactitol-1-phosphate 5-dehydrogenase		gene254	8.5	1.00E-07
SA2981_RS02295			glutamate synthase		gene458	9.7	2.00E-12
SA2981_RS02300			glutamate synthase subunit beta		gene459	47.4	4.30E-38
SA2981_RS03035			dihydrolipoamide dehydrogenase		gene607	183.1	2.50E-41
SA2981_RS03605			glyoxal reductase		gene720	-19	4.40E-19
SA2981_RS03745			7-cyano-7-deazaguanine reductase		gene748	14.6	4.50E-22
SA2981_RS04765			pyridine nucleotide-disulfide oxidoreductase		gene953	13.9	5.20E-13
SA2981_RS05740			monooxygenase IsdI			-1312.9	1.10E-14
SA2981_RS06835			guanosine monophosphate reductase		gene1367	15.5	1.20E-15
SA2981_RS08680			glyceraldehyde-3-phosphate dehydrogenase			-23.3	9.70E-21
SA2981_RS09130			pyridine nucleotide-disulfide oxidoreductase		gene1829	-30.7	
SA2981_RS10750			ketol-acid reductoisomerase		gene2151	9.1	2.70E-10
SA2981_RS10760			3-isopropylmalate dehydrogenase		gene2153	22.3	5.80E-22
SA2981_RS12585			nitrite reductase NAD(P)H small subunit		gene2517	10.1	2.40E-10
SA2981_RS12860			2-dehydropantoate 2-reductase		gene2572	11	2.30E-15
SA2981_RS13510			diapolycopene oxygenase		gene2702	-14.8	8.40E-22
SA2981_RS13615			Baeyer-Villiger flavin-containing monooxygenase		gene2723	878.6	3.30E-07
SA2981_RS13805			ribonucleoside-triphosphate reductase		gene2761	-29	1.20E-19
SA2981_RS13835			glutathione peroxidase		gene2767	16.9	5.30E-20
SA2981_RS14055			methionine sulfoxide reductase A		gene2811	-864.7	1.70E-10

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Supplementary Table 8. Expression of operons in *S. aureus* 04-02981 on exposure to Ag for 80 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
SA2981_RS00310	**operon_0038**	**	transposase				gene61
SA2981_RS00315			DNA-binding protein		350.1	3.90E-03	gene62
SA2981_RS00320			transposase		155.4	4.50E-02	gene63
SA2981_RS00570	**operon_0067**	*	siderophore biosynthesis protein SbnA		50.3	2.40E-03	gene113
SA2981_RS00575			2-2C3-diaminopropionate biosynthesis protein SbnB		211.3	1.00E-02	gene114
SA2981_RS00580			siderophore biosynthesis protein SbnC		-31.2	1.40E-02	gene115
SA2981_RS00585			siderophore biosynthesis protein SbnD				gene116
SA2981_RS00590			siderophore biosynthesis protein SbnE				gene117
SA2981_RS00595			siderophore biosynthesis protein SbnF				gene118
SA2981_RS00600			siderophore biosynthesis protein SbnG				gene119
SA2981_RS00605			diaminopimelate decarboxylase				gene120
SA2981_RS00610			siderophore biosynthesis protein SbnI				gene121
SA2981_RS00625	**operon_0070**	**	diacetyl reductase ((S)-acetoin forming)				gene124
SA2981_RS00635			NAD-dependent dehydratase				gene126
SA2981_RS00640			UDP-phosphate N-acetylgalactosaminyl-1-phosphate transferase		15.2	4.20E-02	gene127
SA2981_RS04455	**operon_0433**	**	terminase				gene891
SA2981_RS04460			terminase				gene892
SA2981_RS04465			phage portal protein				gene893
SA2981_RS04470			phage head morphogenesis protein				gene894
SA2981_RS04480			phage capsid protein				gene896
SA2981_RS04485			major capsid protein				gene897
SA2981_RS04495			phage head-tail adapter protein				gene899
SA2981_RS04515			tail protein				gene903
SA2981_RS04535			phage tail protein				gene907
SA2981_RS04540			peptidase				gene908
SA2981_RS04545			minor structural protein				gene909
SA2981_RS04570			cell wall hydrolase				gene914
SA2981_RS04575			tail protein				gene915
SA2981_RS04585			phage holin				gene917
SA2981_RS04590			amidase				gene918
SA2981_RS06095	**operon_0589**	*	uracil permease				gene1219
SA2981_RS06100			aspartate carbamoyltransferase				gene1220
SA2981_RS06105			dihydroorotase				gene1221
			carbamoyl-phosphate synthase small chain	catalyzes production of carbamoyl phosphate from bicarbonate and glutamine in pyrimidine and arginine biosynthesis pathways 3B forms an octamer composed of four CarAB dimers			
SA2981_RS06110					-3.8	1.00E-03	gene1222
SA2981_RS06115			carbamoyl-phosphate synthase large chain				gene1223
SA2981_RS06120			orotidine 5'-phosphate decarboxylase	type 1 subfamily 3B involved in last step of pyrimidine biosynthesis 3B converts orotidine 5'-phosphate to UMP and carbon dioxide 3B OMP decarboxylase 3B OMPDCase 3B OMPdecase			gene1224
SA2981_RS06125			orotate phosphoribosyltransferase		-2.8	4.60E-02	gene1225
SA2981_RS07375	**operon_0711**	**	amino acid permease		-3.3	1.10E-03	gene1476
SA2981_RS07380			serine/threonine dehydratase		-3.1	6.70E-03	gene1477
SA2981_RS07385			alanine dehydrogenase				gene1478
SA2981_RS10410	**operon_0982**	*	transcriptional activator RinB				gene2083
SA2981_RS10420			membrane protein				gene2085
SA2981_RS10450			Holliday junction DNA helicase				gene2091
SA2981_RS10460			replication protein DnaD				gene2093
SA2981_RS10465			single-stranded DNA-binding protein				gene2094
SA2981_RS10470			MBL fold metallo-hydrolase				gene2095

SA2981_RS13585	**operon_1312**	**	TetR family transcriptional regulator	20.6	1.60E-04	gene2717
SA2981_RS13590			short-chain dehydrogenase	-6.3	4.20E-04	gene2718
SA2981_RS13595			amidohydrolase			gene2719
SA2981_RS13600			hydrolase			gene2720

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Expression of operons in *S. aureus* 04-02981 on exposure to Ag for 120 minutes

Locus Tag	Operon	Rate	Gene product	Note	Fold change	p- value	Gene number
			transcriptional activator				
SA2981_RS10410	**operon_0982**	**	RinB		-25.2	6.60E-03	gene2083
SA2981_RS10420			membrane protein		-381.7	6.80E-05	gene2085
SA2981_RS10450			Holliday junction DNA helicase				gene2091
SA2981_RS10460			replication protein DnaD		-14.2	1.90E-03	gene2093
SA2981_RS10465			single-stranded DNA-binding protein				gene2094
SA2981_RS10470			MBL fold metallo-hydrolase				gene2095
SA2981_RS13620	**operon_1314**	**	type II secretion protein				gene2724
SA2981_RS13630			type VII secretion protein		-2.9	4.60E-02	gene2726

Rate= The rating values (1 to 5) reflect binned values based on: (TopHits/ClassSize) * -log2(adj-pvalue)

Manuscript #5

Research Paper

Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain

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Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain

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Abstract

Multi-drug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) cause nosocomial infections that can have deleterious effects on human health. Thus, it is imperative to find solutions to treat these detrimental infections as well as to control their spread. We tested the effect of two different antimicrobial materials, functionalised graphene oxide (GOX), and AGXX® coated on cellulose fibres, on the growth and transcriptome of the clinical MRSA strain *S. aureus* 04-02981. In addition, we investigated the effect of a third material as a combination of GOX and AGXX® fibres on *S. aureus* 04-02981. Standard plate count assay revealed that the combination of fibres, GOX-AGXX® inhibited the growth of *S. aureus* 04-02981 by 99.98%. To assess the effect of these antimicrobials on the transcriptome of our strain, cultures of *S. aureus* 04-02981 were incubated with GOX, AGXX®, or GOX-AGXX® fibres for different time periods and then subjected to RNA-sequencing. Uncoated cellulose fibres were used as an internal control. The antimicrobial fibres had a huge impact on the transcriptome of *S. aureus* 04-02981 affecting the expression of 2650 genes. Primarily genes related to biofilm formation and virulence (such as *agr, sarA*, and those of the two-component system SaeRS), and genes crucial for survival in biofilms (like arginine metabolism *arc* genes) were repressed. In contrast, the expression of siderophore biosynthesis genes (*sbn*) was induced, a probable response to stress imposed by the antimicrobials and the conditions of iron-deficiency. Genes associated with potassium transport, intracellular survival and pathogenesis (*kdp*) were also differentially expressed. Our data suggest that GOX, AGXX®, and their combination fibres act as efficient antimicrobials against *S. aureus* 04-02981 and are potential candidates for applications in antimicrobial surface coatings.

Keywords: MRSA, GOX, AGXX®, antimicrobial, biofilm, RNA-sequencing

Introduction

Multidrug resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) cause severe nosocomial infections and can develop resistance to all classes of antibiotics (Vestergaard et al., 2019). In addition, *S. aureus* can tolerate starvation, desiccation, and acid stress conditions on a variety of surfaces, making it extremely difficult to combat (Oie et al., 1996; Chaffin et al., 2012). MRSA was also listed as a high priority pathogen in the list published by the World Health Organization (WHO) in 2017 (Tacconelli et al., 2017, WHO, 2017). Nosocomial infections cause around 50,000 deaths per year in Europe (WHO report, 2015). Thus, pathogens, specifically multidrug resistant pathogens like MRSA continue to pose a serious threat to human health (Baptista et al., 2018). This notion has prompted researchers to develop novel antimicrobial strategies to stop the spread of infectious diseases. Some of these strategies include using heavy metal or carbon-based nanomaterials such as gold or silver nanoparticles, carbon nanotubes, and graphene-based antimicrobials (Baptista et al., 2018).

The crucial step in pathogenesis is bacterial adhesion to host cells (Stones and Krachler, 2016). Targeting this adhesion step using certain chemical or biological compounds is an approach that could aid in diminishing the cause and spread of diseases. Graphene oxide (GO) can be used to achieve this aim. GO is derived from graphene and has hydroxyl and epoxy groups on the basal surface and carboxyl groups on the edges. These functional groups impart hydrophilic and dispersing properties to GO making it suitable for medical applications (Liu et al., 2018). GO can readily adsorb metal or inorganic compounds due to its variety of functional groups (Liu et al., 2017). It has an antibacterial effect on Gram-negative pathogens such as *Pseudomonas aeruginosa* (Gurunathan et al., 2012) as well as on Gram-positive bacteria like the dental pathogens *Streptococcus mutans, Porphyromonas gingivalis,* and *Fusobacterium nucleatum* (He et al., 2015). This antibacterial effect is caused by graphene-like parts of GO wrapping the bacterial membranes or by producing reactive oxygen species (ROS) (Akhavan et al., 2011; Gurunathan et al., 2012; Liu et al., 2012; Liu et al., 2018). ROS create oxidative stress and cause damage to bacterial cell membranes, and macromolecules such as lipids, proteins and DNA, ultimately leading to cell death (Malis et al., 2019).

Apart from causing infections, bacterial pathogens also pose a great challenge by contaminating basic sources of water by for instance contaminating sanitary areas and thus

causing water-borne infections. Globally, more than 700 million people lack access to clean drinking water and a total of 1.8 billion people have to obtain their drinking water from sources that are at least temporarily contaminated with faecal matter (WHO report, 2015). Hence, there is an urgent need to develop new strategies to deal with the problem of infection and contamination by multidrug resistant biofilm-forming bacteria such as MRSA. We aim to develop a two-component antimicrobial coating on cellulose where component one is functionalised graphene oxide (GOX) and component two is a carrier material coated with the antimicrobial AGXX®. GO has previously been used in combination with other antimicrobial metals such as silver and titanium dioxide (Jin et al., 2017; Baptista et al., 2018; Jin et al., 2019). AGXX® consists of two transition metals, silver and ruthenium, and has been shown to serve as an antibacterial by generating ROS. It may also play a role in inhibiting biofilm formation in bacteria (Guridi et al., 2015; Clauss-Lendzian et al., 2018; Vaishampayan et al., 2018; Sobisch et al., 2019). The putative mechanism of this two-component antimicrobial coating consisting of GOX and AGXX® is as follows: First, component one, GOX (functionalised GO), binds free bacteria. Adsorption of bacteria (which have an overall negative charge) is ensured by modification of the GO layers by grafting polymers with cationic groups. These flexible and micrometre sized GOX sheets multivalently bind and capture bacteria cells via electrostatic attraction. Second, AGXX® as component two catalytically produces ROS, which will kill the captured bacteria.

In this study, several antimicrobial materials were tested consisting of GOX and AGXX® coated on cellulose fibres. To understand the molecular mechanism of GOX and AGXX®, *S. aureus* 04-02981 (MRSA) was exposed to GOX, AGXX®, or the combination of both GOX and AGXX® and the bacterial response was investigated via RNA sequencing. Our data suggest that GOX, AGXX® and their combination can be considered as candidate antimicrobials as they efficiently inhibit bacterial growth. They mainly affect the expression of genes involved in pathogenesis and virulence and those associated with biofilm formation and metabolism essential for survival in biofilms.

Materials and Methods

Preparation of GOX and AGXX® fibres.

Methylene bisphenyl di-isocyanate (0.1 mg) was dissolved in 1 mL dry dimethyl formamide (DMF). Cellulose fibres (100 mg) were dispersed in 1 mL di-isocyanate/ DMF solution for 15 min. In a separate flask, 10 mg of GOX was dispersed in 9 mL dry DMF. GOX solution was added to the fibres and allowed to react at room temperature for 24 h under constant stirring. The functionalised fibres were washed 10 times by centrifugation at 4000 rpm (Heraeus™ Megafuge 8 benchtop centrifuge) for 5 min to get rid of unattached GOX, di-isocyanate and DMF and lyophilised to obtain dry, functionalised GOX cellulose fibres.

AGXX® fibres were prepared by chemical reduction of silver and ruthenium as previously described (Clauss-Lendzian et al. 2017; Vaishampayan et al. 2018) and then chemically coated on cellulose fibres (patent pending).

Inhibition assay

Different amounts of GOX and AGXX® coated on cellulose fibres of 20x700 µm (Vitacel) and fleece were tested against *S. aureus* 04-02981 (MRSA). 10, 20, 30, 50, and 100 mg of uncoated cellulose, GOX, AGXX®, or GOX-AGXX® were tested. To this end, MRSA was grown overnight in Tryptic Soy Broth (TSB; Carl Roth GmbH and Co. KG, Karlsruhe, Germany) without NaCl. The culture was diluted to ~10^4 colony forming units (CFU) mL^{-1}, after which either uncoated cellulose fibres, or GOX, or AGXX®, or GOX-AGXX® was added to the *S. aureus* 04-02981 culture. The cultures were incubated for 5 h at 37°C with shaking (150 rpm), serially diluted and plated on Tryptic Soy agar (1.5%) plates. After 16-24 h at 37°C the CFU per mL were determined.

RNA extraction

S. aureus 04-02981 was exposed to GOX, AGXX®, and the combination of the two materials and uncoated cellulose fibres as negative control for different time periods, after which the bacterial response was investigated via RNA sequencing. For this purpose, overnight cultures of *S. aureus* 04-02981 were diluted to an optical density at 600 nm (OD_{600}) of 0.05 and then incubated at 37°C, 150 rpm until the mid-exponential phase (4h). Subsequently, either cellulose (30 mg fibres/ 30 mL culture) or GOX (30 mg fibres/ 30 mL culture) or AGXX® (15 mg fibres/ 30 mL culture) or GOX-AGXX® fibres (30 mg GOX fibres plus 15 mg AGXX® fibres/ 30 mL culture) were added to the culture. The cultures were incubated at 37°C, 150 rpm for 0, 30, 60, 120, or 180 min. The cells were harvested by

centrifugation at 10000 rpm in a Heraeus Multifuge X3R Centrifuge (Thermo Electron LED GmbH, Osterode am Harz, Germany). Cell pellets were immediately frozen in liquid nitrogen and stored at -80°C or directly used for RNA extraction using the ZR Fungal/Bacterial RNA MiniPrep™ Kit (ZymoResearch, Freiburg, Germany) following the manufacturer's instructions. To recover total RNA including small RNAs, 1.5 volumes of absolute ethanol were added in step 5 of the protocol. Total RNA was eluted with 50 µL DNase- and RNase-free water and stored at -80°C. RNA quantity and quality were assessed with a NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Scientific, Osterode am Harz, Germany) as well as on agarose gels. Residual contaminating DNA was eliminated with TURBO DNA-free™ Kit Ambion according to the protocol (Life Technologies, Darmstadt, Germany).

RNA sequencing

RNA sequencing was performed by Novogene Company LTD., Cambridge, United Kingdom. The protocol was performed using the following steps; quality control by Agilent 2100; rRNA removal by Ribo-Zero rRNA Removal Kit (Bacteria) (Illumina), followed by strand-specific cDNA library preparation, templating, enrichment and sequencing via Illumina sequencer.

RNA-sequencing data analysis

Raw sequencing reads were aligned to the reference genome of *S. aureus* 04-02981 using Bowtie2 (Langmead and Salzberg, 2012) version 2.2.3 with optimal settings (D 20 -R 3 -N 1 -L 20 -i S,1,0.50 –local) for the IonProton™ Sequence. Post-processing of the SAM files into sorted BAM files was carried out with SAMtools (Li et al., 2009, version 1.2-207). The samples uncoated cellulose, GOX, AGXX®, and GOX-AGXX® were normalised against the untreated control of the respective time-points. *S. aureus* 04-02981 cultures without addition of any fibres served as the untreated control. Length-normalised confidence interval RPKM (= Reads per Kilobase of transcript per Million mapped reads) values were obtained with Cufflinks (Trapnell et al., 2010). For better prediction of the variation in gene expression, an extra sample (Control_0) was added to the input for T-Rex. Finally, statistical analysis was carried out using the T-REx RNA-Seq analysis pipeline (de Jong et al., 2015). A gene was considered significantly differentially expressed when the fold change was $\geq|2.0|$ and the false discovery rate (FDR) adjusted p-value ≤ 0.05. The data presented in this paper have been deposited at NCBI and are accessible through GSE149013.

185

186 **Reverse Transcription quantitative PCR (RT- qPCR)**

187 To verify the results obtained from RNA-sequencing, RT-qPCR was performed on four genes
188 representing a group of genes that was differentially expressed in RNA-seq. To this end, total
189 RNA extracted from MRSA cultures exposed to uncoated cellulose, GOX, AGXX® or GOX-
190 AGXX® for 0 or 120 min was used. Time-points 0 and 120 min were selected for validation
191 of RNA-seq data since GOX-AGXX® affected the expression of most genes at these time-
192 points. cDNA was synthesized with the RevertAid^{TM} First Strand cDNA Synthesis kit
193 (Thermo Fisher Scientific Inc., Walham, Germany) as per the manufacturer's instructions
194 using 200 ng of total RNA as template and random hexamer primers. cDNA was diluted with
195 DNase- and RNase-free water and amplified in a LightCycler®480 II (Roche Diagnostics
196 GmbH, Mannheim, Germany).

197 The genes encoding 6-phospho-β-glucosidase (*bglA*), staphylococcal accessory regulator A
198 (*sarA*), carbamate kinase (*arcC*), and transcriptional regulator protein *kdpE* were selected to
199 verify the data obtained through RNA-seq; gyrase B (*gyrB*) was used as the house-keeping
200 gene. These genes were amplified using TaqMan chemistry according to the instructions
201 provided in LightCycler®480 Probes Master Kit (Roche Diagnostics). All RT-qPCR
202 reactions were carried out in a total volume of 20 µL. The amplification step was performed
203 45 times and with 'Quantification' analysis mode at 95°C for 10 sec, with a ramp rate of
204 4.4°C/sec, followed by annealing at the respective annealing temperature for 50 sec, with a
205 ramp rate of 2.2°C/ sec and finally an extension at 72°C for 1 sec, with a ramp rate of
206 4.4°C/sec. All primers and probes used in the study are listed in Supplementary Table 1. All
207 RT-qPCR experiments were done in triplicate and each experiment was repeated at least
208 twice. Data were analysed by LightCycler® 480 Software release 1.5.0. Data represent fold
209 changes, calculated by normalising to the *gyrB* gene and relative to the untreated culture of
210 MRSA using the Livak method of relative quantification (Livak and Schmittgen, 2001).
211 Means of three Cp values each were used to calculate the fold changes in gene expression.

212

213

214 **Results**

GOX, AGXX® and their combination fibres effectively inhibit the growth of MRSA

To examine the effect of different amounts of GOX and AGXX® materials on *S. aureus* 04-02981, inhibition assays were performed. MRSA cultures were exposed to either cellulose, GOX, AGXX® or GOX-AGXX® for 5 h after which CFU mL^{-1} was determined using standard plate assays. Optimum results were obtained with 30 mg GOX fibres and 15 mg AGXX® fibres per 30 mL culture. The effect of the materials on *S. aureus* 04-02981 growth is presented in Fig. 1; CFU mL^{-1} values are given in Supplementary Table 2.

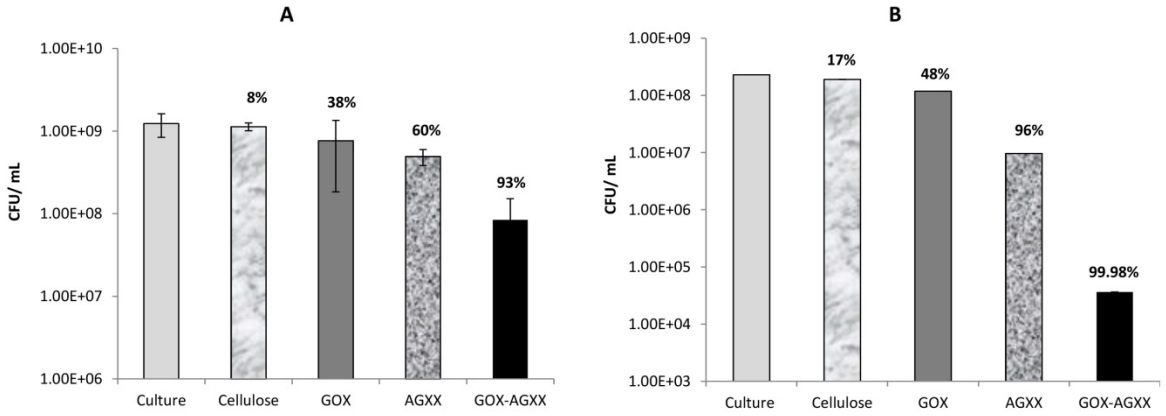


Fig. 1. Effect of GOX-AGXX® on the growth of *S. aureus* 04-02981. A. Effect of GOX and AGXX® coated on cellulose fleece on *S. aureus* 04-02981 liquid cultures. Mean CFU mL^{-1} was calculated from three independent CFU values each. **B.** Effect of the test materials coated on cellulose fibres on *S. aureus* 04-02981 cultures. Error bars indicate standard deviation. The percentage value above each sample column indicates the percent reduction in CFU mL^{-1} in that sample relative to an untreated control (culture without addition of antimicrobials = Culture).

GOX-AGXX® fibres had a more pronounced effect on *S. aureus* 04-02981 than the fleece. The coated fibres resulted in 99.98% growth inhibition of *S. aureus* 04-02981, while the coated fleece inhibited the growth by 93.00%. Therefore, GOX (30 mg/ 30 mL) and AGXX® (15 mg/ 30 mL) coated on cellulose fibres were used in further experiments.

Cellulose-based fibres have a huge impact on the transcriptome of *S. aureus* 04-02981

The raw RNA-seq sequences were aligned to the genome of *S. aureus* 04-02981. All the sequenced samples had satisfactory read depth, ranging from approximately 10 million reads (Control_180) to approximately 20 million reads (GOX-AGXX®). An average sequence depth of approximately 14 million reads was achieved. The library sizes for all the sequenced samples are provided in Supplementary Table 3.

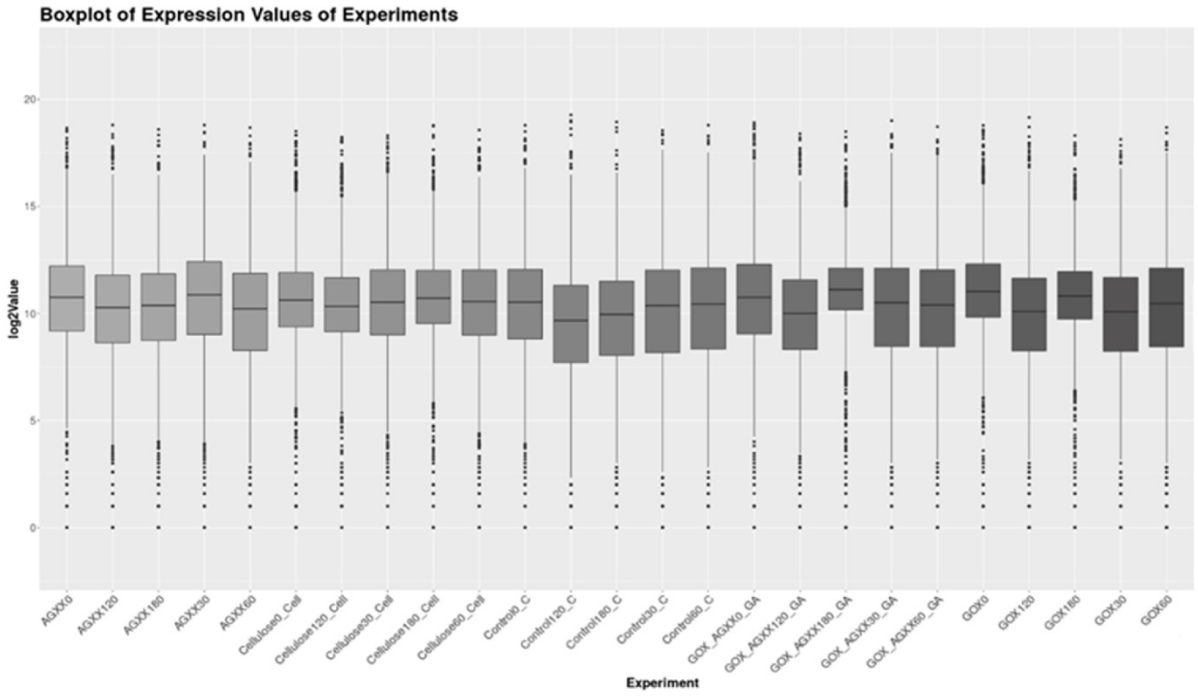


Fig. 2. Box plot of normalised signals. The majority of the log2 normalised gene count values of each sample and experiment are presented as bars. The experiment names indicate the sample and the time (in min) for which the culture was exposed to the particular material. The experiment AGXX®0 represents the *S. aureus* 04-02981 culture exposed to AGXX® fibres for 0 min, AGXX®120 represents the *S. aureus* 04-02981 culture exposed to AGXX® fibres for 120 min, and the same scheme applies to all the experiments. Outliers are represented by dots and the mid-point as a horizontal line.

Fig. 2 shows that after normalisation of the samples to their respective controls (untreated culture) the gene counts were equally distributed. Outliers were mostly present only in the high- or low-fold range. The data from Figs. 1 and 2 clearly indicate the remarkable effect of the cellulose-based fibres on *S. aureus* 04-02981. Intriguingly, cellulose fibres without any

antimicrobial coating also had a tremendous unexpected effect on gene expression. Upon exposure to uncoated cellulose, GOX, AGXX®, or GOX-AGXX® fibres for 0, 30, 60, 120, and 180 min, 2650 genes in *S. aureus* 04-02981 were differentially expressed in total. The numbers of differentially expressed genes per sample and time-point are shown in Fig. 3.

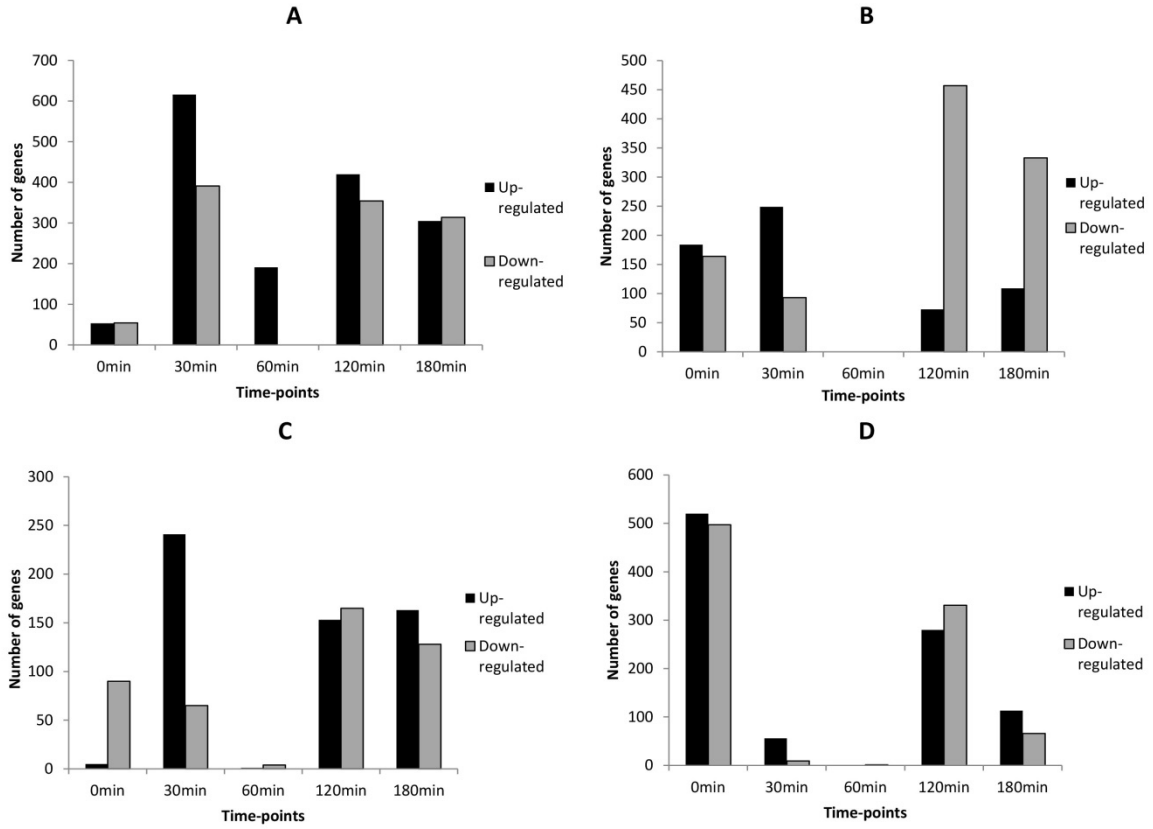


Fig. 3. Number of differentially expressed genes in *S. aureus* 04-02981. Total number of up-regulated (black bars) and down-regulated (grey bars) genes in *S. aureus* 04-02981 on exposure for different times (X-axis) to A) Cellulose (uncoated), B) GOX, C) AGXX®, D) GOX-AGXX®.

A number of unexpected but interesting results are apparent from Fig. 3. First, cellulose hugely impacted gene expression of MRSA at all time-points (Fig. 3A). Second, only a few genes were differentially expressed after 60 min of exposure in any of the samples. Since we did not expect cellulose to significantly change gene expression of *S. aureus* 04-02981, we looked into genes possibly associated with cellulose degradation or utilisation and checked their expression pattern in the RNA-seq data, namely those of aryl-phospho-β-D-glucosidase (*bglA*), 6-phospho-β-galactosidase (*lacG*), α-D-1,4-glucosidase (*malA*), catabolite control

protein A (*ccpA*), and the phosphotransferase system (PTS IIC component, SA2981_2262). The gene products of *bglA* and *lacG* belong to the glycoside hydrolase (GH) 1 family. These enzymes hydrolyse β-glycosides in various carbohydrates. β-D-glucosidase hydrolyses cellulose with the help of PTS by converting cellobiose to glucose (Yang et al., 2013). CcpA uses carbon catabolite repression to regulate transcription of glycolytic pathway genes (Balasubramaniam et al., 2017), while MalA carries out the endohydrolysis of (1->4)-alpha-D-glucosidic linkages (Voet et al., 2008). Differential expression of these genes is presented below in Table 1.

Table 1. Differential expression of genes putatively associated with cellulose degradation in *S. aureus* 04-02981

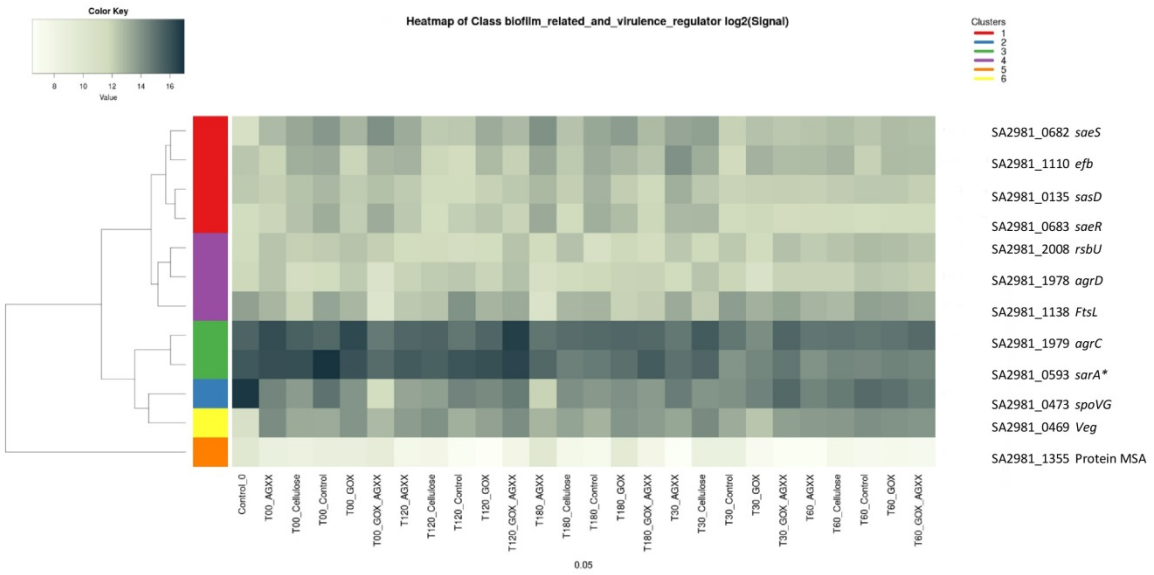
Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_0266	***bglA***	6-phospho-beta-glucosidase activity	GOX at t0	2.9
			GOX-AGXX® at t0	3.9
			Cellulose at t30	4
			Cellulose at t120	3.2
			Cellulose at t180	2.7
SA2981_1694	***ccpA***	global transcription regulator of carbon catabolite repression	Cellulose at t120	-2.7
			GOX-AGXX® at t120	-3.2
			Cellulose at t180	-2
SA2981_2127	***lacG***	6-phospho-beta-galactosidase activity	GOX-AGXX® at t0	3.3
			Cellulose at t30	8.7
			AGXX® at t30	2.2
			Cellulose at t120	2.8
			GOX at t120	2.5
			AGXX® at t120	2.6
			Cellulose at t180	-3
SA2981_1464	***malA***	Endohydrolysis of (1-4)-alpha-D-glucosidic linkages in polysaccharides containing three or more (1-4)-alpha-linked D-glucose units	Cellulose at t0	2
			GOX at t0	2.6
			GOX-AGXX® at t0	3.9
			Cellulose at t30	3.3
			GOX-AGXX® at t30	2.1
			AGXX® at t60	2.5

			GOX at t120	-2.5
			AGXX® at t120	-2.1
			GOX-AGXX® at t120	-11.5
SA2981_2262	**PTS IIC component**	Phosphoenol pyruvate system involved in glucose transport	GOX at t0	2.4
			GOX-AGXX® at t0	2.7
			Cellulose at t30	2.7
SA2981_0194	**RpiR**	transcriptional regulator	GOX-AGXX® at t0	2.8
			Cellulose at t30	6
			GOX-AGXX® at t120	-2.8
			Cellulose at t180	-4.4
			AGXX® at t180	-2.7

Table 1 only shows the samples and time-points at which the respective genes were differentially expressed in RNA-seq.

GOX, AGXX® and their combination fibres repress genes associated with biofilm formation in *S. aureus* 04-02981

We checked the effect of the different fibres (uncoated cellulose, GOX, AGXX®, and GOX-AGXX®) on the expression of genes involved in biofilm formation and those that are essential for survival of MRSA in biofilms. Additionally, the effect on virulence-associated genes was investigated. Several genes associated with biofilm formation, survival in biofilms, and virulence were indeed differentially expressed (See Fig. 4 for an overview). Supplementary Table 4 gives the gene ID, names, and differential expression, as fold changes, of the genes shown in Fig. 4.



299

300 **Fig. 4. Biofilm and virulence-related genes differentially expressed in *S. aureus* 04-02981**
301 **upon exposure to GOX, AGXX®, or GOX-AGXX® fibres.** The genes are clustered as
302 indicated by the dendrogram on the left of the heatmap. Cluster 1 (red) contains the *saeS, efb,*
303 *sasD*, and *saeR* genes. Cluster 2 (purple) represents *rsbU, agrD*, and *ftsL*. Cluster 3 (green)
304 groups *agrC* and *sarA**. *spoVG,* and the genes for the Veg protein and protein MSA are
305 individually represented by blue, yellow, and orange colours, respectively. '*', gene selected
306 for validation through RT-qPCR. The experiment names at the bottom of the heatmap
307 indicate the time-point (Tx) followed by the specific sample. E.g., the experiment
308 T00_AGXX represents the culture exposed to AGXX® fibres for 0 min, T30_AGXX
309 represents the culture exposed to AGXX® fibres for 30 min, etc.

310 Most of the genes depicted in Fig. 4 were differentially expressed at all time-points of
311 exposure to at least one of the antimicrobials except at t60. The genes of the two-component
312 system SaeRS impact biofilm formation by modulating the synthesis of extracellular
313 proteases. Uncoated cellulose fibres at t180 had the highest impact on expression of *saeS,*
314 encoding the sensor histidine kinase and *saeR,* specifying the response regulator of the
315 system (Baker et al., 2010). Both were down-regulated by 3 and 4.5 fold, respectively.
316 Extracellular fibrinogen gene *efb*, an important virulence factor in *S. aureus* (Ko et al., 2016),
317 was affected the most on exposure to AGXX® at t0 while the cell-wall anchored protein gene
318 *sasD* (Muthukrishnan et al., 2011) was highly affected at t180 in the presence of GOX. These
319 two genes were down-regulated 4.7 and 3.6-fold, respectively. No differential expression was
320 observed at t60 and t120 for any of these four genes upon exposure to all different fibres.

Accessory gene regulator (*agr*) genes are essential for biofilm formation in *S. aureus*. The gene *agrC*, specifying a member of the AgrCA two-component system serves as the sensor histidine kinase, while *agrD* is required for production of autoinducer peptide (Baker et al., 2010; Quave and Horswill, 2014). Stage V sporulation gene G *spoVG* is involved in antibiotic resistance and the synthesis of virulence factors in *S. aureus* (Bischoff et al., 2016); it was only differentially expressed at t0, and only in the presence of GOX-AGXX®. The three genes *agrC, agrD* and *spoVG* were down-regulated 2.2, 3.5, and 2.4-fold, respectively. GOX-AGXX® at t0 also had the highest impact on staphylococcal accessory regulator gene *sarA,* a virulence regulator, cell division protein gene *ftsL* (Glas et al., 2015), and on the gene encoding the Veg protein, which stimulates biofilm formation by inducing extracellular matrix genes (Lei et al., 2013). These genes were down-regulated 5.6, 14.5, and approximately 20-fold, respectively.

GOX, AGXX® and their combination fibres repress genes critical for survival of MRSA in biofilms. The genes for arginine metabolism *arcA*, *arcB*, *arcC*, and *arcD* are essential for MRSA to survive in biofilms (Beenken et al., 2004; Qin et al., 2014). Their expression upon exposure to GOX, AGXX® or GOX-AGXX® fibres is presented in Fig. 5 and Supplementary Table 5.

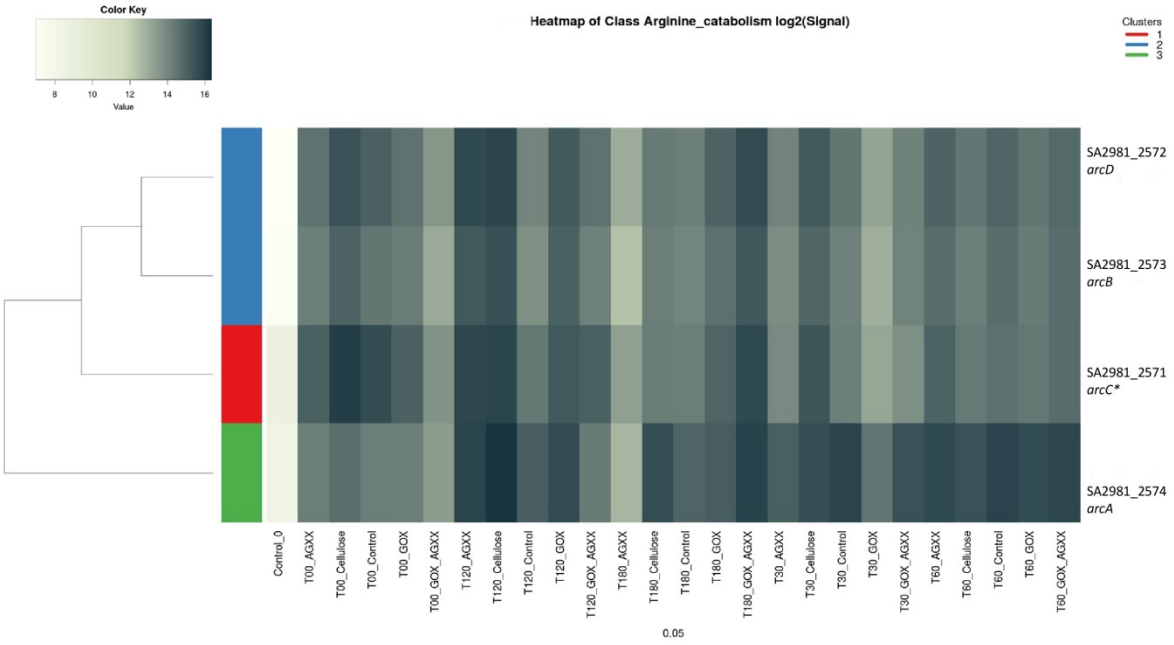


Fig. 5. MRSA genes crucial for survival in biofilm. Arginine-ornithine antiporter gene *arcD*, and the ornithine transcarbamoylase gene *arcB* are present in cluster 1 (blue colour),

while carbamate kinase gene *arcC*, and the arginine deiminase gene *arcA* are present in red and green clusters, respectively. '*', gene selected for validation by RT-qPCR. For further explanation of the experiment names, see the legend to Fig. 4.

The products of *arcABC* catalyse the conversion of arginine to ornithine and are related to intermediary metabolism; ArcD enables arginine uptake and ornithine export. All four genes were most differentially expressed (down-regulated) in the presence of GOX-AGXX®. On one hand, *arcB*, *arcC*, and *arcD* were down-regulated at t0 by 4.8, 4.4, and 4.8 fold, respectively. On the other hand, GOX-AGXX® at t120 had the highest impact on *arcA*, repressing the gene 4 fold. All these genes were repressed in all the samples except *arcC*, which was up-regulated 2.6-fold upon exposure to GOX-AGXX® at t180. Previous studies have reported induced expression of the *arc* genes in *S. aureus* biofilms compared to planktonic cultures (Beenken et al., 2004; Resch et al., 2005). Interestingly, the *arc* genes were down-regulated here as well as several genes associated with biofilm formation namely, *agrD*, *saeRS*, *sarA*, *efb*, and those of the proteins Veg, MSA, and FtsL (See Fig. 4, Supplementary Table 4). Combining these data on genes associated with biofilm formation and virulence, and those essential for survival in biofilms one might speculate that GOX, AGXX®, and GOX-AGXX® assist in moderating biofilm formation in *S. aureus* 04-02981.

GOX, AGXX® and their combination fibres affect the expression of potassium transport (*kdp*) genes in *S. aureus* 04-02981

The RNA-seq data revealed a strong effect of the cellulose-based fibres on the expression levels of the *kdp* genes (Fig. 6 and Supplementary Table 6).

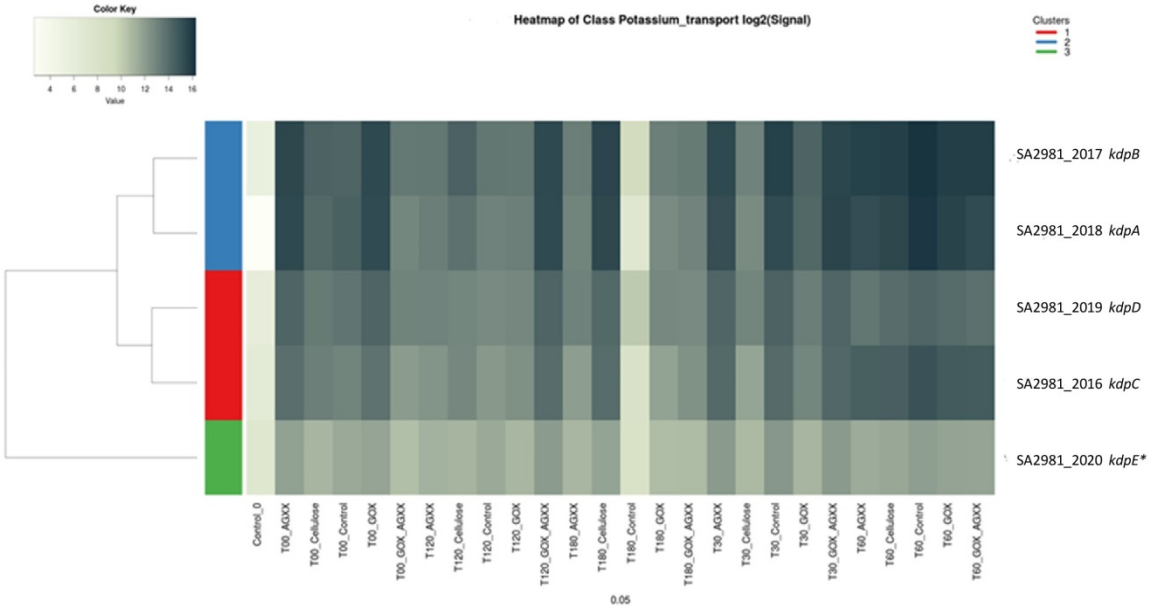


Fig. 6. Heatmap of the expression of potassium transport genes in *S. aureus* 04-02981 upon exposure to GOX, AGXX® or GOX-AGXX® fibres. Differential expression of the genes encoding potassium transport ATPase A (*kdpA*), ATPase B (*kdpB*), and ATPase C (*kdpC*). KdpDE is a two-component system where *kdpD* is the sensor histidine kinase and *kdpE* the response regulator. For the coding of the experiments at the bottom of the figure, see the legend to Fig. 4.

When *S. aureus* 04-02981 was exposed to cellulose, GOX, AGXX®, or GOX-AGXX® fibres, all five *kdp* genes were differentially expressed at some of the time-points (0, 30, 60, and 120 min).

kdpA was remarkably differentially expressed in the presence of cellulose fibres at t180 (187-fold up-regulated). It was up-regulated in the presence of all the fibre materials used at t180 but down-regulated at the earlier time-points, e.g. in the presence of GOX-AGXX® at t0 (by approximately 5 fold) and in the presence of cellulose at t30 (9 fold). A similar pattern of differential gene expression was observed for *kdpC* and *kdpD* where the cellulose fibres at t180 had the strongest effect on expression. The genes were down-regulated at earlier time-points such as t0, and t30, while they were up-regulated at later time-points such as at t120, and t180. The transcription of *kdpB* and *kdpE* was most affected in the presence of GOX-AGXX® at t180 with up-regulation by 17, and 97-fold, respectively. There is a link between the KdpDE system and bacterial stress response which makes its association with survival within a host clear and relevant (Freeman et al., 2013). Since K^+ concentration stimulates the

induction of the *kdp* genes (Freeman et al., 2013) it is no surprise that salt shock also affects the transcription of these genes. Additionally, *kdpDE* is also linked to antimicrobial and oxidative stress response (Freeman et al., 2013). In our experiments, we used growth medium (TSB) devoid of salt which may be one of the reasons why the expression of these genes was affected. We also suspect that the antimicrobial and oxidative stress imposed by the ROS generated in the presence of AGXX® leads to loss of ions from the cell (due to the damage caused to the bacterial membranes) and hence *S. aureus* 04-02981 up-regulates the expression of *kdpABCDE* to recover from the loss of K^+, for its survival.

In addition to genes associated with biofilm formation and virulence (Fig. 4), biofilm survival (Fig. 5), and potassium transport (Fig. 6), other highly affected groups of genes were those involved in antibiotic resistance (Supplementary Table 7), and siderophore biosynthesis (*sbn* genes) the products of which are associated with iron homeostasis (Supplementary Table 8).

Considering that GO and AGXX® have both previously been shown to produce ROS (Akhavan et al., 2011; Clauss-Lendzian et al., 2018), the expression of three oxidative stress genes was not surprising. The genes encoding the alkyl hydroxyperoxidase subunit F (*ahpF*), catalase (*katA*), and thioredoxin A (*trxA*) are all induced in the presence of AGXX® and/or GOX-AGXX® fibres (see Supplementary Table 9).

Validation of RNA-sequencing data

We used RT-qPCR to validate the transcriptomic data obtained by RNA-sequencing. Four genes, namely *bglA, sarA, arcC,* and *kdpE* were selected as representatives of the groups of genes that were highly affected in MRSA upon exposure to GOX, AGXX®, or GOX-AGXX® fibres. Gene *bglA* was chosen to confirm the response of MRSA to cellulose since this sugar polymer unexpectedly seemed to interfere with gene expression in this organism. The gene for SarA - a virulence regulator that enables biofilm formation in MRSA, *arcC* - necessary for survival in biofilms, and *kdpE* - a potassium transporter gene also involved in virulence regulation in MRSA, were the other selected genes. Figs. 7 and 8 summarize the RT-qPCR results and compare them with the RNA-seq data.

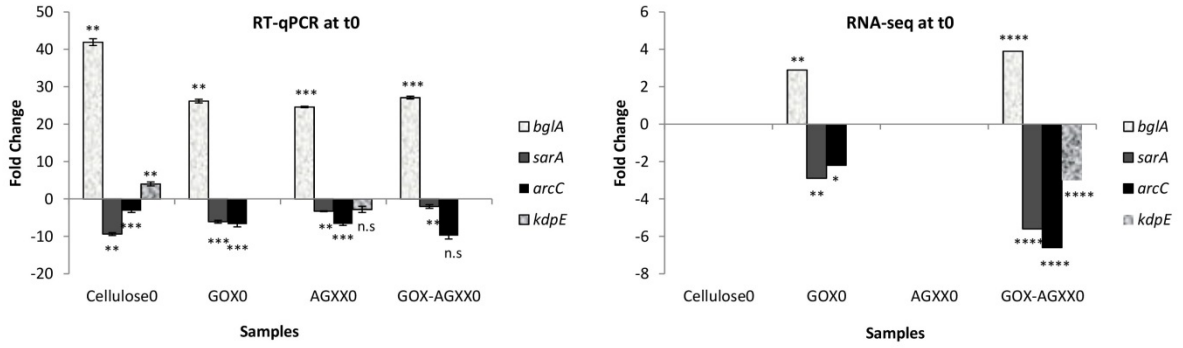
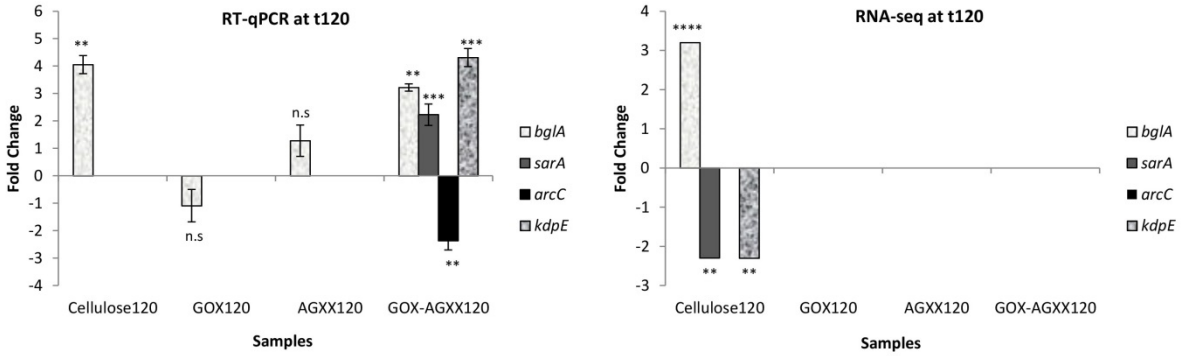


Fig. 7. Differential expression of four selected genes upon exposure to GOX, AGXX®, or GOX-AGXX® fibres at time-point zero min. Expression patterns of the selected genes by RT-qPCR (left panel) and by RNA-seq (right panel). Error bars indicate standard deviation. Asterisks indicate *p*-values showing statistical significance (****p < 0.0001, ***p < 0.001, **p < 0.01, *p < 0.05; n.s., not significant). The samples indicate the antimicrobial or cellulose and the time (in min) that *S. aureus* 04-02981 was exposed to that particular substance.

The trend of differential gene expression was similar between both techniques. Some differences observed in RNA-seq and RT-qPCR were as follows i) expression of *bglA* was highly induced in all the samples in RT-qPCR at t0 especially in the presence of cellulose (41fold), ii) *sarA* was differentially expressed in all the samples in RT-qPCR while in RNA-seq, only in the presence of GOX, and GOX-AGXX®, and iii) *kdpE* was induced in the presence of cellulose in RT-qPCR (4 fold) but was not differentially expressed in RNA-seq. All gene expression values in terms of fold change are presented in detail in Supplementary Table 10.

We also compared the data of both technologies for the selected genes at t120 (see Fig. 8, Supplementary Table 11).



439

440 **Fig. 8. Differential expression of four validation genes at time-point 120 min after**
441 **exposure to GOX, AGXX®, or GOX-AGXX® fibres.** Expression patterns of the selected
442 genes using RT-qPCR (left panel) and RNA-seq (right panel). For further explanation, see
443 legend to Fig. 7.

444 In the presence of cellulose, both methods showed an up-regulation of *bglA*, RNA-seq data
445 revealed a 3-fold, and RT-qPCR data a 4-fold induction, the change of gene expression in the
446 presence of GOX-AGXX® in RT-qPCR was also around 3fold (*bglA*= 3, *sarA*= 2, *kdpE*= 4,
447 and *arcC*= -2 fold).

448 Although the two techniques, RNA-seq, and RT-qPCR cannot be directly compared in terms
449 of absolute gene expression values, the data obtained using both these techniques matched
450 well for some samples, especially for GOX at t0, and GOX, and AGXX® at t120. The
451 expression difference of some of the significantly differentially expressed genes as revealed
452 by RT-qPCR was under the threshold value of 2fold as obtained by RNA-seq (these genes
453 were not expressed OR not differentially expressed in RNA-seq) which is the cut-off value of
454 this technique. Examples are the changes of expression of *sarA* (-1.7fold) in the presence of
455 AGXX®, and that of *arcC* (-1.9fold) in the presence of GOX (Fig. 8).

456

457 **Discussion**

458 Multi-drug resistant pathogens such as MRSA cause lethal nosocomial infections. Thus, it is
459 imperative to find solutions to fight these organisms as well as to control their spread. We
460 tested the effect of two different antimicrobial materials - GOX, and AGXX® - coated on
461 cellulose fibres as well as the combination of both materials on the growth and transcriptome
462 of the clinical MRSA strain *S. aureus* 04-02981.

The RNA-seq data revealed that all the cellulose-based fibres had a remarkable effect on the transcriptome of *S. aureus* 04-02981. Surprisingly, uncoated cellulose fibres also markedly impacted gene expression in *S. aureus* 04-02981 at all time-points examined. This strain was influenced most when exposed to uncoated cellulose fibres for 30 min (1007 genes differentially expressed), with the number of genes going gradually down after longer exposure times. These results were intriguing since uncoated cellulose fibres did not markedly influence the growth of *S. aureus* 04-02981 in the microbiological tests. Cellulose is a linear chain of β-1, 4-glucose monomers linked by β-1, 4-glycosidic bonds (Devendran et al., 2016; Wu et al., 2016). Bacteria degrade it using three types of enzymes: endoglucanases and cellobiohydrolases, which synergistically depolymerize cellulose to cellobiose, followed by β-glucosidases, which hydrolyse cellobiose to glucose (Devendran et al., 2016; Zhang et al., 2017; Zang et al., 2018). The action of the β-glucosidases is considered to be the rate-limiting factor in cellulose degradation, making the study of the expression of β-glucosidase genes imperative to understand cellulose degradation (Zang et al., 2018). Hence, we examined the expression of genes putatively involved in cellulose degradation in *S. aureus* 04-02981. Expression of *bglA*, *malA*, *lacG*, *ccpA*, *rpiR*, and *PTS* (IIA, and IIC components) was affected with uncoated cellulose fibres having a high impact on the expression of these genes among all samples at t30. Interestingly, *bglA*, *malA*, *lacG*, *rpiR*, and *PTS* IIC component were all up-regulated several-fold in the presence of uncoated cellulose fibres at t30; *bglA* and *lacG* were also up-regulated at t120. The RpiR transcriptional regulator and CcpA sense metabolic changes in the environment and modulate the transcription of virulence genes such as *agr* and genes involved in metabolic pathways. CcpA allows bacteria to use the preferred carbon source (Balasubranian et al., 2017). In our experiment, CcpA/RpiR might have been expressed by *S. aureus* 04-02981 to utilize cellobiose as an additional carbon source to glucose present in the growth medium.

Among the genes whose expression was impacted by the antimicrobials were those associated with biofilm formation and virulence. Agr and Sae are among the most studied and best characterized regulators in *S. aureus* with AgrCA and SaeRS two-component systems crucial for virulence in *S. aureus*. Agr is a quorum-sensing system specified by four genes *agrA*, *agrB*, *agrC*, and *agrD*. Agr positively regulates virulence factors and is linked to virulence by the effector molecule RNAIII (Qin et al., 2014; Balasubranian et al., 2017). In our study, the sensor histidine kinase *agrC* and the autoinducing peptide *agrD* are both generally down-regulated. AgrCA and SaeRS positively regulate expression of extracellular

proteins Eap and Emp required for biofilm formation in *S. aureus*. Like AgrCA, SaeRS is also critical for biofilm formation (Baker et al., 2010; Balasubranian et al., 2017). Induction of SaeRS increased biofilm formation in *S. aureus* (Mashruwala et al., 2017). The expression of both *saeR* and *saeS* is down-regulated in our study.

Another global regulator in *S. aureus*, SarA, affects transcription of staphylococcal virulence genes and is critical for biofilm formation (Cheung and Zhang, 2002; Balasubranian et al., 2017). It increases the transcription of *agrBDCA* and RNAIII by binding to the P2 and P3 promoters, respectively, in the *agr* locus (Cheung et al., 1992; Chien et al., 1999; Balasubranian et al., 2017). A study of the biofilm formation characteristics of eight wild type strains of *S. aureus* and their respective *sarA* and *agr* mutants revealed that deletion of *sarA* reduced biofilm formation in six out of eight of the strains (Beenken et al., 2003). Repression of *agrCD, saeRS*, and *sarA* along with other virulence factors such as *rsbU*, and genes encoding protein MSA, and cell division proteins such as Efb, SasD, FtsL, Veg and SpoVG in the presence of GOX, AGXX®, or GOX-AGXX® (Fig. 4 and Supplementary Table 3) suggests that the antimicrobial materials may have a negative effect on biofilm formation in *S. aureus* 04-02981.

Agr encodes RNAIII which is linked to biofilm formation and virulence, and is also associated with biofilm survival. The auto inducer RNAIII-activating protein induces phosphorylation of RNAIII-activating protein (TRAP). Korem et al. (2005) demonstrated that TRAP increases the expression of genes essential for survival in biofilms such as the *arcABCD* genes (Korem et al., 2005). *arcABC* are part of the arginine deiminase pathway and catalyse the conversion of arginine to ornithine, ammonia and carbon dioxide. Bacterial cells also require these genes to survive under stress conditions (Lindgren et al., 2014). The *arcABCD* genes were generally down-regulated in our data. Interestingly, they were repressed in the samples in which the expression of genes associated with biofilm formation was also diminished. This suggests the potential of GOX, AGXX® and their combination fibres in attenuating biofilm formation in *S. aureus* 04-02981, especially when combined with the fact that these fibres inhibit bacterial growth, hence reducing the population density in the culture. We have previously shown reduction of biofilm formation in *S. aureus* 04-02981 in the presence of AGXX® (Vaishampayan et al., 2018).

Another group of genes that were highly differentially expressed were those encoding the Kdp system. These are essentially potassium transporters, but they also act as virulence regulators. K^+ is a vital cation for bacterial growth and survival (Ballal et al., 2007).

Concentration of K^+ is crucial for maintaining turgor of cells, regulating pH and for the infectious status of *S. aureus* (Ballal et al., 2007; Xue et al., 2011). KdpDE, first characterized in *E. coli*, is an important virulence regulator in *S. aureus* (Asha and Gowrishankar, 2007; Xue et al., 2011). The primary role of KdpFABC and KdpDE in *S. aureus* NCTC8325 is proposed not to be in K^+ transport but in the regulation of transcription of virulence genes (Xue et al., 2011). KdpE can directly bind to the promoters of virulence genes and alters their transcription (Xue et al., 2011). *kdpDE* mutants show very low survival rates, indicating that *kdpDE* is essential for survival of *S. aureus* (Xue et al., 2011). KdpDE also influences the expression of biofilm and virulence regulator genes like *arcA, hisB*, Veg protein gene, *ahpC, kdpB, kdpC, agrC, agrD, lrgA, aur, spa, capA*, and thioredoxin genes (Xue et al., 2011).

We observed that at t0, t30, and at t120, the *kdp* genes were downregulated. The opposite trend was seen at t180, where the genes were highly upregulated. One reason behind this trend might be the fact that the transcription of the *kdp* genes is increased under K^+ deficient conditions and in the post-exponential phase in *S. aureus* (Xue et al., 2011). Since KdpDE responds to population density to decide whether or not to elicit a response, the downregulation at earlier time-points such as at t0 and t30 where the population density was lower than at t120 and t180 where these genes were up-regulated is at least partially justified. Up-regulation at t180 especially in the presence of uncoated cellulose fibres further explains this since uncoated cellulose fibres did not inhibit bacterial growth in the growth inhibition experiments.

Iron is another vital nutrient required for growth of many bacteria (Dale et al., 2004). Some strains of *S. aureus* produce siderophores under iron-limited growth conditions; production of siderophores is crucial to virulence of the bacteria (Dale et al., 2004; Perry et al., 2019). A nine-gene operon called *sbn* is responsible for siderophore production in *S. aureus* (Dale et al., 2004). In our study, all the *sbn* genes were up-regulated in the presence of all the materials, at all tested time-points, the highest up-regulation being observed in the presence of uncoated cellulose at t30 (for *sbnA, sbnB,* and *sbnE* genes) at t120 (for *sbnC, sbnD, sbnF*, and *sbnH*), and in the presence of GOX-AGXX at t0 (for *sbnE*, *sbnG, sbnH*, and *sbnI* genes). Since the expression of the *sbn* operon enables prolonged survival of *S. aureus* in the environment (Dale et al., 2004), we speculate that the increased expression of these genes in *S. aureus* 04-02981 is a response to overcome the stress imposed by the antimicrobial materials, a strategy necessary for the survival of the bacterium.

Further, we confirmed the RNA-seq data by performing RT-qPCR on four selected genes, *bglA, sarA, arcC*, and *kdpE* at t0, and t120. *gyrB* was used as the house-keeping gene. The data obtained were normalised to *gyrB* and fold changes were calculated with respect to the untreated control of the respective time-points. The differences in gene expression measured via RT-qPCR and RNA-seq were similar in case of GOX, and GOX-AGXX® at t0 and GOX, AGXX® at t120 (Fig. 7 and 8). The differences between RNA-seq and RT-qPCR data observed were as follows: i) in the presence of uncoated cellulose, RT-qPCR displayed up-regulation of *bglA*, and *kdpE*, and down-regulation of *sarA*, and *arcC* at t0, whereas these genes were not differentially expressed in RNA-seq, ii) in the presence of AGXX® at t0, *bglA* was up-regulated, and *sarA*, and *arcC* were down-regulated in RT-qPCR but were not differentially expressed in RNA-seq, iii) at t120, in the presence of uncoated cellulose, *sarA* and *kdpE* were down-regulated in RNA-seq but were not differentially expressed in RT-qPCR (Fig. 8), and iv) when treated with GOX-AGXX® (at t120), *bglA, sarA*, and *kdpE* were slightly up-regulated, and *arcC* was down-regulated in RT-qPCR but no differential expression was observed in RNA-seq. However, as the fold changes of *bglA, sarA*, and *arcC* in RT-qPCR were close to the threshold of 2, the difference between RT-qPCR and RNA-seq for these genes is not remarkable.

Several studies have examined the combination of GO and metals like silver. A nanocomposite with chitosan, GO and zinc oxide nanoparticles was tested against *E. coli* and *S. aureus*. The nanocomposite had a strong inhibitory effect on both organisms, mediated by production of ROS (Ray Chowdhury et al., 2015). GO was used in combination with silver nanoparticles (AgNPs) coated on polyurethane foil against *E. coli*, *S. aureus*, *S. epidermidis*, and the pathogenic yeast *Candida albicans* (Jaworski et al., 2018). The combination of GO and AgNPs proved to be far more efficient than GO or Ag-NPs alone. The antimicrobials had a stronger effect on Gram-negative than on Gram-positive bacteria (Jaworski et al., 2018). In a similar study, GO-AgNPs conditioned with sodium borohydride were tested against *E. coli*, and *S. aureus* (Truong et al., 2020). Transmission electron microscopy revealed that GO attached to *S. aureus* cells and wrapped the bacteria. On the other hand, AgNPs penetrated the bacterial cells and damaged the cell membrane, leading to cell death (Truong et al., 2020). Severe growth inhibition was seen in both *E. coli* and *S. aureus* (Truong et al., 2020).

Surface-modified materials in combination with biocidal substances can be efficient in fighting multidrug resistant bacteria (Jaworski et al., 2018). The work presented here also shows this potential.

Conclusion

GOX, AGXX®, and their combination act as effective antibacterial agents against *S. aureus* 04-02981. These materials seem to affect the ability of *S. aureus* 04-02981 to form and survive in biofilms. They also affect the transcription of *kdp*, a system that is crucial for intracellular survival and pathogenesis of MRSA. In addition, the cellulose-based fibres influence the expression of siderophore genes suggesting that they impose stress on the bacterial cells and create iron-deficient conditions. GOX, AGXX®, and their combination have numerous potential applications in medical equipment, as novel biocides, and in agriculture.

Conflict of interest

No conflict of interest declared.

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Author contributions

AV performed all the microbiological and molecular experiments, analysed the RNA-sequencing data and drafted the manuscript. RA, OW and RH developed the antimicrobial fibres and RA and OW generated them. AdJ conducted the bioinformatic analyses of RNA sequences and prepared the RNA-seq data for deposition in NCBI. JK edited the manuscript and gave insightful suggestions towards the discussion of the data. EG designed the project,

supervised all the experiments and edited the manuscript. All authors discussed and revised the manuscript.

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Supplementary information

Transcriptomic analysis of stress response to novel antimicrobial coatings in a clinical MRSA strain (submitted)

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Supplementary Table 1. Primers and probes used for Taqman RT-qPCR of *S. aureus* 04-02981

Primers	Sequence (5' → 3')	Annealing temperature (°C)
gyrB-F	TTAGTGTGGGAAATTGTCGATAAT	57
gyrB-R	AGTCTTGTGACAATGCGTTTACA	
gyrB-probe	6FAM-TACTTTGTATCCGCCACCGCCAAATT-TAMRA	
bglA-F	TATCTTTCCGAATGGGGATG	58
bglA-R	GCGCAAAATGCACAAAATAA	
bglA-probe	6FAM-TGCCACTTCATTTAGCGAAA-TAMRA	
sarA-F	CATCAGCGAAAACAAAGAGAAA	57
sarA-R	TGTTTGCTTCAGTGATTCGTTT	
sarA-probe	6FAM-TTCTTGTTAATGCACAACAACG-TAMRA	
arcC-F	TGTCATTGCATTAGGCGGTA	58
arcC-R	GCGTTGTGTCACTGTTCGAT	
arcC-probe	6FAM-GCTATTAGACGTGCGATGCA-TAMRA	
kdpE-F	ACGGTTTGCTAAGCATCGAC	60
kdpE-R	TCTTTTGCGTCCTCATGACA	
kdpE-probe	6FAM-AGGAGGTACATTTGACGCCA-TAMRA	

Supplementary Table 2. CFU values of Inhibition assay of *S. aureus* 04-02981 with cellulose-based antimicrobials

Fibres	30 mg cellulose	30 mg GOX	15 mg AGXX	30mg GOX_15mgAGXX	Culture
	3.80E+08	1.42E+08	1.81E+06	4.70E+04	2.45E+08
	9.30E+07	1.99E+07	1.24E+07	2.90E+04	2.12E+08
	9.90E+07	1.96E+08	1.47E+07	3.30E+04	2.29E+08
Mean CFU	**1.91E+08**	**1.19E+08**	**9.64E+06**	**3.63E+04**	**2.29E+08**
SD	**1.64E+08**	**9.02E+07**	**6.87E+06**	**9.45E+03**	**1.65E+07**
% reduction in CFU	**16.62%**	**47.83%**	**95.79%**	**99.98%**	
Fleece	**20 mg cellulose**	**20 mg GOX**	**20 mg AGXX**	**40 mg GOX_20mg AGXX**	**Culture**
	1.25E+09	1.06E+09	5.95E+08	3.10E+07	1.66E+09
	1.01E+09	9.60E+07	3.80E+08	1.60E+08	9.90E+08
	1.12E+09	1.14E+09	4.98E+08	6.20E+07	1.02E+09
Mean CFU	**1.13E+09**	**7.65E+08**	**4.91E+08**	**8.43E+07**	**1.22E+09**
SD	**1.20E+08**	**5.81E+08**	**1.08E+08**	**6.73E+07**	**3.78E+08**
% reduction in CFU	**8%**	**37%**	**60%**	**93%**	

Supplementary Table 3. Library sizes of all samples of *S. aureus* 04-02981 subjected to RNA-seq

Group	Library Size	Experiment
T00_AGXX	1.82E+07	AGXX0
T120_AGXX	1.35E+07	AGXX120
T180_AGXX	1.34E+07	AGXX180
T30_AGXX	1.80E+07	AGXX30
T60_AGXX	1.39E+07	AGXX60
T00_Cellulose	1.48E+07	Cellulose0_Cell
T120_Cellulose	1.30E+07	Cellulose120_Cell
T180_Cellulose	1.51E+07	Cellulose30_Cell
T30_Cellulose	1.50E+07	Cellulose180_Cell
T60_Cellulose	1.49E+07	Cellulose60_Cell
T00_Control	1.60E+07	Control0_C
T120_Control	1.22E+07	Control120_C
T180_Control	1.10E+07	Control180_C
T30_Control	1.46E+07	Control30_C
T60_Control	1.66E+07	Control60_C
T120_GOX_AGXX	2.05E+07	GOX_AGXX0_GA
T180_GOX_AGXX	1.22E+07	GOX_AGXX120_GA
T00_GOX_AGXX	1.45E+07	GOX_AGXX180_GA
T30_GOX_AGXX	1.68E+07	GOX_AGXX30_GA
T60_GOX_AGXX	1.53E+07	GOX_AGXX60_GA
T00_GOX	1.92E+07	GOX0
T120_GOX	1.37E+07	GOX120
T180_GOX	1.34E+07	GOX180
T30_GOX	1.11E+07	GOX30
T60_GOX	1.60E+07	GOX60

Experiment names indicate the antimicrobial and the time of exposure. AGXX0 refers to the MRSA culture exposed to AGXX fibres for zero minutes and so on.

Supplementary Table 4. Differential expression of biofilm and virulence-associated genes in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_0682	***saeS***	histidine protein kinase involved in biofilm formation and virulence regulation	GOX at t0	-2.8
			AGXX at t0	-2.7
			Cellulose at t30	2.1
			Cellulose at t180	-3

SA2981_1110	**efb**	extracellular fibrinogen binding protein that plays a crucial role in virulence	GOX at t0	-3.3
			AGXX at t0	-4.7
			GOX-AGXX at t0	-2.1
			GOX at t30	2.9
			Cellulose at t80	-4
			GOX at t180	-2.8
			GOX-AGXX at t180	-3.2
SA2981_0135	***sasD***	Cell wall anchored protein	GOX at t0	-2.3
			AGXX at t0	-2.1
			Cellulose at t80	-3.1
			GOX at t180	-3.6
			GOX-AGXX at t180	-2.6
SA2981_0683	***saeR***	response regulator involved in biofilm formation and virulence regulation	GOX at t0	-3
			AGXX at t0	-3.1
			Cellulose at t120	-2.8
			GOX-AGXX at t120	-2.5
			Cellulose at t180	-4.5
			GOX at t180	-2.7
			GOX-AGXX at t180	-2.6
SA2981_2008	***rsbU***	encodes virulence factors	Cellulose at t30	-2
			AGXX at t30	2.4
			GOX-AGXX at t120	-2.4
			Cellulose at t180	3.3
			GOX at t180	-2
SA2981_1978	***agrD***	Accesory gene regulator D, involved in biofilm formation and virulence regulation	GOX-AGXX at t0	-3.3
			AGXX at t120	-2
			GOX-AGXX at t120	-2
			AGXX at t180	-2.2
SA2981_1138	**FtsL**	Cell division protein	Cellulose at t0	-3.3

			GOX at t0	-2.2
			GOX-AGXX at t0	-14.5
			Cellulose at t30	-3.8
			Cellulose at t120	-5.1
			GOX at t120	-2.8
			AGXX at t120	-4.9
			GOX-AGXX at t120	-3.8
			GOX at t180	-4
			AGXX at t180	-7.3
SA2981_1979	***agrC***	Accesory gene regulator C, involved in biofilm formation and virulence regulation	GOX at t120	-2.2
SA2981_0593	***sarA***	Transcription regulator	GOX at t0	-2.9
			GOX-AGXX at t0	-5.6
			GOX-AGXX at t60	-2.3
SA2981_0473	**spoVG**	Involved in antibiotic resistance and in the synthesis of virulence factors	GOX-AGXX at t0	-2.4
SA2981_0469	**Veg Protein**	Stimulates biofilm formation	Cellulose at t0	-2.9
			GOX at t0	-3.1
			GOX-AGXX at t0	-20
			Cellulose at t120	-3.2
			GOX at t0	-3.2
SA2981_1355	**Protein MSA**	Modulates expression of *sarA* and several virulence factors	AGXX at t30	-5.4
			GOX-AGXX at t30	-5.7
			GOX at t180	2.3
			AGXX at t180	4.6

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 5. Differential expression of genes essential for survival in biofilms in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_2572	*arcD*	Arginine-ornithine antiporter	GOX-AGXX at t0	-4.8
			AGXX at t180	-3.1
			GOX-AGXX at t180	-2.5
SA2981_2573	*arcB*	Ornithine carbamoyltransferase	GOX-AGXX at t0	-4.4
			AGXX at t180	-4.3
SA2981_2571	*arcC*	Carbamate kinase	GOX at t0	-2.2
			GOX-AGXX at t0	-6.6
			AGXX at t180	-2.5
			GOX-AGXX at t180	2.6
SA2981_2574	*arcA*	Arginine deiminase	GOX-AGXX at t0	-3
			GOX-AGXX at t120	-4
			AGXX at t180	-6.3

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 6. Differential expression of Potassium transporting genes in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_2017	*kdpB*	Potassium transporting ATPase B	GOX-AGXX at t0	-2.9
			Cellulose at t30	-9
			Cellulose at t180	32.9
			AGXX at t180	4.5
			GOX-AGXX at t180	17.4
SA2981_2018	*kdpA*	Potassium transporting ATPase A	GOX-AGXX at t0	-4.9
			Cellulose at t30	-9
			GOX-AGXX at t120	2.2
			Cellulose at t180	186.7
			GOX at t180	23.1
			AGXXX at t180	41.8
			GOX-AGXX at t180	53.6
SA2981_2019	*kdpD*	Potassium transporting ATPase D	GOX-AGXX at t0	-2.5

			Cellulose at t30	-3.9
			Cellulose at t180	9.2
			GOX at t180	3.2
			AGXX at t180	5.7
			GOX-AGXX at t180	5.5
SA2981_2016	*kdpC*	Potassium transporting ATPase C	GOX-AGXX at t0	-3.1
			Cellulose at t30	-7.3
			Cellulose at t180	35
			GOX at t180	6.5
			AGXX at t180	11
			GOX-AGXX at t180	19
SA2981_2020	*kdpE*	Potassium transporting ATPase E	GOX-AGXX at t0	-3
			Cellulose at t30	-3.7
			Cellulose at t120	-2
			Cellulose at t180	7
			GOX at t180	3
			AGXX at t180	47
			GOX-AGXX at t180	96

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 7. Differential expression of antibiotic resistance genes in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_0039	*mecA*	Penicillin-binding protein PBP2a, methicillin resistance determinant MecA, transpeptidase	Cellulose at t30	-13
			Cellulose at t120	-2.3
			GOX-AGXX at t120	-10.1
			Cellulose at t180	3.6
SA2981_0040	*mecR1*	Methicillin resistance regulatory sensor-transducer MecR1	Cellulose at t30	-2.9
			Cellulose at t120	-6.1
			GOX-AGXX at t120	-14.1
SA2981_0034	*bleO*	Bleomycin resistance protein	GOX-AGXX at t0	4.4
			Cellulose at t30	-7
			Cellulose at t120	-2.2
			GOX-AGXX at t120	-10.3

			GOX at t180	-3.8
			AGXX at t180	-5.5

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 8. Differential expression of siderophore biosynthesis genes in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_0117	*sbnA*	Siderophore staphylobactin biosynthesis protein SbnA	GOX at t0	4.4
			GOX-AGXX at t0	7.3
			Cellulose at t30	20.5
			AGXX at t30	5
			Cellulose at t60	10.9
			Cellulose at t120	11.1
			GOX-AGXX at t120	3.5
			Cellulose at t180	7.7
			GOX at t180	17.4
SA2981_0118	*sbnB*	Siderophore staphylobactin biosynthesis protein SbnB	GOX at t0	4.8
			GOX-AGXX at t0	7.3
			Cellulose at t30	28.8
			Cellulose at t120	9
			GOX-AGXX at t120	4.9
			Cellulose at t120	10.7
			GOX at t180	21
			AGXX at t180	4.9
SA2981_0119	*sbnC*	Siderophore staphylobactin biosynthesis protein SbnC	Cellulose at t0	3.4
			GOX at t0	3.7
			GOX-AGXX at t0	6.6
			Cellulose at t30	13.5
			Cellulose at t60	8.7
			Cellulose at t120	23
			AGXX at t120	9
			GOX-AGXX at t120	9.7
			Cellulose at t180	4.6

			GOX at t180	12
SA2981_0120	*sbnD*	Siderophore staphylobactin biosynthesis protein SbnD	GOX at t0	3.8
			AGXX at t0	7.2
			GOX-AGXX at t0	26.2
			GOX at t30	4.4
			Cellulose at t60	7.9
			Cellulose at t120	37.9
			AGXX at t120	13.3
			GOX-AGXX at t120	12.8
			Cellulose at t180	7.5
			GOX at t180	23.9
SA2981_0121	*sbnE*	Siderophore staphylobactin biosynthesis protein SbnE	Cellulose at t0	3.9
			GOX at t0	4.3
			GOX-AGXX at t0	7.2
			Cellulose at t30	8.3
			Cellulose at t60	3.3
			Cellulose at t120	7.5
			AGXX at t120	3.4
			GOX-AGXX at t120	3.6
			Cellulose at t180	2.6
			GOX at t180	6
SA2981_0122	*sbnF*	Siderophore staphylobactin biosynthesis protein SbnF	GOX at t0	3.5
			Cellulose at t30	6.3
			GOX at t30	6.1
			Cellulose at t60	3.5
			Cellulose at t120	13.7
			AGXX at t120	5.2
			GOX-AGXX at t120	6
			GOX at t180	6.1
SA2981_0123	*sbnG*	Siderophore staphylobactin biosynthesis protein SbnG	GOX-AGXX at t0	5.4
			Cellulose at t30	4.4
			Cellulose at t120	4.1
			GOX at t180	5.4
SA2981_0124	*sbnH*	Siderophore staphylobactin biosynthesis protein	GOX at t0	2.8

		SbnH		
			GOX-AGXX at t0	4.7
			Cellulose at t30	3.8
			Cellulose at t120	4.9
			GOX at t180	3.3
SA2981_0124	*sbnI*	Siderophore staphylobactin biosynthesis protein SbnI	GOX at t0	2.7
			GOX-AGXX at t0	4.8
			Cellulose at t30	3.3
			Cellulose at t120	2.2

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 9. Differential expression of oxidative stress genes in *S. aureus* 04-02981

Gene ID	Gene/ product	Description	Sample	Fold change
SA2981_1289	*katA*	Catalase	Cellulose at t30	-2.9
			GOX at t30	-2.8
			AGXX at t30	-2.3
			GOX-AGXX at t120	2.3
			AGXX at t180	2.4
SA2981_1101	*trxA*	Thioredoxin	GOX at t30	2.6
			GOX-AGXX at t30	4.2
			AGXX at t180	2.7
			GOX-AGXX at t180	2.6
SA2981_0379	*ahpF*	Alkyl hydroperoxide reductase subunit F	GOX-AGXX at t120	3

Note: The table shows only the samples and time-points at which the respective genes were significantly differentially expressed.

Supplementary Table 10. Validation of selected *S. aureus* 04-02981 genes for the samples at t0 via RT-qPCR

Fold Change

Gene	Cellulose at t0	GOX at t0	AGXX at t0	GOX-AGXX at t0
bglA	41.93	26.17	24.59	27.09
sarA	-9.38	-6.06	-3.29	-2.02
arcC	-3.18	-6.72	-6.68	-9.84
kdpE	4	-1.9	-2.78	-1.16

Standard Deviation

Gene	Cellulose at t0	GOX at t0	AGXX at t0	GOX-AGXX at t0
bglA	0.9	0.5	0.19	0.38
sarA	0.4	0.39	0.12	0.49
arcC	0.4	0.72	0.4	0.85
kdpE	0.51	0.85	0.85	0.38

Supplementary Table 11. Validation of selected *S. aureus* 04-02981 genes for the samples at t120 via RT-qPCR

Gene	Cellulose at t120	GOX at t120	AGXX at t120	GOX-AGXX at t120
bglA	4.05	-1.09	1.28	3.22
sarA	1.28	1.61	-1.7	2.23
arcC	1.03	-1.95	-1.09	-2.37
kdpE	1.37	1.82	1.65	4.31

Standard Deviation

Gene	Cellulose at t120	GOX at t120	AGXX at t120	GOX-AGXX at t120
bglA	0.33	0.59	0.57	0.13
sarA	0.17	0.25	0.58	0.39
arcC	0.38	0.21	0.29	0.33
kdpE	0.79	1.1	0.53	0.33