UiT The Arctic University of Norway

Faculty of Health Science
Department of Medical Biology

Bacterial Extracellular vesicles and their cargo

Bishnu Joshi

A dissertation for the degree of Philosophiae Doctor – November 2020

Bacterial Extracellular vesicles and their cargo

Bishnu Joshi

A dissertation for the degree of Philosophiae Doctor

Research Group for Host-Microbe Interactions
Department of Medical Biology
Faculty of Health Sciences
UiT- The Artic University of Norway

November 2020

Acknowledgements

This work (thesis) was carried out at Research Group for Host- Microbe Interactions (HMI), Department of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway. Thank you IBA school for providing travel grants for attending courses and conferences.

First and foremost, I would like to thank the supervisory team (**Prof Mona Johannessen**, **Prof. Kristin Hegstad** and **Dr. Fatemeh Askarian**) for their continuous mentoring and support throughout the path of my PhD.

First, I would like to express my sincere gratitude to my principal supervisor, Mona Johannessen. Immensely thank you for providing me with an opportunity to work under your tutelage and explore the field of bacterial vesicles and their role in host-microbe interaction. Despite your busy schedule, your office's door was always open for me to discuss science, share results and suggest creative ideas and help in designing the experiments. Most importantly, your hardworking nature and devotion to science, also boosts and inspires me to do research. Word are not enough to express my gratitude for your kindness, motivation, patience, optimism, and unflagging supportive nature that were instrumental in accomplishing my goals.

A special thanks go to my co-supervisors Kristin Hegstad and Dr. Fatemeh Askarian. Kristin, I recall the day when you warmly welcomed me at Tromsø airport. I am honorably indebted to your kindness, and your in-depth knowledge of enterococcus and bioinformatics. Whenever I have any doubts about any research questions or experimental procedures, your door was always open to discuss. It was always an easy-going communication with you in the field of science or life in general. I always appreciate your support, creative ideas, enthusiasm, and sharing of knowledge.

Fatemeh, thank you very much for your prompt response whenever I mailed you. I am always amazed by your hard work and critical thinking ability. Huge thanks for your valuable inputs in manuscript and thesis. I had learned a lot from your critical comments and thank you always for sharing interesting and useful articles.

Heartfelt thanks go to our collaborators at Umeå University, especially Prof Sun N. Wai for allowing me to visit your lab to explore and learn various techniques. Thank you for providing

me new ideas and listening to my thoughts. Thank you Aftab, Kyaw, Eric and Si to guide me with laboratory facility/techniques during my stay at Umeå.

My dear three musketeers group, Katya and Ahmed. Thank you for your support throughout my endeavor at UiT. My few words cannot describe your importance in my life. You both always motivated me and made living easier and joyful here. It was nice working with you in lab Ahmed. Katya, thanks a lot for being with me in *fiskeri* to run growth curve assays and to do proof reading of my thesis. I am grateful to have you both as a friend and colleague.

To the lab gang, you people are amazing group. A lot of parties, birthday celebrations and a lot of fun. Theresa, thank you very much for sharing and brainstorming together during manuscript writing. My office mate Adriana, Conny and Diana. You guys are awesome. *Connisita*, I miss you, and your good sense of humor. Thanks for arranging hiking trips. Adriana, the super hard working lady and fast learner, I will always be proud of you for balancing science and family. Jessin thank you very much for the computer related troubleshooting. Sincere appreciation to my friends (Clement, Mushtaq, Ken, Eric, Dorota, Hermoine, Runa, Martin, Jonathan, Maria, Balint, Kashif), it was a pleasure being with you all.

I would like to thank all past (Alena, Aud-Malin, and Mohammad) and current lab engineers (Bhupender and Kjersti) for their excellent support in ordering chemicals/reagents on time and every lab instrument related troubleshooting. Kjersti thank you very much from bottom of my heart for teaching me Norwegian language besides lab assistance. I am so glad that you even provide me your own *PCR master mix* during urgency and experiment work. *Tusen takk fra Hjerte* ☺

Sincere thanks goes to UiT Imaging platform (Augusta Hlin Aspar and Kenneth Bowitz Larsen), Proteomics platform (Jack-Ansgar Bruun and Toril Anne Grønset) and Sequencing platform (Ruth Paulssen, Chris Fenton, Hagar Taman) for their help and support.

Thank you Birendra, Sabin, Anup, and Arpita for sharing lunch and celebrating Nepalese festival together.

Lastly but most importantly, I would like to say a big thank you to my family, especially, to my wife Deepika for trusting and bearing me. Your suggestions and support had always made my life better and better. The delicious food prepared by you always made me motivated to work more but sometimes made sleepy as well☺.

Contents

Abbreviations

ACME	Arginine Catabolic Mobile Element
AFM	Atomic Force Microscopy
AST	Antibiotic Susceptibility Testing
BSRD	Bacterial sRNA Database
CC	Clonal Complex
CA	Community Acquired
CMV	Cytoplasmic Membrane Vesicle
ESP	Enterococci Surface Protein
EV	Extracellular Vesicle
GBS	Group B *Streptococcus*
GIT	Gastrointestinal Tract
HA	Hospital Acquired
HGT	Horizontal Gene Transfer
ICU	Intensive Care Unit
IGV	Integrative Genomics Viewer
ISEV	International Society for Extracellular Vesicles
MDR	Multi Drug Resistant
MGE	Mobile Genetic Elements
MIC	Minimum Inhibitory Concentration
MHB	Muller Hinton Broth
MLST	Multilocus Sequence Typing
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MSSA	Methicillin-Sensitive *Staphylococcus aureus*
NTS	Nanoparticle Tracking Analysis
OMV	Outer Membrane Vesicles
OIMV	Outer-Inner-Membrane Vesicles
PBP	Penicillin Binding Protein
PRR	Pattern Recognition Receptor
PSM	Phenol Soluble Modulins
PFT	Pore Forming Toxins
PVL	Panton Valentine Leukocidin
SCC	Staphylococcal Cassette Chromosomes
ST	Sequence Type
TEM	Transmission Electron Microscopy
TRPS	Tunable Resistive Pulse Sensing
VF	Virulence Factor
VFDB	Virulence Factor Database
VISA	Vancomycin Intermediate-Resistant *Staphylococcus aureus*
VRE	Vancomycin Resistant Enterococci
VREfm	Vancomycin Resistant *Enterococcus faecium*
VRSA	Vancomycin Resistant *Staphylococcus aureus*
WTA	Wall Teichoic Acid

List of papers

Paper I

Wagner T*, **Joshi B***, Janice J, Askarian F, Škalko-Basnet N, Hagestad OC, Mekhlif A, Wai SN, Hegstad K, Johannessen M. 2018. ***Enterococcus faecium*** **produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins.** Journal of Proteomics. 187:28-38.doi: 10.1016/j.jprot.2018.05.017. PMID: 29857065.

*Equally contributed

Paper II

Joshi B, Singh B, Nadeem A, Askarian F, Wai SN, Johannessen M, and Hegstad K. 2020. **Transcriptome profiling of *Staphylococcus aureus* associated Extracellular Vesicles reveals presence of small RNA-cargo.** Under revision by the "Frontiers in Molecular Bioscience - Protein and RNA Networks.

Paper III

Kumaraswamy M, Wiull K*, **Joshi B***, Sakoulas G, Kousha A, Vaaje-Kolstad G, Johannessen M, Hegstad K, Nizet V and Askarian F. **Bacterial Membrane-Derived Vesicles Attenuate Vancomycin Activity against Methicillin-Resistant *Staphylococcus aureus***. Manuscript.

*Equally contributed

Summary

Staphylococcus aureus and *Enterococcus faecium* are opportunistic pathogens that cause a wide range of infections, especially in patients with underlying medical conditions. Multidrug resistant versions of these bugs cause frequent outbreaks in the hospital and community. The emergence of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococcal (VRE) strains severely limits the treatment options. Bacteria release extracellular vesicles, which has been defined in the minimal information for studies of extracellular vesicles guideline as "particles naturally released from the cell that are delimited by a lipid bilayer and cannot replicate". While they are packaged with an armamentarium of virulence factors, resistant determinants, and nucleic acids, the characterization of EVs cargo still is limited in Gram-positive bacteria. sGiven the importance of EVs, this thesis aimed to examine proteome and RNA content of these nanoparticles upon exposure to a diverse range of growth conditions (**Paper I-III**).

Isolation of EVs from clinical *E. faecium* strains (**Paper I**) and MRSA (**Paper III**) was done by series of filtration, concentration, and (ultra) centrifugation, followed by density gradient ultracentrifugation with OptiPrep™. Using a label-free proteomic approach, *E. faecium* EVs proteome was profiled for the first time (**Paper I**), and our data revealed that growth phase and growth conditions (*e.g.*, media and exposure to the sub-MIC dosage of antibiotics) could influence the proteome content (**Paper I**). Aligned with other studies, the EV cargo in *E. faecium* was found to contain a wide range of different proteins including vaccine candidates, antimicrobial resistance determinants and virulence factors (**Paper I**). The sub-inhibitory dosage of vancomycin also influenced the proteome profile of MRSA and revealed relatively increased expression of EV- associated proteins that might be involved in bacterial colonization and antibiotic resistance (*e.g.*, multiple antibiotic resistance regulators, amino acyltransferases, and penicillin binding proteins) (**Paper III**). Supplementation of purified EVs in a typical MIC assay using bacteriologic and tissue-mimicking media, RPMI, attenuated MRSA susceptibility to vancomycin (**Paper III**). This might suggest the act of EVs as decoy, in agreement with several other studies. The release of EVs was increased when the *S. aureus* MSSA476 was grown in iron-depleted BHI in presence of sub-inhibitory concentration of vancomycin (**Paper II**). Intriguingly, transcriptomic analysis of RNA isolated from MSSA476-derived EVs revealed presence of various RNA biotypes including mRNA, rRNA, tRNA, and small RNA (sRNA). Three enriched sRNAs (SsrA, RsaC, and RNAIII) were further validated by qPCR, conventional PCR and Sanger sequencing (**Paper II**).

The obtained transcriptome and proteome data from our research can provide basis for future detailed studies of the EV functions. Further characterization of EV cargos will provide grounds to tailor EVs for the therapeutic purposes such as vaccines and biomarkers for diagnostics and prognostic use.

1. Introduction

1.1 Enterococci

1.1.1. Nomenclature and taxonomy

The term “entérocoque” was coined by Thierscelin during 1899, while he isolated the bacterium from the patients suffering from infective endocarditis[1]. In Greek, the word “entero” (“έντερο”) mean “intestine” and “coccus” (“κόκκος”) mean “spherical particle or granule,”- reflecting the origin of the organisms and its morphology. However, later they were classified as group D streptococci based on Lancefield's precipitin test[2,3]. In 1970, Kalina and colleagues proposed to reclassify enterococci based on detailed studies on biochemical, cultural and molecular techniques[4]. The *Enterococcus* genus belongs to Enterococcaceae family and to date 68 species and two subspecies have been reported. (https://www.bacterio.net/genus/enterococcus#r]

1.1.2. Biology and general characteristics

Enterococci are Gram-positive cocci that usually occur individually, in short chains, or in a group. They are catalase-negative (although a few strains are catalase-positive), non-spore forming, chemo-organotrophic facultative anaerobes and some of the species also produces pigment[5]. Most of the species of *Enterococcus* are non-motile except *Enterococcus gallinarum* and *Enterococcus casseliflavus*. The genome of *Enterococci* is highly malleable with low G+C content (36-40%), but this can vary among species. The average length of *Enterococcus* genomes is ~ 3.20 Mb[6]. They are mesophilic organisms, but can survive in a temperature ranges from 10°C-60°C for about 30 min[7]. They can thrive in a broad pH range (4.8-9.6), but optimal growth has been observed at pH 7.5. They have been isolated from high-salt concentration (6.5% NaCl, 40% bile salt), which would otherwise inhibit other streptococci[8]. Remarkably, they are also able to tolerate high heavy metal concentrations and desiccation[9]. They are ubiquitous and found in plants, soil, water, fermented food and dairy products and the gastrointestinal tract (GIT) of various insects, reptiles, birds and mammals.[10,11].

Approximately 1% of the microbial community of the GIT are constituted of the genus *Enterococcus*[12]. Of note, *Enterococcus faecium*, *Enterococcus faecalis*, *Enterococcus durans,* and *Enterococcus avium* are the species most frequently colonizing the human GIT. These species are capable of utilizing a variety of sugars and generally, one of the predominant end

products of their sugar metabolism is lactic acid. Hence, they are also classified as lactic acid bacteria[13].

1.1.3. Pathogenicity of enterococci

Pathogenicity refers to the "ability of an organism to cause disease (i.e., harm the host)"[14]. Enterococcal colonization of the GIT is considered to be an important risk factor for infection. *E. faecalis* and *E. faecium* are predominantly found in the GIT as commensal microorganisms. However, exposure to broad-spectrum antibiotics, results in skewing of the microbial community referred to as "dysbiosis". Microbial dysbiosis, along with impairment of the host immune system facilitates flourishment of the opportunistic pathogens. They can cause diseases ranging from minor urinary tract infections and soft tissue infections to severe bacteremia, neonatal sepsis and endocarditis[15]. The severity of infection depends upon various factors including virulence factors (VFs) released by the bacteria, host immune status and site of infection[16]. Various epidemiological and observational cohort studies examined the risk factors associated with enterococcal infections[17]. Among them, age of patients, duration of the hospital stay and other co-morbidity such as diabetes, cancer, organ transplant (kidney, heart, and liver), surgery like heart-valve or hip replacement, long-term indwelling catheters, and prior antibiotic exposures are major risk factors for enterococcal infections[18-20]. Upon broad-spectrum antibiotic treatment, antibiotic-resistant bacteria outcompete commensal bacteria, which generally restrict enterococcal proliferation. Specifically, inhibition of Gram-negative organisms via antibiotics will facilitate a downregulation of REGIIIγ- a secreted C-type lectin that keeps the number of Gram-positive bacteria, including enterococci low[21].

1.1.4. Enterococci cause hospital-acquired infections

E. faecalis and *E. faecium* are mainly responsible for infections. During 1980s', *E. faecalis* was the most predominant species (80-90%) identified in the clinical samples. On the other hand, *E. faecium* only constituted less than 10% of enterococcal clinical specimens. Interestingly, there has been a gradual increased ratio of infections by *E. faecium* compared to *E. faecalis* recent years. Vancomycin resistant infection are more challenging, given vancomycin are used as last resort antibiotics to treat multi-drug-resistant (MDR) pathogens[22,23] and the *E. faecium* are more often vancomycin resistant than *E. faecalis*[24,25].

Enterococci can also easily adapt to the hospitals settings due to their unique tolerance to commonly used antiseptics, disinfectants, UV radiation, desiccation and starvation[26,27]. Furthermore, they are intrinsically less susceptible towards most available antibiotics of various

classes such as penicillins, cephalosporins and aminoglycosides[28,29]. The vancomycin resistant enterococci (VRE)-contaminated inanimate objects such as doorknobs of hospitals, over-bed tables, alarm buttons, toilet seats, and medical surfaces (blood-pressure cuffs, catheter devices) are potential hidden reservoirs and high-contact points for the transmission[26]. It is challenging to eradicate major reservoirs of multidrug-resistant strains in hospital wards since *E. faecium* can easily tolerate low concentrations of chemicals such as alcohol and chlorine[27,30,31], which are generally used as disinfectants in hospitals. Another important factor why it is difficult for healthcare workers to control infections caused by enterococci is because enterococci are capable of surviving on inanimate objects 3-5 times longer compared to other Gram-positive nosocomial pathogens. They can even survive in a dry environment without nutrition for more than five years in a hostile ecological niche such as hospital[30]. Moreover, hospital settings also facilitate the selection and/or exchange of antibiotic resistance among strains as well as continuous genomic organization through recombination and horizontal gene transfer (HGT). The latter drives the evolution and clonal expansion of hospital acquired lineages[32].

1.2. *Enterococcus faecium* and antibiotic resistance

Within the last three decades, *E. faecium*, the harmless commensal quickly turned into a notorious pathogen and ranked among frequent causes of bloodstream infections[33]. The burden of diseases is amplified as *E. faecium* turned out to be intrinsically resistant to various classes of antibiotics such as cephalosporins, aminoglycosides, clindamycin and trimethoprim-sulfamethoxazole[34]. In addition, resistance to glycopeptide antibiotics such as vancomycin is spreading rapidly, which is considered as an important hallmark in the evolution of enterococci towards MDR pathogen. *E. faecium* turned out to be resistant towards vancomycin due to acquisitions of *van* gene clusters[35]. Currently, there are eight different acquired *van* clusters, described as *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN* that mediate varying degrees of susceptibility (i.e., resistance) to the glycopeptides antibiotics such as vancomycin[36]. Of note, *vanA* and *vanB* gene clusters are most prevalent because they often are transferable via plasmids and other mobile genetic elements (MGE). Overexpression of the *van* genes results in an alteration in the synthesis of peptidoglycan precursors thereby decreasing the binding affinity of vancomycin[37]. Due to the acquisition of the vancomycin resistance clusters in MGE, the propensity of dissemination of these resistant genes to other isolates via HGT is also enhanced. Enterococci are not only able to exchange resistance gene within species but also show inter-species gene transfer mostly with other Gram-positive organisms such as staphylococci and streptococci thereby worsening the situation[38].

E. faecium is one of the high-risk pathogens belonging to the "***E***SKAPE" group. The acronym "ESKAPE" encompasses a collection of six most troublesome pathogens (***E****nterococcus. faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* species), that are capable of escaping all available conventional antibiotics treatment, thus creating a global health threat. Worldwide, the morbidity and mortality rates caused by ESKAPE pathogens ranging from 30 to 70%[39]. Patients suffered from Vancomycin Resistant *Enterococcus faecium* (VREfm) along with other comorbidities (old age, diabetes, organ transplant) required hospitalization for treatment. VREfm infection in patients with underlying co-morbidity can also cause sepsis. To treat the patients, they need hospitalization and intensive care unit (ICU) facility that create a financial burden especially to the patients from third-world nations[40]. The treatment procedure are often lengthy and during this period the risk of transmission of the infection to other healthcare personnel working in close proximity with infected patients is also potentially higher, leading towards sporadic outbreak in hospital settings[41].

1.2.1. Vancomycin-Resistant *Enterococcus faecium* (VREfm)

Shortly after Leclercq and colleagues reported the isolation of vancomycin resistance *E. faecium* (VREfm) in 1986, it became public health threat[42]. First reported in England, a year after, it was reported in the United States[43] and now is documented to be worldwide[42,44]. According to annual reports published by the European Antimicrobial Resistance Surveillance Network (EARS-Net), VREfm in EU countries increased from 10.2% in 2015 to 17.3% in 2018. The prevalence of VREfm in the United States is reported to be 66%, Australia 48.7-56.8% and Latin America 31%[45-48]. Another prospective study conducted in Brazil among 26 different hospitals reported that more than 60% of *E. faecium* isolates were VRE during 2007-2015[49]. A five year data (2012-2017) analysis obtained from German Antimicrobial Resistance Surveillance reveal that VREfm were higher among population aged 40-59 years compared to young and adolescent groups[50].

Along with an increase in the prevalence of VREfm, the attributable death associated with VRE was also found to be doubled between 2007-2015[51]. Due to the rising trend of VREfm across the globe, the World Health Organization and the U.S. Centers for Diseases Control and Prevention listed VREfm as a high priority pathogen. Appropriate epidemiological surveillance and infection control measure are essential to prevent outbreaks of VREfm.

1.2.2. Evolution of *E. faecium* genome

Before genome sequencing became easily available, Multilocus sequence typing (MLST), an unambiguous procedure for characterizing isolates of bacterial species with a set of seven housekeeping genes (*adk*, *atpA*, *ddl*, *gyd*, *gdh*, *purK*, and *pstS*) was used to determine sequence type (ST) of an isolate. The hospital associated isolates were then clustered into clonal complex 17 (CC17) using the eBURST algorithm. CC17 possess important characteristics such as resistance to antibiotics (ampicillin and quinolone), and harbor a pathogenicity island encoding gene for enterococcal surface protein (Esp) in the majority of isolates[52]. The most important sequence types in the CC17 lineage are ST17, ST18 and ST78 which cluster separately from the community isolates[53]. Recently, Turner and co-workers showed that the reliability of eBURST network to infer information about evolutionary relationship is not accurate for genomes with high recombination rate such as *E. faecium* leading to error prone phylogenetic analyses[54] and inappropriately linkage of lineages ST17, ST18 and ST78 into a clonal complex.

To understand the evolution of *E. faecium* from commensals to hospital-adapted strains, Lebreton *et al.*[55] sequenced the genomes of 51 *E. faecium* strains, which showed that the genomes grouped into two main clades. Clade A consists of hospital associated and animal isolates, while clade B isolates especially are of human commensal origin. With the aid of comparative genomics, clade A was further sub-divided into clade A1 (hospital-associated), which includes the former CC17, and clade A2 (animal-associated) (**Figure 1).**

The diversification of nosocomial clade A from commensal clade B started approximately started 3000 years ago - a time wherein human beings started domestication of animals and urbanization and hygiene practiced were introduced. Moreover, with the introduction of antibiotics 75 years ago in clinical medicine and agriculture, there has been another bifurcation between subclades A1 and A2. This phenomena might reflect that human intervention such as domestication and antibiotic use played a pivotal role in evolution of enterococci[56]. Notably, clade A1 has been circulating all over the globe and distinguished from other lineages as they have some specific features such as resistance to ampicillin and quinolone. The ability to sustain in harsh environment like the hospital environment is probably due to resistance to disinfectants and survival in draught environment. Indeed, hospital-adapted strains have bigger genome size due to acquisition of MGEs as well as antimicrobial resistance and virulence determinants[55,57,58]. The flexible genome complemented with the difference in restriction modification system, presence of an Insertion sequence element and a pathogenicity Island which likely assist *E. faecium* in incorporating genes involved in antimicrobial resistance and

virulence, which enable them to adapt to hostile circumstances, colonizes and cause pathogenicity in their host[59-61].

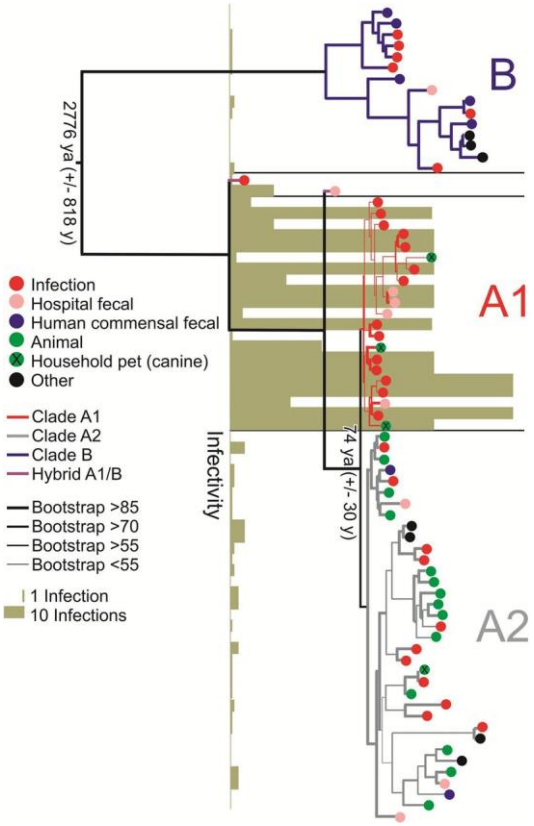


Figure 1: Clade structure of *E. faecium*. The origins of the strains and the dates for the split between the clades are indicated (ya, years ago). Reprinted with permission, modified from mBio[62].

1.2.3. Virulence factors of *E. faecium*

Virulence is derived from Latin word "virulentus", meaning "full of poison" or the capacity of a pathogen to cause disease/infection[14,63]. In other words, the quantitative measurement of pathogenicity is termed as virulence. Bacteria express a multitude of virulence factors (VFs) to enhance their pathogenicity[32,64]. VFs enable pathogens to colonize and proliferate better subsequently leading to tissue damage and infection[65]. In addition, they play a vital role in escaping from host defenses systems[66,67]. They are encoded by genes integrated in the genome both on the chromosome and on extrachromosomal elements, often on MGEs termed plasmids. They can also be encoded on pathogenic islands or bacteriophages. Conventional VFs include non-secreted or secreted proteins involved in adherence, tissue invasions, and escape from host immune surveillance or in forming pores in eukaryotic cell membranes (pore-forming toxins). Moreover, determinants involved in providing nutrients such as iron scavengers (*e.g.*, siderophores), catalases and regulators, also play a pivotal role during infection. There are

comprehensive databases such as VirulentPred[68] and Virulent-GO[69], virulence factor database (VFDB)[70], victors[71] available to predict the virulence factors present in bacteria.

E. faecium harbor a surplus of VFs which reinforce the pathogen to flourish as versatile opportunist mediating nosocomial infections. The characterization and knowledge on the VFs will provide insight on understanding the complex pathogenic process of opportunistic bacteria such as *E. faecium*. The detailed list of virulence factors found in *E. faecium* are described in **Table 1.**

Table 1: Overview of *E. faecium* virulence factors. Virulence factors utilized by *E. faecium* include cell wall components, secreted virulence factors and membrane-bound factors. Table modified from[32].

Virulence factors	**Biological effects**
Lipoteichoic acid (LTA)	Contributes biofilm formation and increased susceptibility to antimicrobial peptides.
Wall teichoic acid (WTA)	Involves in host cell attachment. Protects against complement-mediated killing mechanism of neutrophils.
Enterococcal surface protein (Esp)	Promotes biofilm formation, different variants in nosocomial isolates.
Collagen-binding adhesin (Acm)	Binds to collagen types IV, laminin and dentin, assist in biofilm formation.
Second collagen -binding adhesion (Scm)	Binds to collagen type V and fibrinogen.
Fibronectin-binding protein (fnm)	Binds to fibronectin and play key role in pathogenesis of endocarditis.
Proline-rich protein (PrpA)	Binds to immobilized fibrinogen and fibronectin.
E. faecium collagen binding protein (EcbA)	Binds to the collagen type V and fibrinogen.
Pili (PilA and PilB)	Contributes to adherence during biofilm formation.
Gelatinase (gelE)	Hydrolyzes gelatin, collagen, and other small peptides. Inhibits complement mediated immune responses and contributes biofilm formation.
E. faecium secreted antigen(sagA)	Binds to fibrinogen, collagen type I, collagen type IV, fibronectin, laminin and contributes biofilm formation.
Catabolite control protein A (CcpA)	Affects growth and biofilm formation capacity.
Autolysin (AltA)	Contributes to biofilm stability and Acm localization.

Serine glutamate repeat A (SgrA)	Binds to the extracellular matrix nidogen 1 and nidogen 2. Involved in pathogenesis of catheter related infections and biofilm formation.
TIR-domain-containing proteins (TirEs)	Promotes bacterial proliferation in human blood and may contribute to the pathogenesis.
Cytolysin (Cyl)	Facilitates infection via damaging host cell membrane.
Capsule (CapD)	Contributes in biofilm formation and evade from host immune system such as attack from neutrophil and phagocytosis.
PTS permease	Promotes biofilm formation in presence of human serum and pathogenesis of infective endocarditis
Biofilm and endocarditis-associated permease A (BepA)	Contributes in biofilm formation and infective endocarditis

1.3. Staphylococci

1.3.1. Nomenclature and taxonomy

Historically, Alexander Ogston described *Staphylococcus* in 1880s while he isolated the pathogen from wound infection[72]. The word *Staphylococcus* originate from Greek "Staphyle" **(**σταφυλαί) which means bunch of grapes and "coccus" ("κόκκος") means spherical particles or granule. In 1884, Rosenbach differentiated *S. aureus* from *S. albus* based on colony characteristics on agar plate. *S. aureus* impart golden yellow color (from Latin *aurum*, gold) and *S. albus* white (Latin *albus*, white)[73]. Later, *S. albus* was renamed *S. epidermidis* because they are native flora of human skin. Interestingly, *S. aureus* is one of the first described pathogen and still knowledge on this bacterium is continuously expanding till date[74].

The genus *Staphylococcus* belongs to Staphylococcaceae family and till date 63 species and 30 subspecies have been validated and deposited in Prokaryotic Nomenclature database (https://lpsn.dsmz.de/, report accessed on 11.09.2020).

1.4. *Staphylococcus aureus*

Staphylococcus aureus are Gram-positive bacteria often clustering resembling "bunch of grapes" under microscopic observation[75,76]. Although they can remain as commensals, they are capable of causing infection in humans and animals. *S. aureus* are aerobic and/or facultative anaerobes, catalase positive, oxidase negative, coagulase positive, non-spore forming microorganism. Some of the strains are also capsule formers[77]. The bacterium has a diameter of 0.5-1.0 μm[78]. The thick cell-wall of the bacterium consist of peptidoglycan backbone together with wall teichoic acid (WTA), forming tough insoluble heterogenous matrix[79,80]. In addition, *S. aureus* are differentiated from other members of Staphylococci as it has yellow pigment production and ability to produce coagulase. The secondary metabolites staphyloxanthin secreted during stationary phase impart yellow pigmentation[81]. In addition, staphylococci can tolerate high concentration of salt and grow in presence of 10% NaCl[82]. *S. aureus* is furthermore capable of producing plethora of toxins (α, β, γ, δ, exfoliative, enterotoxin) having wide spectrum of hemolytic and cytolytic activity[83,84].

1.4.1. Colonization and pathogenesis

S. aureus is an opportunistic pathogen and humans are frequently colonized for a short term or throughout the life. Approximately, 20-30 % of healthy adult population carry *S. aureus* in the nostril as a part of persistent normal flora. However, *S. aureus* can also remain a transient flora

of skin, throat, perineum and intestine[85]. To be defined as persistent nasal *S. aureus* carriers, at least two-culture positive isolated from a nasal swab in one-week duration is required[86]. Colonization is important risk factor for subsequent *S. aureus* infection. Multiple factors, including array of adherence as well as immune-modulating proteins expressed by the bacteria, in addition to host factors, and presence of local microbiota influence *S. aureus* colonization and infection. In a host-microbe interface, there is a battle between immune system of host and pathogenicity of the bacteria. Humans are protected against infection by both innate and adaptive immune system, in addition to physical barriers such as skin, epithelial and mucous membrane surfaces. *S. aureus* are inhibited in crossing the skin barrier due to secretion of antimicrobial peptides, such as defensins and cathelicidins[87]. An accidental breach of such barriers poses a risk to human infection. Moreover, immune status of host also affects the severity of the disease that ranges from minor skin infections to life-threatening diseases such as endocarditis, meningitis, osteomyelitis, hemolytic pneumonia and toxic shock syndrome[88].

1.4.2. *S. aureus* genome

The first *S. aureus* genome was sequenced in 2001[89] and till date more than 150 *S. aureus* genomes have been sequenced and are available in NCBI genome database. The staphylococcal genomes are about 2.8 Mbp with GC content of 32.7%. The genome is classified into core genome and accessory genome. Approximately 75% is defined as core genome, while the rest is accessory genome, which may contain MGE including plasmids, parts of bacteriophage genomes, pathogenicity islands, transposons and staphylococcal cassette chromosomes (SCC). The core genome is mostly conserved among the species and accessory genome play a pivotal role in clonal evolution, which provide the beneficial advantage to the strain to escape from available antibiotics, colonization new host and adapt to new ecological niches[90].

1.4.3. Methicillin resistant *Staphylococcus aureus* (MRSA)

Methicillin-resistant *S. aureus* was first reported from clinical isolates from hospitalized patients in England in 1961, only two years after methicillin was introduced into clinical practice to treat infections caused by *S. aureus*[91]. However, treatment with methicillin in hospital settings was discontinued due to its toxicity and replaced by penicillins and their derivatives such as oxacillin, flucloxacillin and dicloxacillin. Nevertheless, the acronym MRSA continues to be used until date. The spread of MRSA probably occurs through disseminations of existing resistant clones or by acquisition of *SCCmec* by a methicillin-sensitive *S. aureus* (MSSA) strain. SCC*mec* is a mobile genetic element that encodes a variant methicillin-resistant penicillin-binding protein absent in MSSA[92]. Due to acquisition of SCC*mec*, MRSA strains are

mostly resistant to all β-lactam antibiotics such as penicillin. During the 1960s', MRSA strains were only confined to the hospital environment, but the prevalence is increasing all over the globe. However, the prevalence of MRSA is highly variable among different geographical regions, ranging from < 1% in some Northern European countries to > 50% in some American and Asian countries[93]. The incidence of MRSA exceeds 20% from the recent data reported from 85 World Health Organization (WHO) member states countries[94].

Community acquired methicillin-resistant *S. aureus* (CA-MRSA) are frequent causes of infection and considered more virulent and pathogenic than hospital acquired MRSA strains (HA-MRSA). Generally, there is no sharp distinction between HA-MRSA and CA-MRSA. Nevertheless, CA-MRSA emerged during late 1990s and is capable of causing skin and soft tissue infections even in healthy and young people. CA-MRSA strains harbor SCC*mec* types IV or V, Panton Valentine Leukocidin (PVL) encoding genes LukS-PV and LukF-PV, as well as arginine catabolic mobile element (ACME) cluster[95-97]. SCC*mec* is a mobile genetic element that harbors resistance genes for beta-lactam antibiotics[98]. PVL is a prophage-encoded toxin involved in white blood cell lysis[99] and ACME assists in enhanced transmission, survival and persistence of the pathogen[100]. Among CA-MRSA clone, USA300 belonging to CC8 lineages was successful in adaptation and spread rapidly across the globe (predominant in the United States, Canada and Europe). They are able to adapt and transmit infection and cause outbreaks in various settings such as prisons[101], children day care centers[102], youth sports center[103], and military camps[104]. Of concern, MRSA isolates are only susceptible to few antibiotics such as vancomycin, linezolid, teicoplanin and clindamycin. However, MRSA isolates have recently been reported to have reduced susceptibility to vancomycin, known as vancomycin intermediate-resistant *S. aureus* (VISA) (MIC=4-8 µg/mL) and complete resistance to vancomycin (MIC≥16 µg/mL) termed vancomycin-resistant *S. aureus* (VRSA)[105]. VISA and VRSA raises prominent public health threat, given vancomycin was last resort antibiotic to treat MDR infections. Due to limited therapeutic options available to treat MRSA, understanding the pathogenesis of MRSA and molecular characterization using omic approaches (*e.g.*, transcriptomic and proteomics) are essential[106].

1.4.4. Virulence factors in *S. aureus*

S. aureus contain diverse set of VF, including in average 24 different cell wall anchored proteins and over 40 secreted proteins and enzymes involved in steps from host colonization to pathogenesis[107]. The pathogenicity of *S. aureus* is driven by a battery of virulence factors involve in adhesion, invasion, as dissemination to the hosts[108]. The cell wall anchored proteins

include microbial surface proteins such as clumping factors, fibronectin binding proteins and Protein A. The secreted proteins involve extracellular enzymes such as protease, hyaluronidase, lipase, coagulase and proteases, as well as a wide range of toxins (pore-forming toxins, exfoliative toxins and super antigens). The combination of the VFs contributes to adhesion, host tissue penetration, immune evasion and proliferation of the bacteria causing the disease (**Figure 2**). The VF are expressed depending on the requirement of pathogens to establish infection. For example, at the early colonization stage, adhesion proteins are expressed while for later onset of infection, toxins play major roles. In addition, VFs also facilitate long-term persistence inside host through *e.g.*, its ability to evade host immunity. The regulation of VFs is tightly controlled and coordinated and varies according to growth factors/and or growth conditions[109]. The innovative proteomics and advanced bioinformatics tools have made it easier to explore VF expressed by organisms, including evaluating their sub-cellular localization and putative biological function.

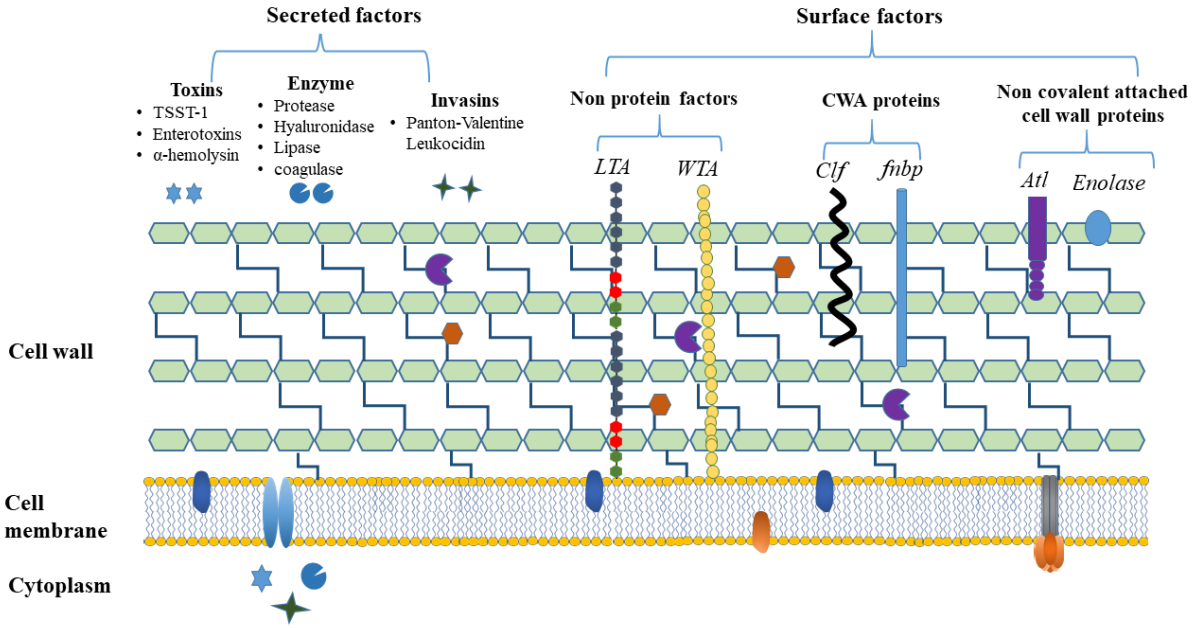


Figure 2: Schematic representation showing examples of *S. aureus* virulence factors including surface factors and secreted factors. Examples of secreted factors include toxins, enzymes and invasins. Surface bound factors include structures such as lipoteichoic acid (LTA), wall teichoic acid (WTA) as well as cell wall-anchored (CWA) proteins including clumping factor (Clf), fibronectin binding protein (FnBP), autolysin (Atl) and enolase. Figure modified from[110].

1.5. Regulatory RNA and virulence

RNA is no longer considered as only an intermediate link as mRNA between genome and proteome. Indeed, there are diverse functions encompassed by the RNA molecules. They can also be involved in gene regulation at the transcriptional/and or translational level[111,112]. Approximately ~20 % of the bacterial genes code for RNAs that have regulatory functions. With few exceptions like RNAIII, most of the regulatory RNA are about 80-200 nucleotides in length [113]. The regulatory RNAs are also described as small RNAs (sRNA).

Generally, prokaryotic sRNAs are non-coding, mainly originate from intergenic regions and are involved in gene regulation[114]. Based on the locations of sRNA genes and their targets, sRNA can be divided into different categories, the main two being *cis*-encoded RNAs and *trans*-encoded RNAs. *Cis*-encoded RNAs base pair with target mRNAs with perfect complementarity and are located in the same DNA region as their target mRNA. *Trans*-encoded RNAs are located in another genomic location and only share partial complementarity to their target mRNA. The hybridization of sRNA with mRNA control gene expression by modulating translation and/or stability of their target mRNA[111,115]. Moreover, Riboswitches are *cis*-regulatory elements involved in gene regulation at the levels of the transcription and translation. Riboswitches consist of two structural domains: a ligand binding aptamer, and an expression platform[113,116]. Upon binding of ligand with aptameric region, the riboswitch undergoes conformational changes and regulate gene expression of downstream protein coding sequence (CDS) region[117,118].

Bacterial sRNAs are involved in diverse biological processes such, carbohydrate metabolism, metabolite transport, synthesis and degradation. They can also contribute in), growth processes (*e.g.*, toxicity, biofilm formation), adaptation to stress and varying culture conditions such as temperature, iron limitation or cell density. Moreover, recent evidence shows that regulatory RNAs play key roles in microbial pathogenesis[116,119,120].

1.6. Bacterial Extracellular vesicles

1.6.1. Nomenclature

Secretion of vesicles is considered as a universal phenomenon which occur in prokaryotes (Gram-positive and Gram-negative bacteria), eukaryotes and archaea. The vesicles are described and termed differently depending upon the cell-type from which they originate. In Gram-negative bacteria, the vesicles are termed outer membrane vesicles (OMVs), while in Gram-positive bacteria, they are commonly referred to as membrane vesicles (MVs), extracellular vesicles (EVs) or cytoplasmic membrane vesicles (CMVs) as they originate from the cytoplasmic membrane[121]. Hence, the International Society for extracellular Vesicles (ISEV), recommend "**Extracellular Vesicle**" as an appropriated generic term for "particles naturally released from the cell that contain lipid bilayer and cannot replicate"[122]. Hereafter, the abbreviation EVs will be used in the thesis for mentioning vesicles from both Gram-positive and Gram-negative bacteria.

1.6.2. Structure and cargo of extracellular vesicles

EVs have spherical shape and are heterogeneous in size ranging from 50-500 nm[123,124]. These nano-sized bubbles are either attached to bacteria or released from bacterial cell surfaces during their growth (**Figure 3**). The maximum yield of EVs released by bacteria occur at late exponential phase and stationary phase of bacterial growth[125]. The EV cargo is packaged with various bioactive molecules such as toxins, enzymes, virulence factors, quorum sensing molecules, metabolites, and nucleic acid (DNA, RNA) (reviewed in[126,127]). Integrated database such as EVpedia (http://evpedia.info), Vesiclepedia (http://microvesicles.org/), and EV track (https://evtrack.org/) provide information on proteins, lipids, and nucleotides (DNA, mRNAs, miRNAs) enclosed in prokaryotic, non-mammalian eukaryotic, and mammalian EVs.

The vesicles may also fuse and form elongated cylindrical shaped nanotube like structures. Such nanotubes are co-purified together with EVs from various Gram-negative and a few Gram-positive bacteria including *S. aureus*[128]. The composition and cargo of bacterial EVs is illustrated in **Figure 3.**

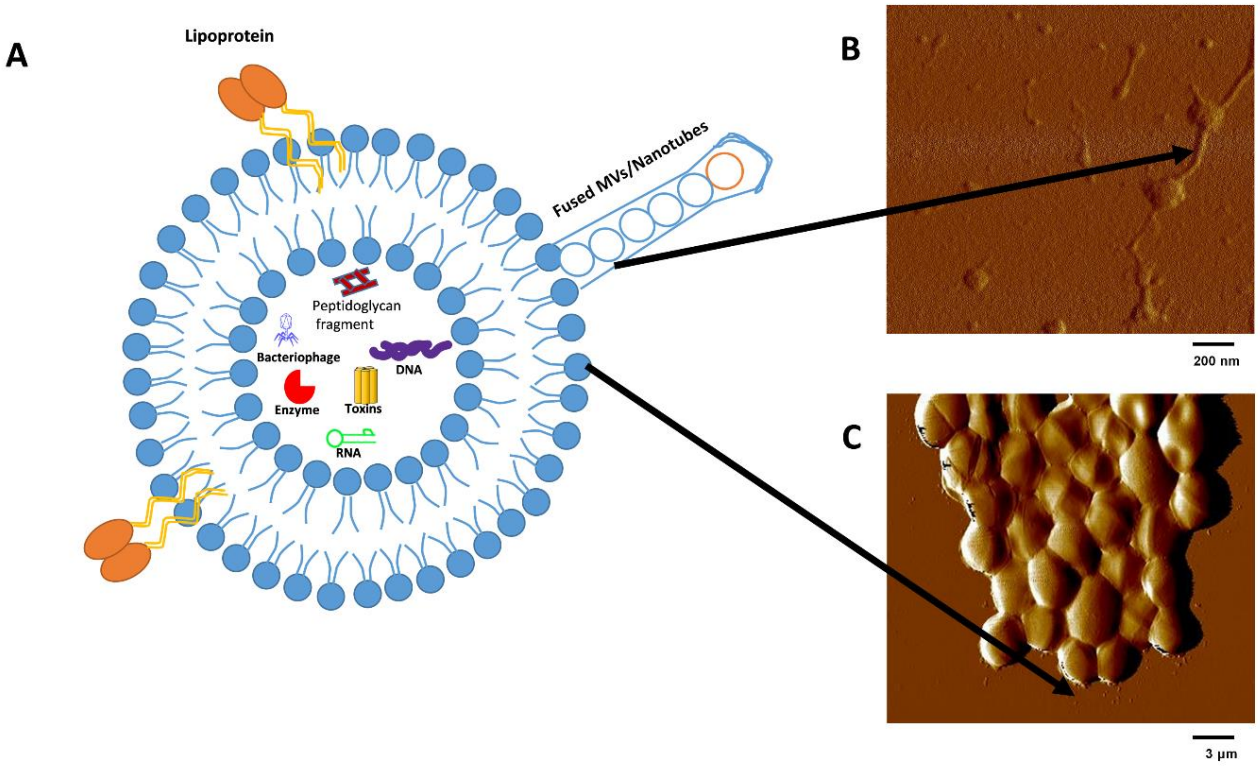


Figure 3: A) Schematic representation of EVs and its cargo, containing bioactive macromolecules. B) Atomic Force Microscopy (AFM) image of EVs forming nanotubes (Black arrow). C) AFM image of *S. aureus* grown in Brain Heart Infusion (BHI) releasing EVs (Joshi, Hegstad, Johannessen, unpublished).

1.6.3. Extracellular vesicles from Gram positive bacteria

The fact that Gram-positive bacteria also release vesicle was proven in the 1990s for *Bacillus cereus*[129]. Researchers were skeptical regarding the possibility of EV release from Gram-positive bacteria as they possess a thick-peptidoglycan layer (50-100 nm) and lack outer membrane. Hence, it took almost 30 years to fully realize the importance of EV release from thick-wall organisms[130]. Lee and coworkers performed characterization of EVs released by *S. aureus* in 2009[131]. Thereafter, multiple researches are ongoing on proteomics characterization of EVs cargo and their function from Gram-positive bacteria (**Table 2**). The total number of EVs associated proteins varies according to growth phase at which EVs have been harvested, use of bacteriological media used for EVs isolation and also producer strains. It has been reported, the number of EVs associated proteins varies from 28 (*Filifactor alocis)* to 617 (*S. aureus*). Most of virulence factors including toxins and cell wall remodeling proteins (autolysin, penicillin binding proteins) have been found to be overrepresented in EVs cargo[132]. There are limited reports available on lipidomics analysis of EVs from Gram-positives bacteria. The

enrichment of short-chain saturated fatty acids were reported from *B. anthracis* and *S. pneumoniae* [133,134].

Table 2: EVs released by Gram-positive bacteria: their size, growth conditions from which they were isolated, number of proteins identified including virulence factors, and some remarks

Species	Size (nm)	Growth phase or OD600	Medium	Virulence factors	No. of protein	Remarks	Year^Reference
S. aureus (ATCC14458)	200 - 100	OD600 ~ 1.0	NB	Penicillin-binding protein (PBP1, PBP2, PBP3), β-lactamases, super antigen (SEQ1, SSAa1, SSAa2)	90	Isolated vesicular protein might play a role in bacterial pathogenesis, antibiotic resistance as well as eliminating competing microbes.	2009[131]
Bacillus anthracis 34F2	*50 - 300*	Late-exponential phase	BHI	Lethal factor (LF), oedema factor (EF; also known as Cya) and protective antigen (PA), anthrolysin (ALO)	36	Toxins were found to be associated with bacterial EVs, protected from degradation and allows to deliver into host cells in a highly concentrated form.	2010[134]
S. aureus	20 - 130	OD600 ~ 1.0	LB	β-lactamases, adhesins, proteolysin, coagulase	143	*S. aureus* EVs delivered their components to host cells through the interactions with lipid raft machinery.	2011[135]
Listeria monocytogenes	20 - 100	OD600 = 2.0	BHI	Internalin B (InlB) and listeriolysin O (LLO)	130	Transcription factor σB hold key role in vesiculation. EV associated virulence factors contribute towards pathogenesis under stressful conditions.	2013[136]
Clostridium perfringens	20 - 400	OD600 ~1.0	(TPG) broth	toxin-Beta2	431	EVs contained many ribosomal proteins, DNA polymerases and several metabolic enzymes, suggesting role of vesicles facilitating functional enzymes transfer.	2014[137]
Streptococcus pneumoniae (ST1)	20 - 80	Late-exponential phase (OD600 = 0.5)	THB	toxin Ply, hyaluronidase (hyl)	208	Pneumococcus- derived EVs enriched in lipoproteins confer protection against infections in animal model.	2014[133]
S. suis	13 - 130	Early stationary phase	THB	SspA, SsnA, Ide, Plr, HtpsC, and Mrp	46	EVs produced from *S. suis* can degrade NETs and thereby escape from the host defense response and also activate NF-κB pathway	2015[138]
S. pyogens	50 - 100	OD600 = 0.4	TSB	Penicillin binding protein1b, Streptolysin O	111	Vesicles associated with lipoprotein were unable to activate TLR2.	2015[139]

S. pyogenes (clinical isolate ISS3348*)*	10 and 272 nm	late-logarithmic-phase	THB	Streptolysin O, Penicillin binding protein	195	GAS EVs contain RNAs, lipids, proteins, and virulence factors. Genetic disruption of two-component regulator CovRS led to hypervesiculation.	2016[140]
B. subtilis	30 - 160	Late exponential phase	BHI	Alkanaine phospatase, superoxide dismutase.	Of 193 identified, 61 protein enriched in sporulating cells	In both vegetative growth phase and sporulation, EVs were detected. Variation in EV protein cargo was observed.	2016[141]
S. agalactiae strains A909 (serotype IA)	150 – 300	OD600 ~ 1.2	THB	extracellular matrix degrading enzymes	ND	GBS associated EVs induced apoptosis of HeLa cells. The collagenase properties of EVs cause preterm births in mouse model.	2016[142]
L. monocytogenes	50 - 350	ND	LB	Listeriolysin O (LLO)	ND	EV- associated toxin (LLO) inhibits autophagy as a survival strategy inside host	2017[143]
Lactobacillus reuteri DSM 17938	50 – 150	ND	MRS	ND	ND	EVs were released by both planktonic and biofilm phenotypes and eDNA was found to be associated with both planktonic and biofilm derived EVs.	2017[144]
L. plantarum WCFS1	31 – 200	Late exponential phase	MRS	ND	158	EVs produced from *L. plantarum* WCFS1 enhance host immune responses via upregulation of the two host genes, *REG3G* and *CTSB.*	2017[145]
Clostridium difficile ATCC 43255	ND	Late-exponential phase	BHI	ND	262	*C. difficile*-derived EVs stimulated the expression of pro-inflammatory cytokine, including interleukin (IL)-1β, IL-6, IL-8, and monocyte chemoattractant protein-1 in Caco-2 cell line	2017[146]
C. perfringens strain 13	ND	Checked EV production every 6 hour	BHI	ND	ND	Sporulation master regulator *spo0A* gene and orphan sensor kinase were required for vesiculogenesis.	2017[125]

S. pneumoniae BAA-255	40 - 200	Late exponential phase	THB with 0.5% yeast extract	ND	104	EV surface proteins can act as antigens that induce adaptive immune responses in the host	2017[147]
S. aureus	25 nm in LB, 50 nm in BHI	Stationary phase	LB and BHI	Catalase, α-hemolysin, Delta-hemolysin, Superoxide dismutase	131 in LB and 617 in BHI	Immunization of mice with *S. aureus* derived EVs confer robust humoral immune response thereby enhanced protection against S. *aureus* infection.	2018[124]
Filifactor alocis	50-270	OD600 = 1.0	Columbia broth	lipoproteins, autolysins,	28	EVs induced the expression of various cytokines (IL-1β, I IL-6, IL-8 etc) and chemokines (CXCL1, CXCL10) in THP-1 cells.	2020[148]
S. aureus strains of humans (CC1), Ovine (CC130), Bovine (CC 97, CC151)	120-170	Early stationary phase	BHI	Bifunctional autolysin Enolase Phenol-soluble modulin Glyceraldehyde-3-phosphate dehydrogenase	Out of 253 proteins, 119 represent EV core proteome	Core proteomes were found to be conserved among phylogenetically different species which strengthen the speculation regarding selective sorting of protein in EVs.	2020[149]
S. mutans	129.0 ± 8.08 nm at pH 7.5 and 105.5 ± 11.6 nm at pH 5.5,	mid-exponential phase	BHI of pH 7.5 and 5.5	Cell surface antigen SpaP, Glucan-binding protein, Dextranase	495 proteins at pH 7.5 and 351 proteins at pH 5.5.	Significant change in ABC transporters proteins were observed in EVs released from *S. mutans* from acidic and neutral pH	2020[150]

[1]

[1] ATCC- American Type Culture Collection
BHI- Brain Heart Infusion
GAS- Group A Streptococci
GBS- Group B Streptococci
LB- Luria Bertany
MRS- De Man, Rogosa and Sharpe agar
PAMP- Pathogen Associated Molecular Pattern
NB- Nutrient Broth
ND- Not Determined
NETs-Neutrophil Extracellular Traps
THB- Todd Hewitt Broth
TLR- Toll Like Receptors
TPG- Trypticase Peptone Glucose
TSB- Tryptic Soy Broth

1.6.4. Factors influencing EV release

The composition of EVs cargo are influenced by growth conditions, strain and the whether the bacteria are exposed to stress. Comparatively, Gram-negative bacteria produced more EVs than Gram-positive bacteria. Moreover, pathogenic bacteria produce more EVs compared to non-pathogenic bacteria[151,152]. The vesiculogenesis (formation of vesicles) is mostly studied in Gram-negative bacteria and route of vesicle formation occurs through budding of the outer membrane and hence termed OMVs. Likewise outer-inner-membrane vesicles (OIMVs)[153], explosive outer-membrane vesicles[154] and tube-shaped membranous structures were also observed from various bacteria, depending upon the inducer used for vesicle formation[155]. The biogenesis of EVs from Gram-positive bacteria despite having thick peptidoglycan layer and lack of outer membrane changed the view on vesicle biogenesis[126]. Toyofuku and colleagues reported that phage encoded endolysin create hole in thick peptidoglycan layer and trigger bubbling cell death, thus assisting release of vesicles from cytoplasmic membrane (**Figure 4**).

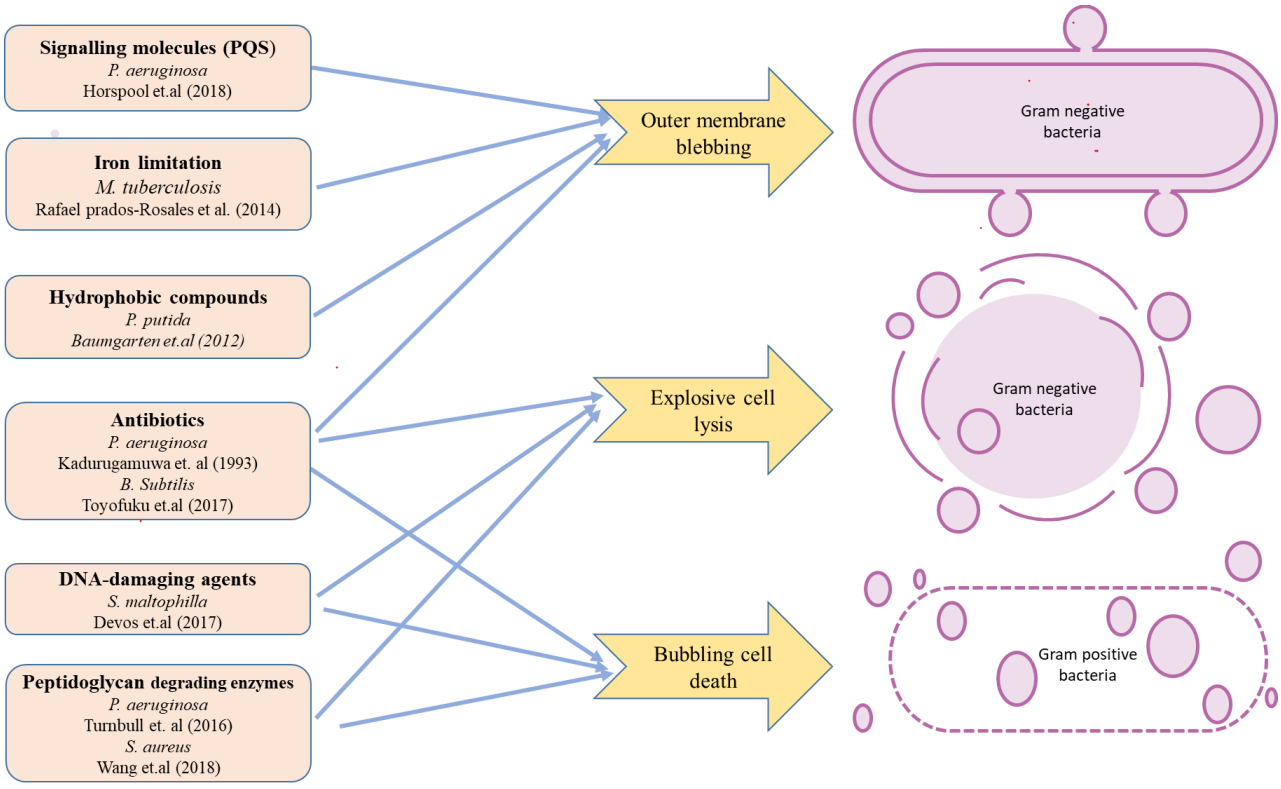


Figure 4: Various mechanisms of EVs formation, depending on bacterial exposure to stress. Figure modified from[127].

Various stress factors trigger bacteria to hypervesiculate. Among them, nutrient stress (iron depletion[156], cysteine depletion[157]), temperature stress (heat shock[158] and cold shock[159]), chemical induced stress (ethylene-diamine-tetra acetic acid[158], sodium chloride[158], hydrogen peroxide[160], 1-octanol[158]), to name a few. Furthermore, changes in growth medium also affects vesiculation. The growth medium affects gene expression pattern in bacteria which subsequently alter the amount and/or content of vesicle release [161]. Bager and colleagues found significant changes in *Gallibacterium anatis* EV production and protein composition upon altering medium, *e.g.*, by adding 1 mM EDTA to BHI medium, or by growing the bacteria in RPMI 1640 HEPES medium[162]. Similarly, *Pseudomonas putida* KT2440 EV production was three-fold higher in LB media than in two other minimal media containing succinate and benzoate[163].

Antibiotics are also considered strong stimulators for hypervesiculation. Mitomycin C, for example, induces genotoxic stress to the bacteria involving the RecA pathway, which then stimulate vesicle release. In addition, stress transcription factor sigB (σB) is responsible for an increment in EV yield in the food-borne pathogen *Listeria monocytogenes*. Lee and colleagues reported nine-fold increase in EV yield in a wild-type strain compared to its isogenic Δ*sigB* mutant of *L. monocytogenes*[136]. The shapes of the vesicles produced from the wild type were intact. Biogenesis of vesicles might be related to stress encountered by the pathogen. There has been observed a three-fold increase in EVs production in *E. faecium* when bacteria are stressed with vancomycin. In addition, under iron restriction, Rafael Prados-Rosales and colleagues observed more vesiculation from *Mycobacterium tuberculosis*[156]. EVs released by *S. aureus* contain cytolytic phenol-soluble modulins (PSMs) facilitating budding of vesicles from the cytoplasmic membrane. Autolysin activity promote EV biogenesis by disrupting the cytoplasmic membrane and altering bacterial cell wall permeability[164]. Collectively, EV biogenesis and release in Gram-positive bacteria seem to be dependent on phages, endolysins or stress that weaken the thick peptidoglycan layer.

1.6.5 Biological Functions of bacterial extracellular vesicles

Bacterial extracellular vesicle is type of secretion system that contain biomolecules required for the broad range of biological activities. The biological functions depend upon the content of the cargo. For example, the EV cargo from pathogenic bacteria is loaded with virulence factors including toxins that can damage host, while the cargo of commensal gut microbiota exchange nutrients via EVs and supports in the maintenance of gut health. In summary, EVs have both beneficial and harmful roles, partly depend on content of the cargo and its specific

pathophysiological trait induced in the host cells. In a microbial world, competition and co-operation are two important microbial traits required for microbial fitness and survival. The survival of *Mycobacterium* in a nutrient limiting environment (*e.g.*, iron limitation) is made possible by enriching iron binding proteins (mycobactin) with in EVs. The mycobatin not only help in uptake of iron from the environment but also support proliferation of iron deficient bacteria[165]. Furthermore, microbial competition is mediated by autolysin that are capable of lysing targeted bacteria. EVs released by Gram-positive bacteria play role in biofilm formation, exchange of nucleic acid mediating resistance, competition with intruders via release of antimicrobial compounds (autolysin lyse bacterial cell wall), supply of nutrients to support and enrich their own microbial community, delivery of toxin, and modulation of host-immune responses including host cell death. During the last decade, remarkable progress has been made in the field of EV from elucidating EV biogenesis to evaluating their functional role[166,167]. The functions are illustrated in **Figure 5.**

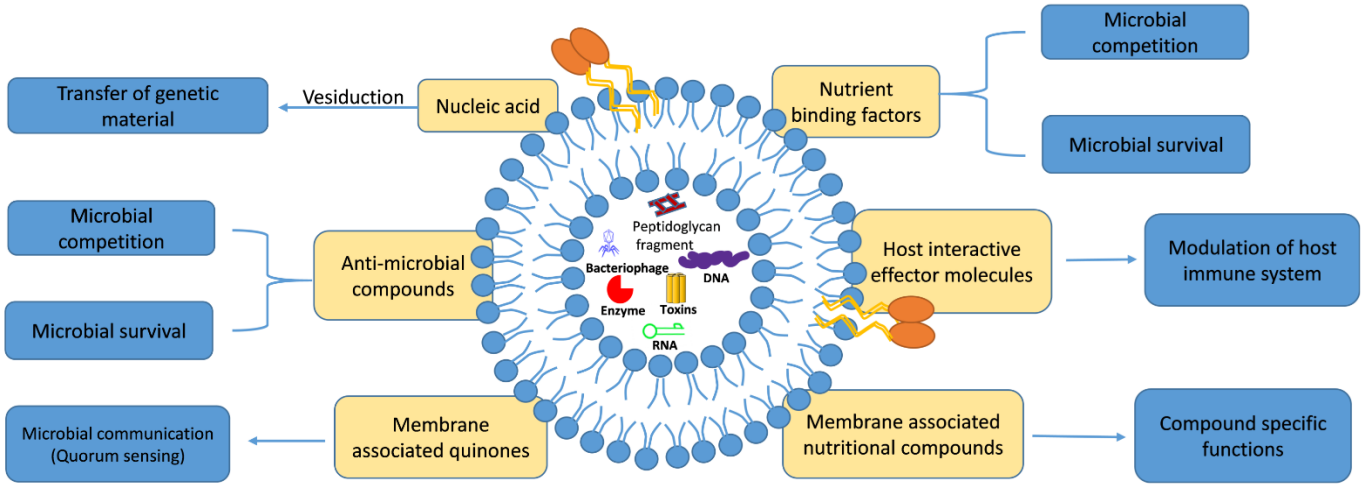


Figure 5: Biological function of bacterial EVs. Figure modified from[167].

1.6.5.1 Biological function of EVs associated toxins

Bacterial toxins have been found to be associated with EVs. Bacterial toxins are classified into exotoxin and endotoxins. Exotoxins are soluble proteins secreted by both Gram-positive and Gram-negative bacteria, while endotoxins are the lipopolysaccharides (LPS) of the Gram-negative outer membrane. Exotoxins are further classified based upon their structure and function as **a**) A-B toxins, **b**) membrane perforating/pore forming toxins (PFTs) and **c**) super antigens. Among them, PFTs are the most common water-soluble toxins that binds to target cells and create pores in the membrane at nanomolar concentration. PFTs including hemolysins

are found to be associated with EVs of *Bacillus, Staphylococcus* and *Streptococcus*[134,142,168]. They support pathogen growth and dissemination and alter plasma membrane permeability of their target cells, leading to target cell death. The pore size formed by toxins are quite variable among Gram-positive bacteria ranging from 2-100 nm. Toxins released by *L. monocytogenes* and *Streptococcus pyogenes* form 30-50 nm heterogeneous pores while alpha-toxin from *S. aureus* creates pores of only ~2 nm in diameter[169]. The packaging of toxins inside vesicles might be an evolutionary defense mechanism allowing bacteria to deliver toxins on distance. The wrapping of toxins inside EVs has multiple advantages such as **i**) a concentrated punch of toxins can be directed to a small surface area, increasing stability and half-life of the toxin and **ii)** toxins delivery can be redirected to discharge the EV content to particular host cell, thereby avoiding degradation from extracellular proteases and maximizing the potency of the toxins[130]. Noteworthy, one of the important toxins, (α-hemolysin; Hla) released by *S. aureus* EVs is delivered to human cells via fusion with the plasma membrane with the assistance of a cholesterol moiety. Group B *Streptococcus* (GBS), an opportunistic pathogen causing 20% neonatal death worldwide [170], is highly virulent due to extracellular matrix degrading proteases concentrated inside vesicles. It has been reported that the intra-amniotic injections of GBS EVs in mouse *in vivo* result in damage of chorio-amnionitis membrane, heightened immune response and released inflammatory molecules leading to subsequent mice death[142].

Bacterial toxins also influence autophagy; which is a mammalian intracellular way of removing invading pathogens/toxins. The pathogen/toxin are engulfed inside double membrane autophagosomes and delivered to a lysosome for degradation. Purified toxins such Hla and Listerolysin (LLO) released by *S. aureus* and *L. monocytogenes*, respectively, are inducing autophagy. Interestingly, Svitlana Vdovikova and colleagues reported that LLO-induced autophagy was abrogated by pre-treatment with EVs[143]. Similar, host cell death caused by pure LLOs was inhibited by pre-treatment with EVs released by same species. EVs not only protect against autophagy, but also against host cell necrosis initiated by pore forming toxins[143].

1.6.6 Membrane vesicles and health benefits

Besides pathogenic bacteria, non-pathogenic Gram-positive bacteria such as lactic acid bacteria (LAB) and species of *Bifidobacterium* also produces EVs. They are considered as good samaritans as they cross feed the commensal bacteria via vesicles to support fitness of gut microbial flora. The probiotic bacteria also outcompete the pathogenic bacteria, maintain the gut flora and prevent diseases such as inflammatory bowel disease[171]. EVs released by

Lactobacillus casei contain effector proteins such as p40 and p75 that has probiotic effects and also protect the intestinal epithelium layer against inflammation[172]. *Lactobacillus rhamnosus* derived EVs induced apoptosis of cancer cells. Moreover, *Bifidobacterium longum* derived EVs induced apoptosis of mast cells and abrogated food allergy[171]. EVs from probiotic bacteria *Lactobacillus plantarum* was found to induced *Caenorhabditis elegans* defense genes *clec-60* and *cpr-1* which resulted in clearance of VRE[145]. Interestingly, EVs released by vaginal microbiota (*Lactobacillus crispatus BC3, Lactobacillus gasseri BC12)* were capable of suppressing HIV-1 replication in cell line study[173].

1.6.7 EVs as therapeutic tools

The bacterial cell machineries that produces EVs have gained a huge attention because of versatile cargo that can be utilized for multiple therapeutics purpose using biotechnological tools. One of the most fascinating and promising areas is to use EVs for vaccine development Besides vaccine research, EVs can be successfully utilized as drug delivery tools, bioreactors for enzyme production and transport, phage therapy and potential as source of disease biomarkers and diagnostics[174].

1.6.7.1 Vaccination

Vaccination has been a remarkable achievement in reducing morbidity and mortality as well as combating infectious diseases. Most vaccines developed are based on the principle developed by Louis Pasteur "isolate, inactivate, and inject'' the disease-causing microorganism. Though these vaccines are safe and efficacious, their potential side effects cannot be ignored completely. To provide improved safety and efficacy, EV-based vaccines are gaining momentum[175-177]. The obvious advantage of using EV-based vaccines is their non-replicative and non–infectious nature and enhanced capacity to modulate innate and adaptive immune response. In addition, EVs contain some especial characteristics (small size, broad distribution of concentrated antigens, stable in human circulation, tolerate room temperature, and easily uptake from immune cells and provide long term memory response) qualifying them as excellent vaccine candidates[178]. The antigenic nature of EV cargo also impart intrinsic adjuvant properties.

MenB-4C (Bexsero) is an EV-based vaccine active against meningococcal disease, a bacterial form of meningitis caused by *Neisseria meningitides* has received license and are available for human use[179-181]. Protective effects of EV-based vaccines in mouse models have also been observed for a *B. anthracis*[134], *S. pneumonia*[133] , *C. perfringens*[137], and *S. aureus*[124,164,182].

Recent report suggested that EVs from *S. aureus* showed promising cell-mediated and humoral immune responses in mouse model suggesting EV-associated proteins confer protecting activity against lethal doses of *S. aureus* without the use of other adjuvants[124,183]. The presence of surface proteins and enrichment of virulence factors and toxins stimulate the immune response and protect against infection thus making EVs as ideal vaccine candidates. Although, there is a potential for EVs based vaccine, their randomized clinical trials in human are still in infancy and need rigorous research to be licensed for human use, ascertaining human safety.

1.6.7.2 Nanosystem for drug delivery

EVs can be utilized as carriers for drugs[184]. They are able to carry not only therapeutics drugs, but also enzymes and nucleic acids, such as mRNA, miRNAs, and small interfering RNAs (siRNAs)[185,186]. Hence, they can also be used as RNA based therapeutics. Similar to liposomes, EVs are efficiently taken up by the host plasma membrane making them efficient candidates for cargo internalization and loading. Furthermore, better physiological properties such as stability and specificity in conjunction with slower release of drugs offer advantageous characteristics compared to liposome mediated drug delivery[187].

One of the limitations of chemotherapeutic drugs is severe toxicity. This problem can be mitigated by loading those anticancer drugs into suitable nano-carries such as liposomes and/or EVs. Liposomes face difficulty in packaging various drugs and disadvantages such as drug leakage and easy degradation upon circulation. EV based nano-carriers are efficient in drug packaging and solve the obstacle associated with drug leakage and degradation encountered by liposome mediated drug delivery[188].

1.6.7.3 Biomarker and Diagnostics

The pathogen-derived EVs contain proteins, mRNAs, and sRNAs that could serve as biomarkers for the diagnosis and prognosis of many infectious diseases[189]. The cargo of EVs reflect the ongoing infection process and provide potential biomarkers that can be used as indicators for the disease progression[190]. The use of biomarkers for disease diagnosis is promising for bacteria in the viable but not cultivable state[191], and for slow growers such as *Mycobacterium tuberculosis*. It has also been reported that *M. tuberculosis* associated peptides and sRNAs are in exosomes released by *M. tuberculosis* infected macrophages[192]. These are excellent candidates for biomarkers as they are stable in the host and remain in circulation due to protection offered by exosomes.

1.6.8 Extracellular vesicles double edge swords: offence as well as defense

In a hostile ecological niche, microorganisms cope with multiple stress, and deploy multiple strategies including both offence and defense to survive. Offensive strategies include release of adhesion factors such clumping factor B and fibronectin binding protein. Similarly, vesicles as a defensive strategy, release antagonistic agents such as peptidoglycan-degrading enzymes, autolysins and antibiotic like compounds such actinorhodin and prodigiosins (found in *Streptomyces coelicolor* EVs), to kill the intruder present in their vicinity[124,193]. In addition, under nutrient limiting condition, *S. aureus* EVs are enriched with iron-binding factors for survival. Proteomics analysis of EVs released by *S. aureus* also highlight the presence of beta-lactamase, an enzyme that degrade beta-lactam antibiotics including ampicillin. The packaging of beta-lactamase inside vesicles might enable ampicillin-susceptible Gram-positive and Gram-negative bacteria to survive in the presence of ampicillin and contribute towards increment of an antibiotic resistant bacterial subpopulation[194].

1.6.9 Extracellular vesicles and horizontal gene transfer (HGT)

Bacteria are constantly exchanging their nucleic acids (DNA, RNA) to other species (interspecies and/or intraspecies) via different routes such as conjugation, transformation and transduction. The presence of nucleic acids inside the vesicles open an avenue for their role in HGT. Recently, 'Vesiduction' has been postulated as a fourth mode of intercellular DNA transfer mediated though vesicles[195]. EV-mediated transfer of bacterial chromosomal DNA was found in *Ruminococcus* species[196]. Short fragments (1-4 kb) of DNA encoding proteins involved in virulence, stress response and antibiotic resistance are also found as EV cargo[197]. Moreover, EV- associated DNA have differences in the restriction/modification pattern[196]. Klieve and coauthors have found that chromosomal DNA from the "parent cell" are easily digested with EcoRI, however, particle-associated DNA from the same species are resistant to digestion with same restriction enzyme-suggesting processing of DNA might occurs while they are destined to EVs. In addition, the presence of phages associated with vesicle strengthen the speculation of a role of EVs in HGT[198].

1.6.10 RNA secreted by microbial extracellular vesicles

In general, microorganisms release RNA and secreted RNAs quickly decay in the surrounding environment during apoptosis or by active secretion systems[199]. However, RNAs in the extracellular environment are prone to degradation by abundant ribonucleases (RNases) present in the surroundings. Hence, bacteria may protect their cargo including RNAs inside EVs. With

the advancement in the technologies like RNA-seq, EV associated RNA from the marine cyanobacterium *Prochlorococcus* was reported in 2014[200]. Thereafter, intensive researches have been conducted to characterize EV associated RNAs and their functions (**Table 3**).

From high-throughput sequencing of EV associated RNA isolated from various Gram-positive and Gram-negative bacteria, it is now well known that bacteria package a large diversity of RNA biotypes including mRNA, sRNA, tRNA, and CRISPR RNAs (reviewed in[201,202]). Interestingly, plasmid encoded transcripts are mostly enriched in EVs of *Borrelia* species[203]. In addition, sRNA secreted by microorganism are of special importance given their role in microbe-microbe and host-microbe interaction[204]. The cargo of EVs including sRNA and mRNA are loaded into recipient cell via fusion of the EVs with host-cell membrane using various routes of endocytosis such as clathrin or caveolin-mediated endocytosis / and or lipid raft dependent endocytosis[205]. The functional delivery of RNA associated with EVs is widely studied in eukaryotes[206,207]. Very few functional studies of EV associated RNA from prokaryotic bacteria has been done[208,209].

Table 3: RNA associated with bacterial EVs and their function. Table modified from[201,210].

Microbial species	Description of RNA	Method used	Functional effect	Year^Reference
Neisseria gonorrheae	EVs associated RNAs	Gel electrophoresis	ND	1989[211]
Prochlorococcus marinus	94% rRNA; 89% of genome represented	RNA-seq	ND	2014[200]
Porphyromonas gingivalis 33277	16S rRNA, mRNAs (mfa1, sod, and fimA)	RT-qPCR	ND	2015[212]
Escherichia coli MG1655	All RNA classes detected, > 90% tRNA, 6.4% rRNA, 0.54% ncRNA, 0.19% mgeRNA	RNA-seq, RT-qPCR	ND	2015[213]
Vibrio cholerae	mRNA and sRNA derived from intergenic regions.	RNA-seq	ND	2015[214]
Streptococcus pyogenes	rRNA and mRNA derived from coding regions	RNA-seq	ND	2016[140]
Pseudomonas aeruginosa PA14	15 - 45 nt sRNA which are stable due to formation of secondary structures, most abundant RNA found were tRNA, ncRNA and tmRNA	RNA-seq, RT-qPCR	Reduction in IL-8 secretion upon transfection of sRNA in HBE cells	2016[215]
Aggregatibacter actinomycetemcomitans ATCC33384	msRNA (<30 nt)	RNA-seq, RT-qPCR	Reduced secretion of cytokines (IL-5, IL-13 and IL-15) after transfection of sRNA in Jurkat cell line after 48 hours.	2017[209]
Salmonella enterica serovar *Typhimurium*	Majority of RNAs are rRNAs, sRNAs, and fragmented mRNAs	RNA-seq, RT-qPCR	ND	2018[216]
Borrelia burgdorferi B31 (ATCC 3521)	Mainly plasmid-encoded mRNA and sRNAs	RNA-seq, RT-qPCR	ND	2018[203]
Streptococcus sanguinis SK36	sRNAs (S.S-439, S.S- 1964) of size less than 30 nt	RNA-seq, RT-qPCR	ND	2018[217]

Listeria monocytogenes EGD-e	RNA < 200 nt, mainly rRNA	RNA-seq	IFN-β expression upon transfection of EV-associated RNA (rli32) in BMDM cell line.	2019[208]
Vibrio cholerae	Enrichment of noncoding and functional RNA such as VrrA, GcvB, tmRNA, RNase P, CsrB2, and CsrB3	RNA-seq	ND	2019[218]
Helicobacter pylori	sncRNAs (sR-2509025 and sR-989262)	RNA-Seq, qRT-PCR	Reduce OMV-induced IL-8 secretion in human gastric adenocarcinoma (AGS) cells line	2020[219]

2

[2] BMDM- Bone-Marrow Derived Macrophages
HBE- Human Bronchial Epithelial Cells
RmsRNA- MicroRNA-size RNA
ND- Not Determined
RT-qPCR- Reverse Transcription Quantitative Real-Time PCR.
rRNAs- Ribosomal RNA
tRNA- Transfer RNA
IFN-β- Interferon -β
mgeRNA- Mobile Genetic Element
ncRNA- Noncoding RNA
RNA-seq- RNA Sequencing
sncRNAs - Small Noncoding RNAs
sRNAs- Small RNA
tmRNA- Transfer-Messenger RNA

2. Methodological considerations

2.1 Extracellular vesicle isolation

EVs are heterogeneous in size and secreted in the extracellular milieu during different stages of the growth phases. Crude vesicles are obtained by sequential ultracentrifugation and ultrafiltration procedure[220]. The workflow for the preparation of vesicles is shown in **Figure 6.** To prepare the crude vesicles, bacteria were pelleted by centrifugation at 6000×g for 30 min at 4°C. Any residual bacterial cells were removed by successive filtration, first with 0.45 µm filter and then with 0.22 µm filter. To concentrate the filtrate, the supernatant was loaded on 100kDa Vivaflow 200 cassettes (Sartorius AG) and centrifuged at 30,000×rpm for 3 h at 4°C. In this study, concentration steps were added for vesicle isolation in *E. faecium* species (**Paper I**), while for *S. aureus*, the bacterial free supernatant was directly ultracentrifuged using 45Ti Rotor at 30,000 ×rpm for 3 h. The obtained vesicle pellets were either resuspended in ice-cold PBS (*e.g.*, proteomics analysis) or RNAlater to protect RNA (*e.g.*, RNA-seq analysis) depending upon the experimental goal (**Paper I and II**). In either case, isolated EVs were confirmed to be free of bacterial cells by plating aliquots of EVs on the blood agar plate, followed by incubation at 37°C for overnight incubation. EVs are generally stable at room temperature for short-term (*e.g.*, overnight) storage; however, preservation at -80°C has been suggested for a long-term storage (*e.g.*, a year) [221].

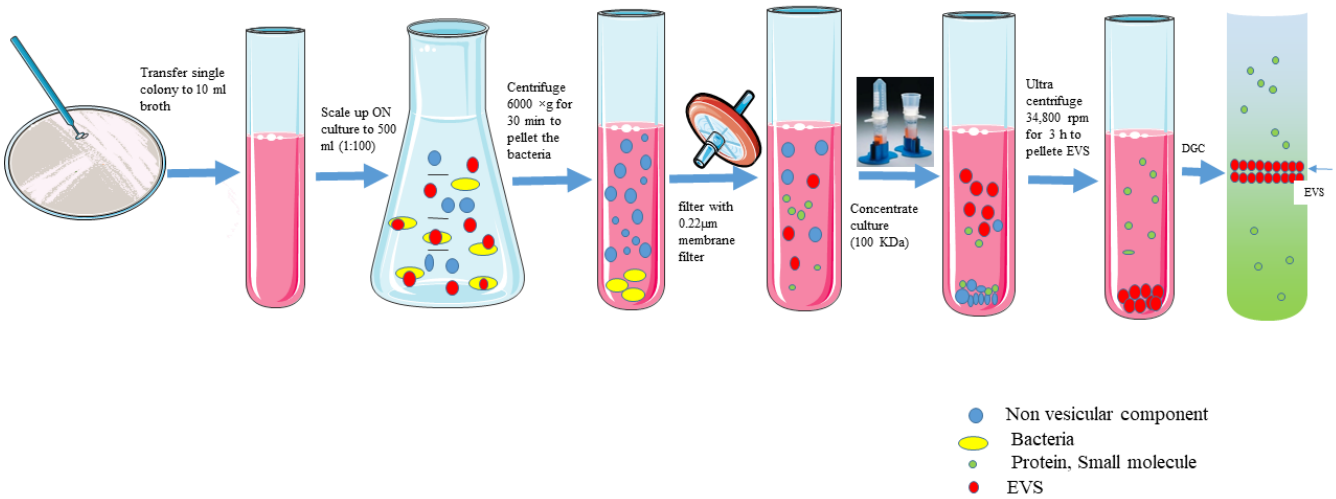


Figure 6: Workflow for EV isolation and purification. Figure modified from[222]. Images adapted from Servier Medical Art Commons (http://smart.servier.com).

One of the major challenges in vesicle isolation from bacteria (*e.g.*, Gram-positive) is that the ultracentrifugation protocol result in a crude pellet that not only contains EVs but also other contaminants such as membrane-bound proteins and nanoscale contaminants [220]. Several washing steps with PBS can help to eliminate a part of this contamination, and density gradient centrifugation can be used for further purification of EVs. Besides, optimization of speed and time during the ultracentrifugation step are of utmost importance in the isolation process. For instance, the excessive ultracentrifugation speed can lead to a high shearing force that might damage EVs.

2.2 Extracellular vesicles purification by density gradient

Density gradient ultracentrifugation is widely used for the separation and purification of EVs, which separates particles based on their density. The gradient can be generated by using sucrose[211] or Optiprep - an iodixanol-based solution[223]. OptiPrep stock solutions (60%) has a density of ~1.3 g/ml and PBS buffer can be used as diluent to prepare various gradients of Optiprep (final % Optiprep, v: v). For better purification, the optimal series of gradients are prepared in the ultracentrifugation tubes. EVs are either loaded on the top or bottom of the gradient depending on the bacterial species. As an example, for *E. faecium* and *S. aureus*[124] a bottom- and top-loading approach were chosen, respectively. One of the biggest advantages of OptiPrep is that it to some extend allow depletion of bacteriophages, which are co-pelleted with EVs during the isolation processes. However, it doesn't completely remove all nano-scale contaminants (*e.g.*, phage filament, intact phage particles or capsid. In our study, presence of vancomycin probably enabled activation of prophages. Electron microscopy analysis revealed presence of several of mentioned nano-scale contaminants including phages in specific fractions after density gradient centrifugation (data not shown). Our results are in line several other studies that reported co-purification of filamentous phages with EVs from *Stenotrophomonas maltophilia* upon exposure to ciprofloxacin stress[153], possibly due to induction of prophages residing in the *S. maltophilia* 44/98 genome[153]. Biller and colleagues also reported co-purification of the filamentous phage particles along with vesicles even post-density gradient ultracentrifugation[200].

However, when high yield or enriched EVs is required and Optiprep is not the best option, ultrafiltration step can be used. The ultrafiltration column partly remove lipoprotein aggregates and thereby help in obtaining enriched EV[224]. This approach was utilized to minimize eventual RNA contamination originated from bacterial cell lysis that could have occurred during EV isolation. RNase treatment of crude EVs was also done to reduce nano-contaminants. Although

addition of RNase treatment of EVs can further ascertain removal of extracellular RNA we cannot rule out the possibility that our EV-associated RNA might contain nanoscale contaminants.

2.3 Physical characterization of bacterial extracellular vesicles

With the advancement of electron microscopy, which utilizes electrons as a source of illumination, one can view the structure of EVs, which are in the range of nanometer scale. The morphology of EVs can be visualized using transmission electron microscopy (TEM) and atomic force microscopy (AFM).We used the latter approach to evaluate the purity and intactness of EVs compared to crude pellet after Optiprep [216]. Both methods have strengths and weaknesses, which are listed in **Table 4.**

Table 4: Advantages and disadvantages with TEM and AFM[224].

Transmission electron Microscopy	**Atomic force microscopy**
Advantages	
Sample preparation is easier to prepare on TEM grids.	Possible to image the particle size range from 10–1000 nm and provides information on morphology.
Less background and better signal to noise ratio.	Possible to image vesicles in contact independent mode (tapping mode).
Disadvantages	
Series of fixation and dehydration steps, direct contact of vesicles with grids during sample preparation, and imaging under vacuum and electron beam radiation might alter the true morphology of vesicles.	The EVs are adsorbed onto support surfaces, such as mica, resulted in substantial background and might modify the structure of the vesicles.

The sizes of EVs are measured by dynamic light scattering or Nanoparticle tracking analysis (NTA). NTA analysis (**Paper II**) can provide the possibility to analyze size distribution and particle number simultaneously [225]. One of the challenges in NTA analysis is to find the "appropriate" dilution factor so that the NTA camera can quantify EV particles. Sometimes, in a heterogeneous population of EVs, the larger vesicles mask the smaller ones, affecting particles' overall concentration. Electron microscopy analysis was used previously for quantification of the particle number. However, the method was time consuming as it requires analysis of multiple microscopic fields where each EV are counted.

In our studies (**Paper I and II**), we have also used fluorescent dyes, DiD and PKH2, to visualize the EVs, as they efficiently bind to the lipid membrane of EVs[226,227]. One of the disadvantages of vesicle characterization using fluorophore conjugate lipophilic dyes is their non-specific binding to other co-pelleted contaminants that may be in a crude EV pellet, which subsequently might result false positive signals. Similarly, to validate the association of RNA with the EVs cargo, the commercially available RNA staining dyes (*e.g.*, acridine orange, Syto84 or Syto-RNASelect)[228] can be used (**Paper II**), although the specificity and efficiency of such dyes has not been validated so far[228].

In addition to particle number, quantification of the EVs yield can be performed based on protein estimation, *e.g.*, Bradford reagent, bicinchoninic-acid assay (BCA), or lipids (FM4–64)[229]. The choice of method might result in different EV yield. Hence, combination of various quantitative techniques (protein estimation, lipid estimation, and particle counts) are useful to assess the comparison of EV production under different growth conditions.

2.4 Identification of proteins associated with vesicles by mass spectrometry

Mass spectrometry (MS)-based approach is increasingly used for proteome identification. It is a powerful analytical technique due to its high-throughput nature, excellent sensitivity and selectivity. The label free proteomics approach was employed to study the EV associated proteome including, virulence factors, vaccine candidates, and antimicrobial resistance determinants, (**Paper I and Paper III**). After gradient centrifugation, aliquots of the fractions were loaded on the SDS-PAGE gel. Fractions with similar protein profiles were pooled together, and the EV- associated proteins were precipitated in TCA/Acetone, before being delivered to the Core-facility for the downstream analysis (**Paper I and III**). Next, the precipitated proteins went through urea re-solubilization and proteolytic digestion. Trypsin is generally used to cleave complex proteins into peptides due to its unique cleavage pattern at the C-terminal side of lysine and arginine residues[230]. Once the peptides are formed, they are separated on a nano-Liquid chromatography system based on differences in hydrophobicity and polarity of the peptides. Each peptide elutes at a specific retention time and is analyzed by MS/MS. The obtained spectra are matched to *in silico*-generated spectra using a database of known proteins and bioinformatics software such as Proteome discoverer with an inbuilt SEQUEST algorithm. To identify the accurate number of proteins, the database must be updated with correct protein sequence information. One of the disadvantages associated with the proteomics analysis is that the high amount of sample loss due to a series of interlaced steps

such as reduction, alkylation, dehydration, and rehydration during protein extraction and digestions[231].

One of the challenges associated with EVs' proteomic analyses is low protein yield in pure EV fractions obtained after OptiPrep. The proteomics can also be performed on crude EV pellets. However, the membrane debris and lipoprotein aggregate co-pelleted during ultracentrifugation will affect the purity, thus questioning the validity of proteome profiling[232]. The choice of purification level of EVs for proteomic characterization depends upon the research question. For instance, if the purpose of the study is to identify vaccine candidates or to discover biomarkers, vesicles of high purity are needed.

2.5 RNA sequencing

RNA-sequencing (RNA-seq) produces millions of short (15-150 nt) sequences reads that constitute fragments of RNA. The RNA may be protein-coding or non-coding and incudes small RNA, which have regulatory functions. With these powerful techniques, it is possible to study gene expression and characterize novel transcripts at single-nucleotide resolution. To obtain proper sequencing results, the purity and yield of RNA is of utmost importance. The process typically consists of isolation of RNA, enrichment of non-ribosomal RNA, conversion of RNA to cDNA and assembly of cDNA library before sequencing on a high-through platform such as Illumina.

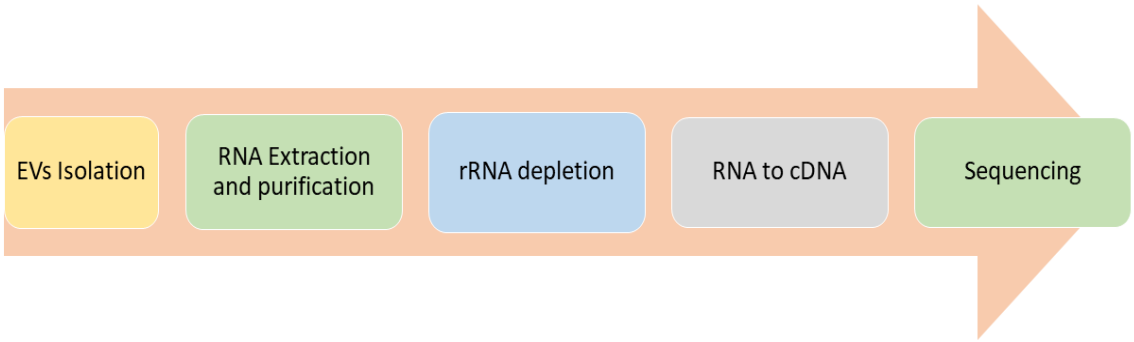


Figure 7: Schematic representation of RNA-seq workflow for isolated EVs.

The extraction of RNA from Gram-positive-derived EVs is challenging due to their release of little amount of EVs, which is consequently resulted in a low yield of RNA. To obtain a sufficient amount of EVs-derived RNA for downstream sequencing analysis, we pooled EVs isolated from several rounds of purification (500 mL cultures of *S. aureus* grown under stress condition) (**Paper II)**. Next, sRNA was isolated using miRNA extraction kit and went through rRNA depletion process to further enrich small RNA. Despite the availability of several

depletion strategies **(Table 5**), Ribo-zero rRNA depletion were employed due to efficient elimination of 16S and 23S rRNA from most of the bacterial species and its compatibility with fragmented RNA.

2.5.1 Bioinformatics analysis of RNA-seq data

The output of EVs-derived RNA-sequencing from Illumina consists of FASTQ files. FASTQ files contain information about the total number of sequences, sequence length distribution and percentage of GC content, which are important measurements for quality control. Based on % GC content, it is possible to predict the sequence reads carries any contamination originated from other species. The quality of sequence reads is based on the Phred scores. Generally, in the context of sequencing, Phred-scaled quality scores of 20 or above is acceptable.

FASTQ files can be aligned to a reference genome by various alignment tool such as TOPHAT, BowTie or others. In our study (**Paper II**), BowTie 2 aligner was used since it is ultrafast, memory-efficient alignment program widely used to align sequence reads to large genomes[233]. Upon alignment, the software generates a Sequence Alignment Map (SAM), a text-based format suitable for storing aligned biological sequences. As SAM file requires huge space for storage, thus they converted to binary version, also called BAM files (.bam), which are memory efficient, faster to process, and importantly can be indexed. Several visualization/analysis tools can be used for downstream analysis, including Tablet[234], EagleView [235], Artemis[236], and Integrative Genomics Viewer (IGV)[237]. An important feature of Artemis compared to other tools, is the possibility of annotating novel RNA. Similarly, IGV is useful for creating Sashimi plots, which provide information about transcript isoforms and splice junction variation commonly seen in eukaryotes. We also used Artemis (**Paper II**) due to its user-friendly web-based graphical interface that provides a comprehensive set of reading alignment views as well as an insight on coverage. Moreover, the sRNA information available from database such as Bacterial sRNA Database (BSRD) can be crossed checked manually using Artemis.

Table 5 : ribosomal RNA depletion techniques used for bacterial transcriptomic[238].

Name	Supplier	Depletion strategy	Compatibility (Fragmented RNA)	Remarks
Ribo-Zero™ rRNA	Illumina	Subtractive hybridization	Yes	Recently discontinued
MICROBExpress Bacterial mRNA Enrichment kit	ThermoFisher AM1905	Subtractive hybridization	No	Targets short conserved regions of rRNA
mRNA-ONLY Prokaryotic mRNA Isolation kit	Epicentre MOP51010	Exonuclease digestion	No	Based on 5-monophosphate-dependent rRNA degradation
RiboMinus™ Transcriptome Isolation Kit, bacteria	ThermoFisher K155004	Subtractive hybridization	No	Subtractive hybridization
Pan-Prokaryote riboPOOL	siTOOLs Biotech	Subtractive hybridization	No	Targets partial regions of rRNA

3. Objective of the study

The **overall aim** of this PhD study was to characterize the EV cargo of two Gram-positive nosocomial pathogens, *S. aureus* and *E. faecium*. The study specifically aimed to explore whether stress and growth conditions influence the EV cargo.

Specific objectives of paper I

1) To evaluate whether clinical *E. faecium* strains release EVs
2) To explore whether the enterococcal proteinaceous cargo is influenced by growth conditions
3) To identify virulence factors and vaccine candidates associated with EVs

Specific objectives of paper II

1) To evaluate whether small RNAs are associated with *S. aureus* derived EVs
2) To characterize the sRNAs associated with the EVs

Specific objectives of paper III

1) To evaluate the proteome profile of MRSA (USA300)-derived EVs grown in the absence or presence of sub-therapeutic concentrations of vancomycin.
2) To explore the interactions between exogenously administered MRSA-derived EVs with cell wall targeting antibiotics using an *in vitro* antimicrobial susceptibility testing

4. Summary of results

Paper I: *Enterococcus faecium* produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins.

E. faecium release membrane vesicles, which are spherical in shape and heterogenous in size. The size of vesicles ranges from 37 ± 23 nm in E155 strain to 83 ± 29 nm in the K59-68 strain. The vesicles were negatively charged and contained lipids and fragments of peptidoglycan. The EV-associated proteins varied among the strains and growth conditions. AtlA and SagA, involved in biofilm formation, were associated with EVs of all strains and both examined growth conditions (stationary and exponential growth phase). In addition, several immunogenic proteins such as penicillin-binding protein (PBP5), peptidoglycan-binding protein (LysM), D, D-carboxypeptidase (DdcP, and peptidyl-prolyl-cis-trans-isomerase (PpiC) were found in the EVs isolated from the four strains. In addition, 11 antimicrobial resistance-related proteins, *e.g.* the *vanA* cluster contributing to vancomycin resistance, was associated with EVs from *E. faecium*.

Paper II*:* Transcriptome profiling of *Staphylococcus aureus* associated extracellular vesicles reveals presence of small RNA-cargo.

Iron-depletion and sub-inhibitory concentration of vancomycin resulted in five folds increase in EVs obtained from *S. aureus* MSSA476. The presence of RNA associated with EVs was confirmed by confocal microscopy using RNA and lipid specific dyes. In RNase treated EVs, ~ 73% co-localization of RNA and lipid stained particles was found. RNA-seq data showed presence of sRNA, tRNA, mRNA and various riboswitches including T-boxes. The most enriched sRNAs (SsrA, RsaC and RNAIII) identified by RNA-seq were further validated by qPCR, conventional PCR and Sanger sequencing.

Paper III: Bacterial membrane-derived vesicles attenuate vancomycin activity against Methicillin-Resistant *Staphylococcus aureus.*

We found that exogenous administration of EVs resulted in increment of the minimum inhibitory concentration (MIC) of vancomycin (VAN), a cell wall targeting antibiotics, up to two to four fold against MRSA (USA300 strain) grown in convenient laboratory media (CA-MHB) and physiological media (RPMI+5%BHI), respectively. The exogenous addition of EVs did not increase the MICs of nafcillin (NAF), cefazolin (CFZ) or daptomycin (DAP) in either media. In addition, increased survival of MRSA was found in presence of whole blood and human neutrophils upon treatment with sub-MIC VAN along with exogenous addition of MVs.

Proteomic analyses revealed upregulation of multiple antibiotic resistance regulators, amino acyltransferases, and proteins known to facilitate resistance to β-lactams and quinolones upon isolating MVs under sub-MIC dosage of antibiotics.

5. General discussion

Every species on the planet, from prokaryotes to eukaryotes, release extracellular vesicles. The EVs are involved in the maintenance of bacterial physiology and ecology, including nutrients transfer and release of antimicrobial compounds for survival and competitions. EVs also play a role in host-microbe interactions, *e.g.*, during infection. Therefore, characterization of EVs released by these bacteria is worthwhile to research endeavor.

The thesis aimed to get insight on extracellular vesicles cargoes shed by *S. aureus* and *E. faecium*. In the **Paper I,** we explored the proteome of the *E. faecium*-derived EVs and identified various EV-associated proteins that might be involved in promoting virulence, antimicrobial resistance and pathogenicity. In the **Paper II**, we identified the sRNAs associated with EVs (both embedded in the membrane and inside lumen) released by *S. aureus* strain MSSA476. Three sRNAs (SsrA, RNAIII, RsaC), were among the most enriched. These have molecular targets associated with metabolism, virulence and transport. In the **Paper III**, we found that exogenous addition of EVs derived from parental strain (MRSA USA300) increased the MIC up to 4-fold in physiological media, and thereby seemed to protect MRSA against killing by vancomycin, which is probably due to the decoy action of EVs.

4.1 Size and morphology of EVs from *S. aureus* and *E. faecium*

Three nosocomial *E. faecium* blood-stream isolates (DO, K59-68, and K60-39) and one fecal isolate (E155) and two *S. aureus* strains (MSSA476 and MRSA USA300) were selected for the studies. Although the morphology of EVs was found to be spherical in all selected species, their size, yield, and proteinaceous cargo were influenced by the species and growth conditions (*e.g.* media) (**Paper I-III**).

The sizes of *E. faecium*-derived EVs varied between 37 nm (E155 strain) to 83 nm (K59-68 strain) (**Paper I**). This is in concordance with another study where EVs were isolated from a vancomycin-resistant fecal isolate (*E. faecium* ATCC 700221) and reported spherical EVs with a size of ≤50 nm[239]. Similarly, we found spherical EVs with a heterogenous size (100-150 nm) in *S. aureus* MSSA476, which is consistent with others studies[124,164,239] showing the mean size of EVs produced by various *S. aureus* strains (MW2, N305, RF122, O11, and O46) of diverse origins (human, bovine and ovine) was reported be less than ≤ 200 nm[128]. Notably, we should not rule out that the techniques used for size measurement, including tunable resistive pulse sensing (TRPS), NTA, AFM, and EM[240] can influence at low extent on the outcome of measurement. The characterization of EVs size is an important parameter to consider when

considering using EVs as drug delivery tools[132]. The small size of EVs provides flexibility to cross the major biological membrane, even blood-brain barriers[241], and EV loaded drugs can have the potential to treat neurological disorders.

4.2 Extracellular vesicles associated proteins involved in vesicle biogenesis

The ability to form EVs from thick-wall Gram-positive bacteria was neglected for a long period. Although our understanding of bacterial EVs begun soon after their discovery around 1960, research on the characterization of EV cargo from *S. aureus* was only started in 2009[131]. Afterward, multiple studies have been performed to characterize EVs cargo from other Gram-positive bacteria using different high-throughput omics technology, *e.g.* proteomics, transcriptomic, and lipidomics. Moreover, functional assessment of EV- associated virulence factors and nucleic acid (RNA) upon interaction with host cell has also been studied[124,135,164,208,242]. It is still debatable how vesicles are formed from Gram-positive organisms with complex and thick cell walls. There were three hypotheses postulated for the biogenesis of EVs i) turgor pressure mediated vesicles release; ii) cell wall remodeling enzyme creating holes in peptidoglycan, thereby facilitating EVs release; iii) EVs release via protein channels[126].Schlatterer and colleagues demonstrated that EV biogenesis depends upon the turgor pressure and presence of PSM peptides, which enhances the membrane fluidity[243]. Taken together and considering the thick peptidoglycan cell wall, it is conceivable to support the notion that enzymes interfering with peptidoglycan crosslinking (*e.g.*, penicillin-binding protein (PBP), autolysin) and enzymes that enhances membrane fluidity (*e.g.*, PSM) are key factors in vesiculogenesis. Using proteomics approach, we found that PBP and autolysin were associated with the EVs generated by nosocomial clade of *E. faecium* and *S. aureus* (**Paper I and Paper III**). We also detected PSM in the EVs cargo of *S. aureus*. The involvement of PBP, PSM and endolysin in release of EVs from *S. aureus* with higher yield has been previously reported[183,244]. Interestingly, PBPs and autolysins were also detected in EVs released from other Gram-positive bacteria such as *Listeria monocytogenes*[136], *Propionibacterium acnes*[245], *Bacillus anthracis*[134], and *Filifactor alocis*[148].

Antibiotics such as vancomycin, a cell wall targeting antibiotics, and other stress factors such as temperature and nutrient limitation are known to be triggers for vesiculation[127]. Considering the vancomycin mechanism of action, this might be mediated by weakening the cell wall via inhibiting cross linking of peptidoglycan. We found a fivefold increase in the EV particle count and higher protein concentrations in EV preps when growing *S. aureus* in iron-depleted BHI in

presence of sub-MIC of vancomycin (**Paper II**). In addition, we observed that exposure to the stress was also induced the release of bacteriophages (**Paper II**). Interestingly, EV release triggered by phage endolysin is well demonstrated in *B. subtilis* and *S. aureus*[198,244].

4.3 EV associated proteins involved in virulence

Using a proteomics approach, we found that several virulence factors were associated with the *S. aureus* or *E. faecium* -derived EVs (**Paper I and III**). This is also well-documented in several other studies performed on *S. aureus*[124,128,131,135]. Various adhesins (CapD and PrpA), collagen-binding proteins (Acm and Scm), and fibronectin-binding protein (Fnm) are involved in host cell invasion leading to the pathogenesis of *E. faecium*[32]. In the **Paper I**, we found several of these virulence factors associated with EVs. We could hypothesize that the EVs containing these virulence factors might influence *E. faecium* pathogenesis; however, this remains to be investigated. Askarian and colleagues reported that exogenous addition of bacterial EVs at a concentration of 20µg can augment bacterial survival in presence of human blood, however the bacterial survival is abolished by exposure to sonicated/and or proteinase K treated EVs[124]. In addition, in all four isolates belonging to the nosocomial clade of *E. faecium,* the virulence factors AtlA and SagA were found to be EV-associated. AtlA and SagA are both involved in biofilm formation[246], and might do similar as part of EVs.

Biofilm is usually formed by aggregation of single or multispecies bacterial population surrounded by self-produced extracellular matrix. In addition, other microbial components such as pili, flagella, phage and EVs have been reported within microbial biofilms[121]. EVs not only support microbial community within biofilm by sharing nutrients to the colonizing microorganisms, but also send signaling compounds such as 2-heptyl-3-hydroxy-4-quinolone (PQS) for co-aggregation of bacterial populations. PQS is essential for microbial communication (quorum sensing), biofilm formation[247] and EV formation for *Pseudomonas aeruginosa*[248].

Both *S. aureus* and *E. faecium* are biofilm producers and can form biofilm on human body parts such as in the urinary tract and deep-seated wounds and on indwelling medical devices like catheter. Organisms within biofilms are resilient towards antibiotics and may survive within an encapsulated sheath of exopolysaccharides and proteins as persister cells[249]. Persisters are mostly resistant towards antibiotics, even after the disruption of biofilm by antimicrobials, viable persister cells are capable of repopulating and reforming biofilms thus ultimately resulting in difficult-to-treat chronic infections. Identification of novel compounds that target

biofilm, persister cells and EV formation could be novel strategies for hard to treat chronic infections [250].

4.4 EV-associated nucleic acid

EV-associated RNA released by *S. aureus* is challenging to work with, as Gram-positive bacteria shed very little amounts of EVs. We made a first attempt to validate RNA as part of the EV cargo of *S. aureus* **(Paper II).** The bacteria were grown under stress (iron-depleted BHI in presence of sub-inhibitory concentration of vancomycin) as this enhance vesicles yield. Various literature underscores the importance of stress for hypervesiculation and modulation of regulatory RNA in bacteria[127,251]. We found fragments of sRNA and tRNA enriched in EVs protected from exogenous RNase (**Paper II**). Interestingly, we have identified and validated a few sRNA fragments (*e.g*., SsrA, RNAIII, and RsaC) associated with *S. aureus* EVs. RNAIII is the longest sRNA (514 nt) known in *S. aureus*. It is also known as dual RNA because due to its regulatory function and encoding a 26 amino acid long δ-endotoxin, which the latter seems to play a role in lysis of host cell membranes[252]. RNAIII also acts as an effector of the Agr quorum-sensing system in *S. aureus* cells and is involved in biofilm formation. Whether EV-associated RNA also contributes to biofilm formation, analogous to EV-associated eDNA that is upregulated during biofilm formation by *Strepotocccus mutans*, is yet to be proven[253] .

One might speculate that EVs act as a "postman" to deliver the cargo, including RNA molecules, to the target cells as a sole purpose for communication. In one of the studies, alignment of EV-associated sequence reads to the human genome using bioinformatics resulted in unique alignment to 14 different chromosomes, including X and Y chromosomes. The alignment of bacterial reads to human reads is not a very common phenomenon and more surprisingly, most of the aligned regions are on human long non-coding RNA involved in epigenetic modulation[202,254]. Similarly, some exciting results on human as well as plant- derived exosomal RNA prove that they can be involved in shaping the composition of gut microbiota[255,256]. *E. faecium* is part of commensal microbiota and might also modulate gene expression of other gut microflora residing in the same ecological niche or modulate gene expression of the host cells via the transfer of sRNA. The reciprocal regulation of gene expression between bacteria and host cells through exchange of sRNA via EVs and/or exosome open up a novel research area within host microbe interactions[257].

For future studies, it will be interesting to validate whether EVs can pack full-length transcript and to get a better understanding on the functional role of extracellular RNA, including

fragmented ones. So far, no studies have addressed whether *S. aureus* and *E. faecium* EVs are associated with DNA. Nevertheless, there are few reports showing EV mediated gene transfer in Gram-positive bacteria[195,258]. One study described encapsulation of genetic material up to 370 Kbp by EVs[259]. Since *E. faecium* are continuously evolving via the exchange of genetic material through horizontal gene transfer, other non-canonical pathways as "vesiduction" merits further investigations[195]. The transfer of genetic material via EVs have been reported from various bacterial species found from diverse ecologies like the deep sea and anaerobic environments such as the gut of ruminants (*Ruminococcus* species) and inner tooth cavities (*Porphyromonas gingivalis*)[212,260].

The interplay between host and microbe is a very dynamic process. During the infection process, pathogenic bacteria release EVs and deliver their cargo to host cells. To protect against infections, host cell also releases exosomes[261]. Indeed, EVs released by infected host cells contained pathogen-derived factors, *e.g.* lipoarabinomannan (LAM), CFP-10 (Rv3874), and circulating sRNA (ASdes) during *Mycobacterium* infection[262,263]. sRNA-associated with EVs is stable in human circulation for a considerable time and can resist over 100 ng/mL of RNase, which is present in tissue and body fluids[195], implicating their potential as biomarkers.

Generally, small RNA present in bacteria are not always constitutively expressed by the bacterial cells, as particular conditions including nutrient limitation, antibiotic exposure, change in pH, or oxidative stress for transcriptional induction[264] are required. The stress condition (chelex treated BHI +antibiotics) that employed for vesicles isolation in **Paper II** mimic an infection condition in host. It would be interesting to explore other physiological relevant media such as RPMI1640 supplemented with bacteriologic media to support the bacterial growth for EV isolation and compare the yield and analyze the differentially enriched RNA in the presence of host components such as immune cells and/or mammalian exosomes.

4.5 EVs and influence on spread of AMR and sensitivity to antimicrobials

The detection of antimicrobial resistance determinants in EVs of *E. faecium* described in the **Paper I** (ribosome methylase ErmB, the aminoglycoside modifying enzymes Aac6′-Ie-Aph2″-Ia and Aph3′-III, and vanA cluster proteins involved in vancomycin resistance) might play an important role in drug resistance between commensal *E. faecium* and other gut microflora. It will be interesting to explore EV-mediating transfer of the above-mentioned antimicrobial resistance determinants to antibiotic sensitive commensal *E. faecium* strains. In a microbial community, the transfer of a resistant determinant and valuable enzyme (glycosyl hydrolase) to

other species offers a survival advantage by sharing of the "public goods"[265] and EVs play a key role in this phenomenon. For instance, it was shown that EV could mediate transfer of a carbapenem resistance (*bla*$_{OXA-24}$) gene to a susceptible strain of *Acinetobacter baumannii* , thereby providing protection against carbapenems[266]. The presence of resistant determinants and virulence factors in the bacterial EVs may serve as an efficient mean of dissemination of resistant genes, enzymes (β-lactamase, methyltransferase), and antimicrobial compounds. In a polymicrobial infection, exchange of resistance genes and protein through EV adds an extra layer of complexity to the understanding of host-microbe interactions and the development of therapeutic strategies.

Vancomycin is considered as one of the last resort antibiotic for the treatment for MRSA and several other Gram-positive strains[267]. The glycopeptide antibiotic vancomycin inhibits peptidoglycan chain elongation by binding to the D-Ala-D-Ala residue of peptidoglycan precursors, which interferes with peptidoglycan crosslinking and eventually hinder cell wall biosynthesis and result in bacterial death. Vancomycin is still the drug of choice for over 50 years to treat MDR infections in clinical settings. The treatment failure associated with organ toxicity (nephrotoxicity, ototoxicity) compounded by the emergence of resistant pathogens has questioned the therapeutic utility of this drug[268].

In **Paper II and Paper III**, we used vancomycin to stress the bacteria and characterize EV cargo. In **Paper III**, we found that the MIC of vancomycin was enhanced to 4 mg/L, when growing clinical MRSA strain in a more physiological medium (RPMI) in the presence of exogenously administered EVs. EUCAST has recommended the MIC breakpoint for *S. aureus* to be 2 mg/L against vancomycin when tested in Mueller Hinton Broth (MHB) (https://www.eucast.org/clinical_breakpoints/). Under these conditions, a MIC value above 2 mg/L is determined as resistance towards vancomycin in MRSA. Hence, the observed MIC enhancement may contribute to therapeutic failure of vancomycin in clinical settings. While studying host-microbe interaction *in vitro*, it is beneficial to use physiological media such as RPMI instead of lab-based conventional media. For MIC testing, the Muller Hinton Broth (MHB) has been implemented in the diagnostic laboratory since 1941 and is still used in hospitals. Although MHB has made a substantial contribution to antibiotic susceptibility testing (AST) and drug discovery, it is high time to rethink and modify the chemical composition to resemble more host like conditions. The AST performed on conventional media like MHB were found to be ineffective, however, supplementation of sodium bicarbonate in MHB simulate

more host like condition and revealed specific antibiotics that caused bacterial clearance although standard MIC testing suggested these antibiotics were not effective. The MIC values obtained from host mimic media (RPMI, MHB+$NaHCO_3$) may be more reliable/comparable to the infection condition[269].

One of the possible mechanisms of augmented MIC (≥ 2 mg/L) of vancomycin in presence of EVs could be the decoy action of EVs. We observed that stress can induce increased shedding of EV from *S. aureus in vitro* (**Paper II)**. Induction of vesiculation has been reported in *S. aureus* upon exposure to several cell-wall targeting antibiotics such as vancomycin[270], and daptomycin[271] which supports the decoy action of EVs[272,273]. Moreover, the absorption or entrapment of antibiotics by EVs from *E. coli*[274] and *Shigella flexneri*[272] has been previously suggested. Thus, one might speculate that upon co-incubation of MRSA with EVs and vancomycin, the increased MIC might be due to either the role of EVs as decoy or due to entrapment of vancomycin by EVs thereby offering protection to bacteria. To follow up the study, it would be interesting to visualize vancomycin localization in bacteria and/or EVs in a co-culture condition using fluorescence dye such as vancomycin BODIPY[275]. This will help to ascertain if it is only the decoy action of EVs that protects MRSA killing against vancomycin or the decoy action can result in entrapment of the antibiotic. Additional experiments should be done to address the influence of other confounding factors such as strain to strain variation, laboratory set condition for MIC testing, slight changes in media composition and fluctuation in incubation temperatures[276].

Besides MIC and checkerboard assay, other *ex vivo* assays (such as whole blood killing assay, neutrophil killing assay and serum killing assay) were also studied in **Paper III**. Literature suggests bacteria can release EVs *in vivo*[277]. EVs were found to increase the survival of MRSA co-incubated with sub-MIC dosage of vancomycin. To understand the mechanism of survival, we have done proteomics assay to reveal differentially expressed proteins associated with EVs isolated under normal and vancomycin stress. Interestingly, we found substantial increase of catalase and Alkyl peroxide reductase under vancomycin stress conditions. One might speculate that ROS mediated killing mechanism of phagocytic cells such as neutrophils might be nullified by concentrated punch of detoxifying enzyme present in EVs elevated under vancomycin stress[278].

4.6 Therapeutics use of EVs as vaccine candidate and antimicrobial agent

Interestingly, the EV- associated virulence factors isolated from four different hospital-associated enterococci showed an enrichment of peptidoglycan associated surface-anchored proteins, including PBP5 (Penicillin-binding protein), LysM (Peptidoglycan binding protein), DdcP (D,D-carboxypeptidase), and PpiC (Peptidyl-prolyl-cis-trans-isomerase) (**Paper I**). All of these could be utilized as vaccine candidates because they are surface exposed and suitable for easy recognition by immune cells for enhancing immune responses[279]. Saavedra and colleagues reported that rabbit antisera raised against all four recombinant proteins was efficient in killing not only *E. faecium* belonging to CC17 but also multiple *E. faecalis*[280]. The EV cargo released by *E. faecium* also contains peptidoglycan hydrolase SagA, which was very recently reported as a promising vaccine candidate in a glycoconjugate form consisting of SagA and capsular polysaccharide Diheteroglycan. Unlike polysaccharide antigen, which are considered a poor immunogen [281], the glycoconjugates with carrier proteins can elicit long lasting humoral immune responses via activation of T-cells[282].

For enterococcal vaccine design, attention should be paid to design a vaccine in such a way that it should be effective against the nosocomial strains but must safeguard asymptomatic carriers where enterococci are part of normal gut flora. Furthermore, vaccination against *E. faecium* is useful for patients having underlying diseases and belong to high-risk groups and/or are already infected with VRE. Commensal *E. faecium* SagA protected worms against *Salmonella* pathogenesis and boosted the efficiency of probiotic bacterial activity against *Clostridium difficile* pathogenesis *in vivo*[283,284]. In addition, the SagA also possessed anti-candida activity[285]. The secretion and packaging of SagA and other proteins inside EV cargo protect them against degradation by extracellular protease and assist in long-distance delivery. Further research should be conducted to study the influence of bacterial EVs on microbe-microbe and host-microbe interactions.

6. Conclusion and future perspectives

The discovery of EV production by Gram-positive bacteria is relatively new. Both *E. faecium* and *S. aureus* have ambiguous positions in terms of causing infection. Scientific investigations on bacteria and their EVs are required to understand more regarding the mechanism of pathogenesis. Given the importance of characterizing the EV cargos, which are released by pathogenic bacteria, we isolated EVs from *E. faecium* and *S. aureus* and characterized their cargoes using transcriptomics and proteomics approaches. These descriptive studies of the proteome and transcriptome of EVs provide basis for future studies of their functions. Our data revealed that several virulence factors, immunogenic proteins, and small RNAs were associated with the EVs. The mechanism by which EV cargos are shuttled between same or different kingdoms and regulate gene expression in recipient cells is still in infancy. Considering the presence of virulence factors, targeting EVs can be a putative anti-virulence therapy approach to "disarm" the pathogen. The detection of immunogenic antigen (SagA) and small RNA (SsrA) associated with EVs holds promises for the development of vaccine and biomarkers for diagnostics and prognostic purposes.

The recently explored arena of extracellular vesicles among Gram-positive bacteria has knowledge gaps and hopefully will be addressed in near future. Some of them includes 1) Which genes and/or proteins trigger extracellular vesicles formation? 2) Why do bacteria pack several bioactive molecules (proteins, nucleic acids and virulence factors) inside EVs? 3) Is the packaging of EVs cargos stochastic / random process or a conserved mechanism regulated by specific set of genes or proteins? 4) Are the EVs-associated sRNAs modulating the response of human-host? With the advancement in the largescale EV isolation and purification techniques, biophysical characterization using advanced imaging (super resolution microscopy), high-throughput sequencing technology and advanced bioinformatics pipelines, it will be possible to decipher fundamental biology of EVs in the future. Improving our understanding within this field will provide an asset to utilize EVs for therapeutics purposes including as vaccines, novel anti-infectives and as biomarkers.

7. References

1. Thiercelin, M. & Jouhaud, L. Sur un diplocoque saprophyte de l'intestin susceptible de devenir pathogene. *CR Soc Biol* **5**(1899).
2. Sherman, J.M. The streptococci. *Bacteriological reviews* **1**, 3 (1937).
3. Sherman, J.M. The enterococci and related streptococci. *Journal of bacteriology* **35**, 81 (1938).
4. Kalina, A. The taxonomy and nomenclature of enterococci. *International Journal of Systematic and Evolutionary Microbiology* **20**, 185-189 (1970).
5. Fisher, K. & Phillips, C. The ecology, epidemiology and virulence of Enterococcus. *Microbiology* **155**, 1749-1757 (2009).
6. Zhong, Z., *et al.* Comparative genomic analysis of the genus Enterococcus. *Microbiological research* **196**, 95-105 (2017).
7. Vu, J. & Carvalho, J. Enterococcus: review of its physiology, pathogenesis, diseases and the challenges it poses for clinical microbiology. *Frontiers in Biology* **6**, 357 (2011).
8. Devriese, L., Baele, M. & Butaye, P. The genus Enterococcus: taxonomy. *Prokaryotes* **4**, 163-174 (2006).
9. De Niederhäusern, S., *et al.* Antibiotics and heavy metals resistance and other biological characters in enterococci isolated from surface water of Monte Cotugno Lake (Italy). *Journal of Environmental Science and Health, Part A* **48**, 939-946 (2013).
10. Lebreton, F., Willems, R.J. & Gilmore, M.S. Enterococcus diversity, origins in nature, and gut colonization. in *Enterococci: from commensals to leading causes of drug resistant infection [Internet]* (Massachusetts Eye and Ear Infirmary, 2014).
11. Mundt, J. Enterococci. *Bergey's manual of systematic bacteriology* **2**, 1063-1065 (1986).
12. Eckburg, P.B., *et al.* Diversity of the human intestinal microbial flora. *science* **308**, 1635-1638 (2005).
13. Švec, P. & Franz, C. The genus Enterococcus. *Lactic Acid Bacteria, Biodiversity and Taxonomy, eds WH Holzapfel and BJB Wood (Chichester: Wiley Blackwellpp)*, 175-211 (2014).
14. Casadevall, A. & Pirofski, L.a. Host-pathogen interactions: the attributes of virulence. *The Journal of infectious diseases* **184**, 337-344 (2001).
15. Higuita, N.I.A. & Huycke, M.M. Enterococcal disease, epidemiology, and implications for treatment. in *Enterococci: From commensals to leading causes of drug resistant infection [Internet]* (Massachusetts Eye and Ear Infirmary, 2014).
16. Yuen, G.J. & Ausubel, F.M. Enterococcus infection biology: lessons from invertebrate host models. *Journal of microbiology* **52**, 200-210 (2014).
17. Tornieporth, N.G., Roberts, R.B., John, J., Hafner, A. & Riley, L.W. Risk factors associated with vancomycin-resistant Enterococcus faecium infection or colonization in 145 matched case patients and control patients. *Clinical Infectious Diseases* **23**, 767-772 (1996).
18. Sakka, V., *et al.* Risk-factors and predictors of mortality in patients colonised with vancomycin-resistant enterococci. *Clinical Microbiology and Infection* **14**, 14-21 (2008).
19. Kheir, M.M., *et al.* Periprosthetic joint infections caused by enterococci have poor outcomes. *The Journal of arthroplasty* **32**, 933-947 (2017).
20. Djembi, L.F., *et al.* Factors associated with Vancomycin-resistant Enterococcus acquisition during a large outbreak. *Journal of infection and public health* **10**, 185-190 (2017).
21. Brandl, K., *et al.* Vancomycin-resistant enterococci exploit antibiotic-induced innate immune deficits. *Nature* **455**, 804-807 (2008).
22. Pericás, J., *et al.* Enterococcal endocarditis revisited. *Future microbiology* **10**, 1215-1240 (2015).
23. Huycke, M.M., Sahm, D.F. & Gilmore, M.S. Multiple-drug resistant enterococci: the nature of the problem and an agenda for the future. *Emerging infectious diseases* **4**, 239 (1998).
24. Elstrøm, P., *et al.* The fight to keep resistance at bay, epidemiology of carbapenemase producing organisms (CPOs), vancomycin resistant enterococci (VRE) and methicillin resistant Staphylococcus aureus (MRSA) in Norway, 2006-2017. *PloS one* **14**, e0211741 (2019).

25. Prevention, E.C.f.D. & Control. Surveillance of antimicrobial resistance in Europe 2018. (ECDC Stockholm, 2019).
26. Arias, C.A. & Murray, B.E. The rise of the Enterococcus: beyond vancomycin resistance. *Nature Reviews Microbiology* **10**, 266-278 (2012).
27. Wendt, C., Wiesenthal, B., Dietz, E. & Rüden, H. Survival of vancomycin-resistant and vancomycin-susceptible enterococci on dry surfaces. *Journal of Clinical Microbiology* **36**, 3734-3736 (1998).
28. Boyce, J., *et al.* Emergence and nosocomial transmission of ampicillin-resistant enterococci. *Antimicrobial agents and chemotherapy* **36**, 1032-1039 (1992).
29. Peyvasti, V.S., *et al.* High-level aminoglycoside resistance and distribution of aminoglycoside resistance genes among Enterococcus spp. clinical isolates in Tehran, Iran. *Journal of Global Antimicrobial Resistance* **20**, 318-323 (2020).
30. Wagenvoort, J., *et al.* Environmental survival of vancomycin-resistant Enterococcus faecium. *Journal of Hospital Infection* **77**, 282-283 (2011).
31. Weinstein, R.A. & Hota, B. Contamination, disinfection, and cross-colonization: are hospital surfaces reservoirs for nosocomial infection? *Clinical infectious diseases* **39**, 1182-1189 (2004).
32. Gao, W., Howden, B.P. & Stinear, T.P. Evolution of virulence in Enterococcus faecium, a hospital-adapted opportunistic pathogen. *Current opinion in microbiology* **41**, 76-82 (2018).
33. Weiner-Lastinger, L.M., *et al.* Antimicrobial-resistant pathogens associated with adult healthcare-associated infections: summary of data reported to the National Healthcare Safety Network, 2015–2017. *Infection Control & Hospital Epidemiology* **41**, 1-18 (2020).
34. García-Solache, M. & Rice, L.B. The Enterococcus: a model of adaptability to its environment. *Clinical microbiology reviews* **32**(2019).
35. Courvalin, P. Vancomycin resistance in gram-positive cocci. *Clinical Infectious Diseases* **42**, S25-S34 (2006).
36. O'Driscoll, T. & Crank, C.W. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infection and drug resistance* **8**, 217 (2015).
37. Bugg, T.D., *et al.* Molecular basis for vancomycin resistance in Enterococcus faecium BM4147: biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry* **30**, 10408-10415 (1991).
38. de Niederhäusern, S., *et al.* Vancomycin-resistance transferability from VanA enterococci to Staphylococcus aureus. *Current microbiology* **62**, 1363-1367 (2011).
39. Giamarellou, H. Multidrug-resistant Gram-negative bacteria: how to treat and for how long. *International Journal of Antimicrobial Agents* **36**, S50-S54 (2010).
40. Cheah, A., *et al.* Enterococcal bacteraemia: factors influencing mortality, length of stay and costs of hospitalization. *Clinical Microbiology and Infection* **19**, E181-E189 (2013).
41. Bonten, M.J., Willems, R. & Weinstein, R.A. Vancomycin-resistant enterococci: why are they here, and where do they come from? *The Lancet infectious diseases* **1**, 314-325 (2001).
42. Leclercq, R., Derlot, E., Duval, J. & Courvalin, P. Plasmid-mediated resistance to vancomycin and teicoplanin in Enterococcus faecium. *New England Journal of Medicine* **319**, 157-161 (1988).
43. Uttley, A. C., CH Collins, J. Naidoo, and RC George. 1988. Vancomycin-resistant enterococci. *Lancet i*, 57-58.
44. Ayobami, O., Willrich, N., Reuss, A., Eckmanns, T. & Markwart, R. The ongoing challenge of vancomycin-resistant Enterococcus faecium and Enterococcus faecalis in Europe: an epidemiological analysis of bloodstream infections. *Emerging Microbes & Infections* **9**, 1180-1193 (2020).
45. Mendes, R.E., Sader, H.S., Castanheira, M. & Flamm, R.K. Distribution of main Gram-positive pathogens causing bloodstream infections in United States and European hospitals during the SENTRY Antimicrobial Surveillance Program (2010–2016): concomitant analysis of oritavancin in vitro activity. *Journal of Chemotherapy* **30**, 280-289 (2018).
46. Coombs, G., Daley, D. & Bell, J. The Australian Group on Antimicrobial Resistance (AGAR). *Microbiology Australia* **40**, 69-72 (2019).

47. Safety, A.C.o. & Care, Q.i.H. AURA 2017: second Australian report on antimicrobial use and resistance in human health. (ACSQHC Sydney, 2017).
48. Panesso, D., *et al.* Molecular epidemiology of vancomycin-resistant Enterococcus faecium: a prospective, multicenter study in South American hospitals. *Journal of clinical microbiology* **48**, 1562-1569 (2010).
49. Sacramento, A.G., *et al.* Changed epidemiology during intra and interhospital spread of high-risk clones of vanA-containing Enterococcus in Brazilian hospitals. *Diagnostic Microbiology and Infectious Disease* **88**, 348-351 (2017).
50. Markwart, R., *et al.* The rise in vancomycin-resistant Enterococcus faecium in Germany: data from the German Antimicrobial Resistance Surveillance (ARS). *Antimicrobial Resistance & Infection Control* **8**, 147 (2019).
51. Cassini, A., *et al.* Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *The Lancet infectious diseases* **19**, 56-66 (2019).
52. Jureen, R., *et al.* Molecular characterization of ampicillin-resistant Enterococcus faecium isolates from hospitalized patients in Norway. *Journal of clinical microbiology* **41**, 2330-2336 (2003).
53. Leavis, H.L., Willems, R.J., Top, J. & Bonten, M.J. High-level ciprofloxacin resistance from point mutations in gyrA and parC confined to global hospital-adapted clonal lineage CC17 of Enterococcus faecium. *Journal of clinical microbiology* **44**, 1059-1064 (2006).
54. Turner, K.M., Hanage, W.P., Fraser, C., Connor, T.R. & Spratt, B.G. Assessing the reliability of eBURST using simulated populations with known ancestry. *BMC microbiology* **7**, 30 (2007).
55. Lebreton, F., *et al.* Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. *MBio* **4**, e00534-00513 (2013).
56. Lebreton, F., *et al.* Tracing the enterococci from Paleozoic origins to the hospital. *Cell* **169**, 849-861. e813 (2017).
57. Leavis, H., *et al.* A novel putative enterococcal pathogenicity island linked to the esp virulence gene of Enterococcus faecium and associated with epidemicity. *Journal of bacteriology* **186**, 672-682 (2004).
58. Rice, L.B., *et al.* A potential virulence gene, hyl Efm, predominates in Enterococcus faecium of clinical origin. *The Journal of infectious diseases* **187**, 508-512 (2003).
59. Palmer, K.L. & Gilmore, M.S. Multidrug-resistant enterococci lack CRISPR-cas. *MBio* **1**, e00227-00210 (2010).
60. van Schaik, W., *et al.* Pyrosequencing-based comparative genome analysis of the nosocomial pathogen Enterococcus faecium and identification of a large transferable pathogenicity island. *BMC genomics* **11**, 239 (2010).
61. Arredondo-Alonso, S., *et al.* Plasmids shaped the recent emergence of the major nosocomial pathogen Enterococcus faecium. *MBio* **11**(2020).
62. Lebreton, F., *et al.* Emergence of epidemic multidrug-resistant Enterococcus faecium from animal and commensal strains. *MBio* **4**(2013).
63. Wain, H. *The story behind the word: some interesting origins of medical terms*, (Thomas, 1958).
64. Powers, M.E. & Wardenburg, J.B. Igniting the fire: Staphylococcus aureus virulence factors in the pathogenesis of sepsis. *PLoS pathogens* **10**(2014).
65. Weiss, R.A. Virulence and pathogenesis. *Trends in microbiology* **10**, 314-317 (2002).
66. Tuchscherr, L., *et al.* Staphylococcus aureus phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection. *EMBO molecular medicine* **3**, 129-141 (2011).
67. Mundy, L., Sahm, D. & Gilmore, M. Relationships between enterococcal virulence and antimicrobial resistance. *Clinical microbiology reviews* **13**, 513-522 (2000).
68. Garg, A. & Gupta, D. VirulentPred: a SVM based prediction method for virulent proteins in bacterial pathogens. *BMC bioinformatics* **9**, 62 (2008).
69. Tsai, C.-T., Huang, W.-L., Ho, S.-J., Shu, L.-S. & Ho, S.-Y. Virulent-GO: prediction of virulent proteins in bacterial pathogens utilizing gene ontology terms. *development* **1**(2009).

70. Chen, L., *et al.* VFDB: a reference database for bacterial virulence factors. *Nucleic acids research* **33**, D325-D328 (2005).
71. Sayers, S., *et al.* Victors: a web-based knowledge base of virulence factors in human and animal pathogens. *Nucleic acids research* **47**, D693-D700 (2019).
72. Ogston, A. Report upon micro-organisms in surgical diseases. *British medical journal* **1**, 369. b362 (1881).
73. Rosenbach, A.J.F. *Mikro-organismen bei den Wund-infections-krankheiten des Menschen*, (JF Bergmann, 1884).
74. Licitra, G. Etymologia: Staphylococcus. *Emerging Infectious Diseases* **19**, 1553 (2013).
75. Gnanamani, A., Hariharan, P. & Paul-Satyaseela, M. Staphylococcus aureus: Overview of bacteriology, clinical diseases, epidemiology, antibiotic resistance and therapeutic approach. *Frontiers in Staphylococcus aureus*, 4-28 (2017).
76. Foster, T. Staphylococcus. in *Medical Microbiology. 4th edition* (University of Texas Medical Branch at Galveston, 1996).
77. O'Riordan, K. & Lee, J.C. Staphylococcus aureus capsular polysaccharides. *Clinical microbiology reviews* **17**, 218-234 (2004).
78. Harris, L.G., Foster, S. & Richards, R.G. An introduction to Staphylococcus aureus, and techniques for identifying and quantifying S. aureus adhesins in relation to adhesion to biomaterials: review. *Eur Cell Mater* **4**, 39-60 (2002).
79. Rajagopal, M. & Walker, S. Envelope structures of Gram-positive bacteria. in *Protein and Sugar Export and Assembly in Gram-positive Bacteria* 1-44 (Springer, 2015).
80. Pasquina-Lemonche, L., *et al.* The architecture of the Gram-positive bacterial cell wall. *Nature*, 1-4 (2020).
81. Liu, G.Y., *et al.* Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *The Journal of experimental medicine* **202**, 209-215 (2005).
82. Parfentjev, I. & Catelli, A.R. Tolerance of Staphylococcus aureus to sodium chloride. *Journal of bacteriology* **88**, 1-3 (1964).
83. Dings, M.M., Orwin, P.M. & Schlievert, P. Exotoxins of Staphyloccocus aureus. *Clin. Microbiol. Rev* **13**, 16-34 (2000).
84. Otto, M. Staphylococcus aureus toxins. *Current opinion in microbiology* **17**, 32-37 (2014).
85. Foster, T.J. The Staphylococcus aureus "superbug". *The Journal of clinical investigation* **114**, 1693-1696 (2004).
86. Liu, G.Y. Molecular pathogenesis of Staphylococcus aureus infection. *Pediatric research* **65**, 71-77 (2009).
87. Kang, J., Dietz, M.J. & Li, B. Antimicrobial peptide LL-37 is bactericidal against Staphylococcus aureus biofilms. *PloS one* **14**, e0216676 (2019).
88. Thomer, L., Schneewind, O. & Missiakas, D. Pathogenesis of Staphylococcus aureus bloodstream infections. *Annual Review of Pathology: Mechanisms of Disease* **11**, 343-364 (2016).
89. Kuroda, M., *et al.* Whole genome sequencing of meticillin-resistant Staphylococcus aureus. *The Lancet* **357**, 1225-1240 (2001).
90. Lindsay, J.A. & Holden, M.T. Staphylococcus aureus: superbug, super genome? *Trends in microbiology* **12**, 378-385 (2004).
91. Jevons, M. Letter Br. *Med J* **1**, 124-125 (1961).
92. Katayama, Y., Ito, T. & Hiramatsu, K. A new class of genetic element, staphylococcus cassette chromosome mec, encodes methicillin resistance in Staphylococcus aureus. *Antimicrobial agents and chemotherapy* **44**, 1549-1555 (2000).
93. Stefani, S., *et al.* Meticillin-resistant Staphylococcus aureus (MRSA): global epidemiology and harmonisation of typing methods. *International journal of antimicrobial agents* **39**, 273-282 (2012).
94. Álvarez, A., *et al.* Methicillin-resistant Staphylococcus aureus in hospitals: latest trends and treatments based on bacteriophages. *Journal of Clinical Microbiology* **57**(2019).
95. Kong, E.F., Johnson, J.K. & Jabra-Rizk, M.A. Community-associated methicillin-resistant Staphylococcus aureus: an enemy amidst us. *PLoS pathogens* **12**(2016).

96. Tenover, F.C. & Goering, R.V. Methicillin-resistant Staphylococcus aureus strain USA300: origin and epidemiology. *Journal of Antimicrobial Chemotherapy* **64**, 441-446 (2009).
97. Miller, L.G. & Diep, B.A. Colonization, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant Staphylococcus aureus infection. *Clinical Infectious Diseases* **46**, 752-760 (2008).
98. Smyth, D.S., Wong, A. & Robinson, D.A. Cross-species spread of SCCmec IV subtypes in staphylococci. *Infection, Genetics and Evolution* **11**, 446-453 (2011).
99. Bamberger, D.M. The Role of Panton-Valentine Leukocidin: The Pendulum Swings. (Oxford University Press US, 2017).
100. McManus, B.A., O'Connor, A.M., Egan, S.A., Flanagan, P.R. & Coleman, D.C. First description of arginine catabolic mobile element (ACME) type VI harboring the kdp operon only in Staphylococcus epidermidis using short and long read whole genome sequencing: Further evidence of ACME diversity. *Infection, Genetics and Evolution* **71**, 51-53 (2019).
101. Control, C.f.D. & Prevention. Methicillin-resistant Staphylococcus aureus infections in correctional facilities---Georgia, California, and Texas, 2001-2003. *MMWR. Morbidity and mortality weekly report* **52**, 992 (2003).
102. Fergie, J.E. & Purcell, K. Community-acquired methicillin-resistant Staphylococcus aureus infections in south Texas children. *The Pediatric infectious disease journal* **20**, 860-863 (2001).
103. Begier, E.M., *et al.* A high-morbidity outbreak of methicillin-resistant Staphylococcus aureus among players on a college football team, facilitated by cosmetic body shaving and turf burns. *Clinical infectious diseases* **39**, 1446-1453 (2004).
104. Frickmann, H. Impact of MRSA on the military medical service and diagnostic point-of-care options for the field setting. *European Journal of Microbiology and Immunology* **8**, 31-33 (2018).
105. Howden, B.P., Davies, J.K., Johnson, P.D., Stinear, T.P. & Grayson, M.L. Reduced vancomycin susceptibility in Staphylococcus aureus, including vancomycin-intermediate and heterogeneous vancomycin-intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clinical microbiology reviews* **23**, 99-139 (2010).
106. Choe, D., *et al.* Genome-scale analysis of Methicillin-resistant Staphylococcus aureus USA300 reveals a tradeoff between pathogenesis and drug resistance. *Scientific reports* **8**, 1-13 (2018).
107. Lacey, K.A., *et al.* The Staphylococcus aureus cell wall-anchored protein clumping factor A is an important T cell antigen. *Infection and immunity* **85**(2017).
108. Cheung, A.L., Bayer, A.S., Zhang, G., Gresham, H. & Xiong, Y.-Q. Regulation of virulence determinants in vitro and in vivo in Staphylococcus aureus. *FEMS Immunology & Medical Microbiology* **40**, 1-9 (2004).
109. Foster, T.J., Geoghegan, J.A., Ganesh, V.K. & Höök, M. Adhesion, invasion and evasion: the many functions of the surface proteins of Staphylococcus aureus. *Nature Reviews Microbiology* **12**, 49 (2014).
110. Ajayi, C. Determinants of Staphylococcus aureus Colonization and Infection."Exploring the Role of Cell Wall Anchored Proteins in Adhesion and Immune Evasion". (2018).
111. Caldelari, I., Chao, Y., Romby, P. & Vogel, J. RNA-mediated regulation in pathogenic bacteria. *Cold Spring Harbor perspectives in medicine* **3**, a010298 (2013).
112. Lalaouna, D., Eyraud, A., Chabelskaya, S., Felden, B. & Masse, E. Regulatory RNAs involved in bacterial antibiotic resistance. *PLoS pathogens* **10**, e1004299 (2014).
113. Waters, L.S. & Storz, G. Regulatory RNAs in bacteria. *Cell* **136**, 615-628 (2009).
114. Wagner, E.G.H. & Vogel, J. Noncoding RNAs encoded by bacterial chromosomes. *Noncoding RNAs: molecular biology and molecular medicine. Kluwer Academic/Plenum Publishers, New York*, 242-258 (2003).
115. Westermann, A.J. Regulatory RNAs in Virulence and Host-Microbe Interactions. *Microbiology spectrum* **6**(2018).
116. Romby, P. & Charpentier, E. An overview of RNAs with regulatory functions in gram-positive bacteria. *Cellular and Molecular Life Sciences* **67**, 217-237 (2010).

117. Breaker, R.R. Prospects for riboswitch discovery and analysis. *Molecular cell* **43**, 867-879 (2011).
118. McCown, P.J., Corbino, K.A., Stav, S., Sherlock, M.E. & Breaker, R.R. Riboswitch diversity and distribution. *Rna* **23**, 995-1011 (2017).
119. Tomasini, A., *et al.* The importance of regulatory RNAs in Staphylococcus aureus. *Infection, Genetics and Evolution* **21**, 616-626 (2014).
120. Papenfort, K. & Vogel, J. Regulatory RNA in bacterial pathogens. *Cell host & microbe* **8**, 116-127 (2010).
121. Vitse, J. & Devreese, B. The Contribution of Membrane Vesicles to Bacterial Pathogenicity in Cystic Fibrosis Infections and Healthcare Associated Pneumonia. *Frontiers in Microbiology* **11**, 630 (2020).
122. Théry, C., *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *Journal of extracellular vesicles* **7**, 1535750 (2018).
123. Nadeem, A., Oscarsson, J. & Wai, S.N. Delivery of Virulence Factors by Bacterial Membrane Vesicles to Mammalian Host Cells. in *Bacterial Membrane Vesicles* 131-158 (Springer, 2020).
124. Askarian, F., *et al.* Staphylococcus aureus Membrane-Derived Vesicles Promote Bacterial Virulence and Confer Protective Immunity in Murine Infection Models. *Frontiers in microbiology* **9**, 262 (2018).
125. Obana, N., *et al.* Immunoactive clostridial membrane vesicle production is regulated by a sporulation factor. *Infection and immunity* **85**(2017).
126. Brown, L., Wolf, J.M., Prados-Rosales, R. & Casadevall, A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nature Reviews Microbiology* **13**, 620 (2015).
127. Toyofuku, M., Nomura, N. & Eberl, L. Types and origins of bacterial membrane vesicles. *Nature Reviews Microbiology*, 1 (2018).
128. Tartaglia, N.R., *et al.* Extracellular vesicles produced by human and animal Staphylococcus aureus strains share a highly conserved core proteome. *Scientific Reports* **10**, 1-13 (2020).
129. Dorward, D.W. & Garon, C.F. DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria. *Applied and Environmental Microbiology* **56**, 1960-1962 (1990).
130. Coelho, C. & Casadevall, A. Answers to naysayers regarding microbial extracellular vesicles. *Biochemical Society Transactions* **47**, 1005-1012 (2019).
131. Lee, E.Y., *et al.* Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of Staphylococcus aureus-derived membrane vesicles. *Proteomics* **9**, 5425-5436 (2009).
132. Palacios, A., *et al.* Biogenesis and Function of Extracellular Vesicles in Gram-Positive Bacteria, Mycobacteria, and Fungi. in *Bacterial Membrane Vesicles* 47-74 (Springer, 2020).
133. Olaya-Abril, A., *et al.* Characterization of protective extracellular membrane-derived vesicles produced by Streptococcus pneumoniae. *Journal of proteomics* **106**, 46-60 (2014).
134. Rivera, J., *et al.* Bacillus anthracis produces membrane-derived vesicles containing biologically active toxins. *Proceedings of the National Academy of Sciences*, 201008843 (2010).
135. Gurung, M., *et al.* Staphylococcus aureus produces membrane-derived vesicles that induce host cell death. *PloS one* **6**, e27958 (2011).
136. Lee, J.H., *et al.* Transcription factor σ B plays an important role in the production of extracellular membrane-derived vesicles in Listeria monocytogenes. *PloS one* **8**, e73196 (2013).
137. Jiang, Y., Kong, Q., Roland, K.L. & Curtiss III, R. Membrane vesicles of Clostridium perfringens type A strains induce innate and adaptive immunity. *International journal of medical microbiology* **304**, 431-443 (2014).
138. Haas, B. & Grenier, D. Isolation, characterization and biological properties of membrane vesicles produced by the swine pathogen Streptococcus suis. *PLoS One* **10**, e0130528 (2015).

139. Biagini, M., *et al.* The human pathogen Streptococcus pyogenes releases lipoproteins as lipoprotein-rich membrane vesicles. *Molecular & Cellular Proteomics* **14**, 2138-2149 (2015).
140. Resch, U., *et al.* A two-component regulatory system impacts extracellular membrane-derived vesicle production in group A Streptococcus. *MBio* **7**, e00207-00216 (2016).
141. Kim, Y., Edwards, N. & Fenselau, C. Extracellular vesicle proteomes reflect developmental phases of Bacillus subtilis. *Clinical proteomics* **13**, 6 (2016).
142. Surve, M.V., *et al.* Membrane vesicles of group B streptococcus disrupt feto-maternal barrier leading to preterm birth. *PLoS pathogens* **12**, e1005816 (2016).
143. Vdovikova, S., *et al.* A novel role of Listeria monocytogenes membrane vesicles in inhibition of autophagy and cell death. *Frontiers in cellular and infection microbiology* **7**, 154 (2017).
144. Grande, R., *et al.* Detection and physicochemical characterization of membrane vesicles (MVs) of Lactobacillus reuteri DSM 17938. *Frontiers in microbiology* **8**, 1040 (2017).
145. Li, M., *et al.* Lactobacillus-derived extracellular vesicles enhance host immune responses against vancomycin-resistant enterococci. *BMC microbiology* **17**, 66 (2017).
146. Nicholas, A., *et al.* Clostridium difficile-derived membrane vesicles induce the expression of pro-inflammatory cytokine genes and cytotoxicity in colonic epithelial cells in vitro. *Microbial pathogenesis* **107**, 6-11 (2017).
147. Choi, C.-W., *et al.* Potential usefulness of Streptococcus pneumoniae extracellular membrane vesicles as antibacterial vaccines. *Journal of immunology research* **2017**(2017).
148. Kim, H.Y., Lim, Y., An, S.J. & Choi, B.K. Characterization and immunostimulatory activity of extracellular vesicles from Filifactor alocis. *Molecular Oral Microbiology* **35**, 1-9 (2020).
149. Tartaglia, N.R., *et al.* Staphylococcus aureus extracellular vesicles elicit an immunostimulatory response in vivo on the murine mammary gland. *Frontiers in cellular and infection microbiology* **8**, 277 (2018).
150. Cao, Y., *et al.* Proteomic and metabolic characterization of membrane vesicles derived from Streptococcus mutans at different pH values. *Applied Microbiology and Biotechnology*, 1-16 (2020).
151. Lai, C.H., Listgarten, M.A. & Hammond, B.F. Comparative ultrastructure of leukotoxic and non-leukotoxic strains of Actinobacillus actinomycetemcomitans. *Journal of periodontal research* **16**, 379-389 (1981).
152. Wai, S.N., Takade, A. & Amako, K. The release of outer membrane vesicles from the strains of enterotoxigenic Escherichia coli. *Microbiology and immunology* **39**, 451-456 (1995).
153. Devos, S., *et al.* Membrane vesicle secretion and prophage induction in multidrug-resistant Stenotrophomonas maltophilia in response to ciprofloxacin stress. *Environmental microbiology* **19**, 3930-3937 (2017).
154. Turnbull, L., *et al.* Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. *Nature communications* **7**, 1-13 (2016).
155. Toyofuku, M., Nomura, N. & Eberl, L. Types and origins of bacterial membrane vesicles. *Nature Reviews Microbiology* **17**, 13-24 (2019).
156. Prados-Rosales, R., *et al.* Role for Mycobacterium tuberculosis membrane vesicles in iron acquisition. *Journal of bacteriology* **196**, 1250-1256 (2014).
157. van de Waterbeemd, B., *et al.* Cysteine depletion causes oxidative stress and triggers outer membrane vesicle release by Neisseria meningitidis; implications for vaccine development. *PloS one* **8**, e54314 (2013).
158. Baumgarten, T., *et al.* Membrane vesicle formation as a multiple-stress response mechanism enhances Pseudomonas putida DOT-T1E cell surface hydrophobicity and biofilm formation. *Applied and environmental microbiology* **78**, 6217-6224 (2012).
159. McMahon, K.J., Castelli, M.E., Vescovi, E.G. & Feldman, M.F. Biogenesis of outer membrane vesicles in Serratia marcescens is thermoregulated and can be induced by activation of the Rcs phosphorelay system. *Journal of bacteriology* **194**, 3241-3249 (2012).
160. MacDonald, I.A. & Kuehn, M.J. Stress-induced outer membrane vesicle production by Pseudomonas aeruginosa. *Journal of bacteriology* **195**, 2971-2981 (2013).
161. Lee, E.Y., *et al.* Global proteomic profiling of native outer membrane vesicles derived from Escherichia coli. *Proteomics* **7**, 3143-3153 (2007).

162. Bager, R.J., *et al.* Outer membrane vesicles reflect environmental cues in Gallibacterium anatis. *Veterinary microbiology* **167**, 565-572 (2013).
163. Choi, C.-W., *et al.* Proteomic characterization of the outer membrane vesicle of Pseudomonas putida KT2440. *Journal of proteome research* **13**, 4298-4309 (2014).
164. Wang, X., Thompson, C.D., Weidenmaier, C. & Lee, J.C. Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nature communications* **9**, 1379 (2018).
165. Rodriguez, G.M. & Prados-Rosales, R. Functions and importance of mycobacterial extracellular vesicles. *Applied microbiology and biotechnology* **100**, 3887-3892 (2016).
166. Liu, Y., Defourny, K.A., Smid, E.J. & Abee, T. Gram-positive bacterial extracellular vesicles and their impact on health and disease. *Frontiers in microbiology* **9**, 1502 (2018).
167. Liu, Y., Alexeeva, S., Defourny, K.A., Smid, E.J. & Abee, T. Tiny but mighty: bacterial membrane vesicles in food biotechnological applications. *Current opinion in biotechnology* **49**, 179-184 (2018).
168. Thay, B., Wai, S.N. & Oscarsson, J. Staphylococcus aureus α-toxin-dependent induction of host cell death by membrane-derived vesicles. *PloS one* **8**(2013).
169. Dal Peraro, M. & Van Der Goot, F.G. Pore-forming toxins: ancient, but never really out of fashion. *Nature reviews microbiology* **14**, 77-92 (2016).
170. Sendi, P., Johansson, L. & Norrby-Teglund, A. Invasive group B streptococcal disease in non-pregnant adults. *Infection* **36**, 100-111 (2008).
171. Kim, J.-H., *et al.* Extracellular vesicle–derived protein from Bifidobacterium longum alleviates food allergy through mast cell suppression. *Journal of Allergy and Clinical Immunology* **137**, 507-516. e508 (2016).
172. Domínguez Rubio, A.P., *et al.* Lactobacillus casei BL23 Produces Microvesicles Carrying Proteins That Have Been Associated with Its Probiotic Effect. *Frontiers in microbiology* **8**, 1783 (2017).
173. Palomino, R.A.Ñ., *et al.* Extracellular vesicles from symbiotic vaginal lactobacilli inhibit HIV-1 infection of human tissues. *Nature communications* **10**, 1-14 (2019).
174. Toyofuku, M., Tashiro, Y., Hasegawa, Y., Kurosawa, M. & Nomura, N. Bacterial membrane vesicles, an overlooked environmental colloid: biology, environmental perspectives and applications. *Advances in Colloid and Interface Science* **226**, 65-77 (2015).
175. Acevedo, R., *et al.* Bacterial outer membrane vesicles and vaccine applications. *Frontiers in immunology* **5**, 121 (2014).
176. Gerritzen, M.J., Martens, D.E., Wijffels, R.H., van der Pol, L. & Stork, M. Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnology advances* **35**, 565-574 (2017).
177. Kunjantarachot, A. & Phanaksri, T. Effective platform for the production of recombinant outer membrane vesicles in gram-negative bacteria. *Journal of Microbiology and Biotechnology* **30**(2020).
178. van der Pol, L., Stork, M. & van der Ley, P. Outer membrane vesicles as platform vaccine technology. *Biotechnology journal* **10**, 1689-1706 (2015).
179. de Kleijn, E.D., *et al.* Immunogenicity and safety of a hexavalent meningococcal outer-membrane-vesicle vaccine in children of 2–3 and 7–8 years of age. *Vaccine* **18**, 1456-1466 (2000).
180. O'Ryan, M., Stoddard, J., Toneatto, D., Wassil, J. & Dull, P.M. A multi-component meningococcal serogroup B vaccine (4CMenB): the clinical development program. *Drugs* **74**, 15-30 (2014).
181. Beernink, P.T., Vianzon, V., Lewis, L.A., Moe, G.R. & Granoff, D.M. A meningococcal outer membrane vesicle vaccine with overexpressed mutant FHbp elicits higher protective antibody responses in infant rhesus macaques than a licensed serogroup B vaccine. *MBio* **10**(2019).
182. Choi, S.J., *et al.* Active immunization with extracellular vesicles derived from Staphylococcus aureus effectively protects against staphylococcal lung infections, mainly via Th1 cell-mediated immunity. *PloS one* **10**(2015).

183. Wang, X., Thompson, C.D., Weidenmaier, C. & Lee, J.C. Release of Staphylococcus aureus extracellular vesicles and their application as a vaccine platform. *Nature communications* **9**, 1-13 (2018).
184. Beaulac, C., Sachetelli, S. & Lagace, J. In-vitro bactericidal efficacy of sub-MIC concentrations of liposome-encapsulated antibiotic against gram-negative and gram-positive bacteria. *The Journal of antimicrobial chemotherapy* **41**, 35-41 (1998).
185. MacDiarmid, J.A., *et al.* Bacterially derived 400 nm particles for encapsulation and cancer cell targeting of chemotherapeutics. *Cancer cell* **11**, 431-445 (2007).
186. Johnsen, K.B., *et al.* On the use of liposome controls in studies investigating the clinical potential of extracellular vesicle-based drug delivery systems–A commentary. *Journal of controlled release* **269**, 10-14 (2018).
187. Kim, M.W., Kwon, S.-H., Choi, J.H. & Lee, A. A promising biocompatible platform: lipid-based and bio-inspired smart drug delivery systems for cancer therapy. *International Journal of Molecular Sciences* **19**, 3859 (2018).
188. Li, R. & Liu, Q. Engineered bacterial outer membrane vesicles as multifunctional delivery platforms. *Front. Mater. 7: 202. doi: 10.3389/fmats* (2020).
189. Chen, Y., Tang, Y., Fan, G.-C. & Duan, D.D. Extracellular vesicles as novel biomarkers and pharmaceutic targets of diseases. *Acta Pharmacologica Sinica* **39**, 499-500 (2018).
190. Wang, J., Tang, L.J. & Garcia, R.C. Extracellular vesicles in mycobacterial infections: their potential as molecule transfer vectors. *Frontiers in Immunology* **10**, 1929 (2019).
191. Xu, J., *et al.* Membrane vesicle protein PagC as a novel biomarker for detecting pathogenic Salmonella in the viable but not culturable state. *Journal of Veterinary Medical Science*, 17-0164 (2017).
192. Kruh-Garcia, N.A., *et al.* Detection of Mycobacterium tuberculosis peptides in the exosomes of patients with active and latent M. tuberculosis infection using MRM-MS. *PloS one* **9**, e103811 (2014).
193. Schrempf, H., Koebsch, I., Walter, S., Engelhardt, H. & Meschke, H. Extracellular Streptomyces vesicles: amphorae for survival and defence. *Microbial biotechnology* **4**, 286-299 (2011).
194. Lee, J., *et al.* Staphylococcus aureus extracellular vesicles carry biologically active beta-lactamase. *Antimicrobial agents and chemotherapy*, AAC. 00522-00512 (2013).
195. Soler, N. & Forterre, P. Vesiduction: the fourth way of HGT. *Environmental Microbiology* (2020).
196. Klieve, A.V., *et al.* Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic Ruminococcus spp. of ruminal origin. *Applied and environmental microbiology* **71**, 4248-4253 (2005).
197. Bitto, N.J., *et al.* Bacterial membrane vesicles transport their DNA cargo into host cells. *Scientific reports* **7**, 1-11 (2017).
198. Toyofuku, M., *et al.* Prophage-triggered membrane vesicle formation through peptidoglycan damage in Bacillus subtilis. *Nature communications* **8**, 481 (2017).
199. Abdullah, Z., *et al.* RIG-I detects infection with live Listeria by sensing secreted bacterial nucleic acids. *The EMBO journal* **31**, 4153-4164 (2012).
200. Biller, S.J., *et al.* Bacterial vesicles in marine ecosystems. *science* **343**, 183-186 (2014).
201. Dauros-Singorenko, P., Blenkiron, C., Phillips, A. & Swift, S. The functional RNA cargo of bacterial membrane vesicles. *FEMS microbiology letters* **365**, fny023 (2018).
202. Badi, S.A., *et al.* Small RNAs in Outer Membrane Vesicles and Their Function in Host-Microbe Interactions. *Frontiers in Microbiology* **11**(2020).
203. Malge, A., *et al.* mRNA transcript distribution bias between Borrelia burgdorferi bacteria and their outer membrane vesicles. *FEMS microbiology letters* **365**, fny135 (2018).
204. Whitworth, D.E. Interspecies conflict affects RNA expression. *FEMS microbiology letters* **365**, fny096 (2018).
205. O'Donoghue, E.J. & Krachler, A.M. Mechanisms of outer membrane vesicle entry into host cells. *Cellular microbiology* **18**, 1508-1517 (2016).
206. Valadi, H., *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nature cell biology* **9**, 654-659 (2007).

207. Colombo, M., Raposo, G. & Théry, C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annual review of cell and developmental biology* **30**, 255-289 (2014).
208. Frantz, R., *et al.* The secRNome of Listeria monocytogenes harbors small noncoding RNAs that are potent inducers of beta interferon. *MBio* **10**, e01223-01219 (2019).
209. Choi, J.-W., Kim, S.-C., Hong, S.-H. & Lee, H.-J. Secretable small RNAs via outer membrane vesicles in periodontal pathogens. *Journal of dental research* **96**, 458-466 (2017).
210. Tsatsaronis, J.A., Franch-Arroyo, S., Resch, U. & Charpentier, E. Extracellular vesicle RNA: a universal mediator of microbial communication? *Trends in microbiology* **26**, 401-410 (2018).
211. Dorward, D.W., Garon, C.F. & Judd, R.C. Export and intercellular transfer of DNA via membrane blebs of Neisseria gonorrhoeae. *Journal of bacteriology* **171**, 2499-2505 (1989).
212. Ho, M.-H., Chen, C.-H., Goodwin, J.S., Wang, B.-Y. & Xie, H. Functional advantages of Porphyromonas gingivalis vesicles. *PloS one* **10**(2015).
213. Ghosal, A., *et al.* The extracellular RNA complement of Escherichia coli. *Microbiologyopen* **4**, 252-266 (2015).
214. Sjöström, A.E., Sandblad, L., Uhlin, B.E. & Wai, S.N. Membrane vesicle-mediated release of bacterial RNA. *Scientific reports* **5**, 15329 (2015).
215. Koeppen, K., *et al.* A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. *PLoS pathogens* **12**, e1005672 (2016).
216. Malabirade, A., *et al.* The RNA complement of outer membrane vesicles from Salmonella enterica serovar Typhimurium under distinct culture conditions. *Frontiers in microbiology* **9**, 2015 (2018).
217. Choi, J.-W., Kwon, T.-Y., Hong, S.-H. & Lee, H.-J. Isolation and Characterization of a microRNA-size secretable Small RNA in Streptococcus sanguinis. *Cell biochemistry and biophysics* **76**, 293-301 (2018).
218. Langlete, P., Krabberød, A.K. & Winther-Larsen, H.C. Vesicles from Vibrio cholerae contain AT-rich DNA and shorter mRNAs that do not correlate with their protein products. *Frontiers in Microbiology* **10**, 2708 (2019).
219. Zhang, H., *et al.* sncRNAs packaged by Helicobacter pylori outer membrane vesicles attenuate IL-8 secretion in human cells. *International Journal of Medical Microbiology* **310**, 151356 (2020).
220. Dauros Singorenko, P., *et al.* Isolation of membrane vesicles from prokaryotes: a technical and biological comparison reveals heterogeneity. *Journal of extracellular vesicles* **6**, 1324731 (2017).
221. Schulz, E., Karagianni, A., Koch, M. & Fuhrmann, G. Hot EVs–how temperature affects extracellular vesicles. *European Journal of Pharmaceutics and Biopharmaceutics* **146**, 55-63 (2020).
222. Jain, S. & Pillai, J. Bacterial membrane vesicles as novel nanosystems for drug delivery. *International journal of nanomedicine* **12**, 6329 (2017).
223. Van Veldhoven, P.P., Baumgart, E. & Mannaerts, G.P. Iodixanol (Optiprep), an improved density gradient medium for the iso-osmotic isolation of rat liver peroxisomes. *Analytical biochemistry* **237**, 17-23 (1996).
224. Ramirez, M.I., *et al.* Technical challenges of working with extracellular vesicles. *Nanoscale* **10**, 881-906 (2018).
225. Maas, S.L., *et al.* Possibilities and limitations of current technologies for quantification of biological extracellular vesicles and synthetic mimics. *Journal of Controlled Release* **200**, 87-96 (2015).
226. Heinrich, L., *et al.* Confocal laser scanning microscopy using dialkylcarbocyanine dyes for cell tracing in hard and soft biomaterials. *Journal of Biomedical Materials Research Part B: Applied Biomaterials: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials* **81**, 153-161 (2007).
227. Chuo, S.T.-Y., Chien, J.C.-Y. & Lai, C.P.-K. Imaging extracellular vesicles: current and emerging methods. *Journal of biomedical science* **25**, 1-10 (2018).

228. Mateescu, B., *et al.* Obstacles and opportunities in the functional analysis of extracellular vesicle RNA–an ISEV position paper. *Journal of extracellular vesicles* **6**, 1286095 (2017).
229. McBroom, A.J., Johnson, A.P., Vemulapalli, S. & Kuehn, M.J. Outer membrane vesicle production by Escherichia coli is independent of membrane instability. *Journal of bacteriology* **188**, 5385-5392 (2006).
230. Olsen, J.V., Ong, S.-E. & Mann, M. Trypsin cleaves exclusively C-terminal to arginine and lysine residues. *Molecular & Cellular Proteomics* **3**, 608-614 (2004).
231. Abramowicz, A., Widlak, P. & Pietrowska, M. Proteomic analysis of exosomal cargo: the challenge of high purity vesicle isolation. *Molecular BioSystems* **12**, 1407-1419 (2016).
232. Klimentová, J. & Stulík, J. Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiological research* **170**, 1-9 (2015).
233. Langmead, B. & Salzberg, S.L. Fast gapped-read alignment with Bowtie 2. *Nature methods* **9**, 357 (2012).
234. Milne, I., Bayer, M., Stephen, G., Cardle, L. & Marshall, D. Tablet: visualizing next-generation sequence assemblies and mappings. in *Plant Bioinformatics* 253-268 (Springer, 2016).
235. Huang, W. & Marth, G. EagleView: a genome assembly viewer for next-generation sequencing technologies. *Genome research* **18**, 1538-1543 (2008).
236. Rutherford, K., *et al.* Artemis: sequence visualization and annotation. *Bioinformatics* **16**, 944-945 (2000).
237. Robinson, J.T., *et al.* Integrative genomics viewer. *Nature biotechnology* **29**, 24-26 (2011).
238. Huang, Y., Sheth, R.U., Kaufman, A. & Wang, H.H. Scalable and cost-effective ribonuclease-based rRNA depletion for transcriptomics. *Nucleic acids research* **48**, e20-e20 (2020).
239. Kim, M.H., *et al.* Production of membrane vesicles by Enterococcus faecium cultured with or without subinhibitory concentrations of antibiotics and their pathological effects on epithelial cells. *Frontiers in cellular and infection microbiology* **9**, 295 (2019).
240. Van der Pol, E., *et al.* Particle size distribution of exosomes and microvesicles determined by transmission electron microscopy, flow cytometry, nanoparticle tracking analysis, and resistive pulse sensing. *Journal of Thrombosis and Haemostasis* **12**, 1182-1192 (2014).
241. Stentz, R., Carvalho, A.L., Jones, E.J. & Carding, S.R. Fantastic voyage: the journey of intestinal microbiota-derived microvesicles through the body. *Biochemical Society Transactions* **46**, 1021-1027 (2018).
242. Kim, M.R., *et al.* S taphylococcus aureus-derived extracellular vesicles induce neutrophilic pulmonary inflammation via both T h1 and T h17 cell responses. *Allergy* **67**, 1271-1281 (2012).
243. Schlatterer, K., *et al.* The mechanism behind bacterial lipoprotein release: Phenol-soluble modulins mediate toll-like receptor 2 activation via extracellular vesicle release from Staphylococcus aureus. *MBio* **9**(2018).
244. Andreoni, F., *et al.* Antibiotics stimulate formation of vesicles in Staphylococcus aureus in both phage-dependent and-independent fashions and via different routes. *Antimicrobial agents and chemotherapy* **63**, e01439-01418 (2019).
245. Jeon, J., *et al.* Proteomic analysis of extracellular vesicles derived from Propionibacterium acnes. *PROTEOMICS–Clinical Applications* **11**, 1600040 (2017).
246. Paganelli, F.L., *et al.* Enterococcus faecium biofilm formation: identification of major autolysin AtlAEfm, associated Acm surface localization, and AtlAEfm-independent extracellular DNA Release. *MBio* **4**(2013).
247. Cooke, A.C., *et al.* PQS-Induced Outer Membrane Vesicles Enhance Biofilm Dispersion in Pseudomonas aeruginosa. *bioRxiv* (2020).
248. Mashburn-Warren, L., Howe, J., Brandenburg, K. & Whiteley, M. Structural requirements of the Pseudomonas quinolone signal for membrane vesicle stimulation. *Journal of bacteriology* **191**, 3411-3414 (2009).
249. Wood, T.K. Strategies for combating persister cell and biofilm infections. *Microbial biotechnology* **10**, 1054-1056 (2017).
250. Wang, W., Chanda, W. & Zhong, M. The relationship between biofilm and outer membrane vesicles: a novel therapy overview. *FEMS microbiology letters* **362**(2015).

251. Westermann, A.J. Regulatory RNAs in Virulence and Host-Microbe Interactions. *Regulating with RNA in Bacteria and Archaea*, 305-337 (2018).
252. Novick, R.P., *et al.* Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *The EMBO journal* **12**, 3967-3975 (1993).
253. Liao, S., *et al.* Streptococcus mutans extracellular DNA is upregulated during growth in biofilms, actively released via membrane vesicles, and influenced by components of the protein secretion machinery. *Journal of bacteriology* **196**, 2355-2366 (2014).
254. Celluzzi, A. & Masotti, A. How our other genome controls our epi-genome. *Trends in microbiology* **24**, 777-787 (2016).
255. Liu, S., *et al.* The host shapes the gut microbiota via fecal microRNA. *Cell host & microbe* **19**, 32-43 (2016).
256. Teng, Y., *et al.* Plant-derived exosomal microRNAs shape the gut microbiota. *Cell host & microbe* **24**, 637-652. e638 (2018).
257. Anfossi, S. & Calin, G.A. Gut microbiota: a new player in regulating immune-and chemo-therapy efficacy. *Cancer drug resistance (Alhambra, Calif.)* **3**, 356 (2020).
258. Fulsundar, S., *et al.* Gene transfer potential of outer membrane vesicles of Acinetobacter baylyi and effects of stress on vesiculation. *Applied and environmental microbiology* **80**, 3469-3483 (2014).
259. Chiura, H.X., Kogure, K., Hagemann, S., Ellinger, A. & Velimirov, B. Evidence for particle-induced horizontal gene transfer and serial transduction between bacteria. *FEMS microbiology ecology* **76**, 576-591 (2011).
260. Domingues, S. & Nielsen, K.M. Membrane vesicles and horizontal gene transfer in prokaryotes. *Current opinion in microbiology* **38**, 16-21 (2017).
261. Keller, M.D., *et al.* Decoy exosomes provide protection against bacterial toxins. *Nature*, 1-5 (2020).
262. Fu, Y., *et al.* Detection of mycobacterial small RNA in the bacterial culture supernatant and plasma of patients with active tuberculosis. *Biochemical and biophysical research communications* **503**, 490-494 (2018).
263. Dahiya, B., *et al.* Detection of Mycobacterium tuberculosis lipoarabinomannan and CFP-10 (Rv3874) from urinary extracellular vesicles of tuberculosis patients by immuno-PCR. *Pathogens and disease* **77**, ftz049 (2019).
264. Holmqvist, E. & Wagner, E.G.H. Impact of bacterial sRNAs in stress responses. *Biochemical Society Transactions* **45**, 1203-1212 (2017).
265. Rakoff-Nahoum, S., Coyne, M. & Comstock, L. An ecological network of polysaccharide utilization among human intestinal symbionts. *Current Biology* **24**, 40-49 (2014).
266. Rumbo, C., *et al.* Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii. *Antimicrobial agents and chemotherapy* **55**, 3084-3090 (2011).
267. Jacob, J.T. & DiazGranados, C.A. High vancomycin minimum inhibitory concentration and clinical outcomes in adults with methicillin-resistant Staphylococcus aureus infections: a meta-analysis. *International Journal of Infectious Diseases* **17**, e93-e100 (2013).
268. Lodise, T., *et al.* Relationship between vancomycin MIC and failure among patients with methicillin-resistant Staphylococcus aureus bacteremia treated with vancomycin. *Antimicrobial agents and chemotherapy* **52**, 3315-3320 (2008).
269. Ersoy, S.C., *et al.* Correcting a fundamental flaw in the paradigm for antimicrobial susceptibility testing. *EBioMedicine* **20**, 173-181 (2017).
270. He, X., Yuan, F., Lu, F., Yin, Y. & Cao, J. Vancomycin-induced biofilm formation by methicillin-resistant Staphylococcus aureus is associated with the secretion of membrane vesicles. *Microbial pathogenesis* **110**, 225-231 (2017).
271. Cafiso, V., *et al.* Modulating activity of vancomycin and daptomycin on the expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. *PLoS One* **7**, e29573 (2012).
272. Kadurugamuwa, J.L. & Beveridge, T.J. Delivery of the Non-Membrane-Permeative Antibiotic Gentamicin into Mammalian Cells by Using Shigella flexneriMembrane Vesicles. *Antimicrobial agents and chemotherapy* **42**, 1476-1483 (1998).

273. Roszkowiak, J., *et al.* Interspecies outer membrane vesicles (OMVs) modulate the sensitivity of pathogenic bacteria and pathogenic yeasts to cationic peptides and serum complement. *International journal of molecular sciences* **20**, 5577 (2019).
274. Manning, A.J. & Kuehn, M.J. Functional advantages conferred by extracellular prokaryotic membrane vesicles. *Journal of molecular microbiology and biotechnology* **23**, 131-141 (2013).
275. Hildebrandt, P., *et al.* Alternative fluorescent labeling strategies for characterizing gram-positive pathogenic bacteria: Flow cytometry supported counting, sorting, and proteome analysis of Staphylococcus aureus retrieved from infected host cells. *Cytometry Part A* **89**, 932-940 (2016).
276. Mouton, J.W., Meletiadis, J., Voss, A. & Turnidge, J. Variation of MIC measurements: the contribution of strain and laboratory variability to measurement precision. *Journal of Antimicrobial Chemotherapy* **73**, 2374-2379 (2018).
277. Prados-Rosales, R., *et al.* Mycobacteria release active membrane vesicles that modulate immune responses in a TLR2-dependent manner in mice. *The Journal of clinical investigation* **121**, 1471-1483 (2011).
278. Hébrard, M., Viala, J.P., Méresse, S., Barras, F. & Aussel, L. Redundant hydrogen peroxide scavengers contribute to Salmonella virulence and oxidative stress resistance. *Journal of bacteriology* **191**, 4605-4614 (2009).
279. Kalfopoulou, E. lmu (2019). Novel active and passive immunotherapy regimens against nosocomial infections caused by multidrug resistant enterococci. Dissertation, LMU München: Faculty of Medicine
280. Romero-Saavedra, F., *et al.* Identification of peptidoglycan-associated proteins as vaccine candidates for enterococcal infections. *PLoS one* **9**, e111880 (2014).
281. Theilacker, C., Krueger, W.A., Kropec, A. & Huebner, J. Rationale for the development of immunotherapy regimens against enterococcal infections. *Vaccine* **22**, S31-S38 (2004).
282. Romero-Saavedra, F., *et al.* Conjugation of different immunogenic enterococcal vaccine target antigens leads to extended strain coverage. *The Journal of infectious diseases* **220**, 1589-1598 (2019).
283. Kim, B., *et al.* Enterococcus faecium secreted antigen A generates muropeptides to enhance host immunity and limit bacterial pathogenesis. *Elife* **8**, e45343 (2019).
284. Rangan, K.J., *et al.* A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens. *Science* **353**, 1434-1437 (2016).
285. Roy, U., Chalasani, A.G. & Shekh, M.R. The anti-Candida activity by Ancillary Proteins of an Enterococcus faecium strain. *Frontiers in microbiology* **6**, 339 (2015).

PAPER I

Journal of Proteomics 187 (2018) 28–38

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

Enterococcus faecium produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins

Wagner T.[a,1], Joshi B.[a,1], Janice J.[a,b], Askarian F.[a], Škalko-Basnet N.[c], Hagestad O.C.[a], Mekhlif A.[a], Wai S.N.[d], Hegstad K.[a,b,*,1], Johannessen M.[a,*,1]

[a] Research Group of Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway, Norway
[b] Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway
[c] Drug Transport and Delivery Research Group, Department of Pharmacy, Faculty of Health Sciences, UiT-The Arctic University of Norway, Tromsø, Norway
[d] The Laboratory for Molecular Infection Medicine Sweden (MIMS), Department of Molecular Biology, Umeå University, Umeå, Sweden

ARTICLE INFO

Keywords:
Enterococcus
E. faecium
Membrane vesicles
Proteomics
Virulence factors
Antimicrobial resistance

ABSTRACT

Enterococcus faecium is a commensal but also a bacteremia causing pathogen, which is inherently resistant to several antimicrobials and has a great ability to acquire new traits. Bacterial membrane vesicles (MVs) are increasingly recognized as a mode of cell-free communication and a way to deliver virulence factors and/or antimicrobial resistance determinants. These features make MVs interesting research targets in research on critical hospital pathogens. This study describes for the first time that *E. faecium* strains produce MVs. It presents a morphological as well as a proteomic analysis of MVs isolated from four different, clinically relevant *E. faecium* strains grown under two different conditions and identifies MV-associated proteins in all of them. Interestingly, 11 virulence factors are found among the MV-associated proteins, including biofilm-promoting proteins and extracellular matrix-binding proteins, which may aid in enterococcal colonization. Additionally, 11 antimicrobial resistance-related proteins were MV-associated. Among those, all proteins encoded by the *vanA*-cluster of a vancomycin resistant strain were found to be MV-associated. This implies that *E. faecium* MVs may be utilized by the bacterium to release proteins promoting virulence, pathogenicity and antimicrobial resistance.

Significance: Enterococcal infections, especially bacteremia and endocarditis, are challenging to treat because *E. faecium* have acquired resistance to multiple classes of antimicrobials, including ampicillin, aminoglycosides, and glycopeptides. Thus, research on different modes of enterococcal pathogenicity is warranted. This study utilized a proteomic approach to identify MV-associated proteins of different nosocomial *E. faecium* strains representing four clinically relevant sequence types (STs), namely ST17, ST18, ST78, and ST192. The presented data suggest that *E. faecium* MVs are involved in virulence and antimicrobial resistance.

1. Introduction

Enterococci are Gram-positive, ubiquitous, facultative anaerobic cocci. They are known to survive hostile conditions such as a saline environment and wide temperature ranges and also for their ability to persist long-term in the hospital environment [1]. *Enterococcus faecalis* and *Enterococcus faecium* naturally colonize the human gut as commensals. However, *E. faecium* in particular has undergone a pronounced transition towards a multi-drug resistant pathogen. The most common infection caused by *E. faecium* is urinary tract infection, but they may also cause life-threatening infections such as endocarditis and bacteremia, especially in debilitated patients [2]. The genetic clade structure of *E. faecium* is characterized by a distinct split of commensal lineage (clade B) and hospital-associated lineage (clade A1) [3]. The nosocomial A1 clade includes sequence types (STs) of the clonal complex 17 (CC17), a globally spread genetic complex characterized by ampicillin resistance, possession of a pathogenicity island and association with hospital outbreaks [4].

Extracellular vesicles are suggested as a mechanism for cell-free intercellular communication across all domains of life. They are crucial components of the bacterial secretome, as these 20–200 nm sized spheres contain lipopolysaccharides, soluble membrane-associated proteins, virulence factors and nucleic acids [5, 6].

Bacterial membrane vesicles were first described in the Gram-

* Corresponding authors at: Research Group of Host-Microbe Interactions, Department of Medical Biology, Faculty of Health Sciences, UiT-The Arctic University of Norway, Norway.
E-mail addresses: Kristin.Hegstad@uit.no (K. Hegstad), Mona.Johannessen@uit.no (M. Johannessen).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.jprot.2018.05.017
Received 8 February 2018; Received in revised form 25 April 2018; Accepted 29 May 2018
Available online 30 May 2018

negative *Escherichia coli* in the 1960s [7, 8], and later in several other Gram-negative species such as *Shigella* sp. [9], *Salmonella* sp. [10], and *Vibrio* sp. [11]. In Gram-negative bacteria, the vesicles are called outer-membrane vesicles (OMVs), as they derive from the outer membrane (OM). The OMVs contain OM components as well as inner membrane constituents and cytoplasmic elements. The role of OMVs in bacterial physiology and pathogenesis, stress responses, biofilm formation as well as secretion and delivery of biomolecules has been demonstrated [12]. The mechanism of vesiculogenesis is poorly understood but seems to involve phospholipid accumulation in the outer leaflet of the outer membrane, whereupon vesicles pinch off from the outer membrane among Gram-negative bacteria [13].

It used to be a long-standing assumption that the thick cell wall of Gram-positive bacteria precluded the existence of vesicles, as they could not escape such a barrier. Gram-positive MVs were discovered in a study from the early 1990s in *Bacillus cereus* and *Bacillus subtilis* [14], but not further characterized for the next 20 years. Finally, in 2009 MVs were described in *Staphylococcus aureus* [15] and have since gained increased attention, i.e. in *Bacillus anthracis* [16], *Mycobacterium tuberculosis* [17] and others, as reviewed by Brown et al. [6]. MVs are key players in host-pathogen interactions, as they can cause disease without the living bacterial cell [18] and may induce strong host responses [19].

MV production in enterococci has not been described previously. The aim of this study was therefore to explore the potential of MVs release from *E. faecium*. In addition, we investigated whether different cultural conditions and strain backgrounds may account for variation in proteinaceous cargo. Four strains representing different, clinically important sequence types (STs) within CC17, the major disease causing clonal complex [20]: ST17, ST18, ST78, and ST192 respectively, were therefore chosen for the study. To the best of our knowledge, this is the first report describing MV release by clinical strains of enterococci and their proteomics-based characterization using an in-solution approach.

2. Materials and methods

2.1. Strains and growth conditions

In the present study, the *E. faecium* strains DO (PRJNA71), E155 (PRJNA192879), K59-68 (in-house sequenced, under submission) and K60-39 (PRJNA407052), representing ST 18, 17, 78 and 192, respectively, were used. They are hospital isolates either from the US or Norway. Their properties and additional information are presented in Table 1.

E. faecium strains were routinely cultured on brain heart infusion (BHI) or Luria Bertani (LB) agar or in liquid BHI or LB at 37 °C.

2.2. Isolation of membrane vesicles

Vesicles were isolated as described for *S. aureus* [16, 21] with a few modifications.

First, to isolate vesicles from bacteria mainly in a viable state, cultures in mid-exponential growth phase grown in nutrient-rich BHI were used (Fig. S1A). Therefore, 1 L of BHI broth were inoculated with enterococci grown overnight in BHI broth (1100) and grown at 37 °C with shaking (230 rpm) to mid-exponential phase (optical density at 600 nm [OD_{600}], approximately 1.5). The cultures were centrifuged at 6000 × *g* for 30 min with a JLA 9.1000 rotor Beckman Instruments Inc., USA) and the supernatant was transferred to a clean Erlenmeyer flask and filtered through a 0.22 µm pore size filter (Merck Millipore, USA). The obtained bacterial-free filtrate was concentrated using Amicon tubes (Merck Millipore, USA, cut off 100 kDa) in a "Beckman" centrifuge at 4000 × *g* for 30 min at 4 °C and the concentrate was ultracentrifuged using a SW 40-TI rotor (20,000 x g for 3 h at 4 °C) to pellet MVs. The MVs were washed in phosphate-buffered saline (PBS) followed by another ultracentrifugation, before re-suspention in PBS. Purified MVs were stored at −80 °C until further analysis. Purified MVs were streaked onto BHI agar

Table 1
Strain characteristics and morphological characterization of MVs.

Strain	Accession	Phylogeny	Isolation site	Country of origin	Ref.	Genes involved in resistance	Genome size in bp	Proteome size in number of coding sequences	Size of MVs in nm ± SD	Charge (ζ potential)	No. MV proteins	% proteins from core	No. MV proteins	% proteins from core
											Exponential growth BHI		Stationary growth LB	
DO	PRJNA71	ST18 (line 18)	Blood-stream isolate	Texas, USA	[98]	*aph(3')-III, ermB, msrC, tetM*	2,848,380	2721	42 ± 18	−3,43 ± 0.15	445	88	660	88
E155	PRJNA192879	ST17 (line 17)	human feces	Chicago, USA	[99]	*aac(6')-Ie-aph(2'')-Ia, aph (3')-III, dfrG, ermB, msrC, vanR-A, vanS-A, vanH-A, vanA, vanX-A, vanY-A, vanZ-A, vanR-B, vanS-B, vanY-B vanW-B, vanH-B, vanB, vanX-B*	3,066,426	3013	37 ± 23	−19.63 ± 1.33	362	90	674	91
K59-68	Under submission to PRJNA407052	ST78 (line 78)	Blood-stream isolate	Tromsø, Norway	[100]	*aph(3')-III, ermB, lnuB, msrC*	3,370,597	3272	83 ± 29	−21.57 ± 1.45	162	88	424	89
K60-39	PRJNA407052	ST192 (line 78)	Blood-stream isolate	Oslo, Norway	[100]	*aac(6')-Ie-aph(2'')-Ia, aph (3')-III, ermB, msrC, tetM*	3,071,612	2879	51 ± 21	−24.90 ± 1.60	605	88	754	90

to confirm the sterility of isolated MVs.

Second, in order to isolate vesicles from bacteria under stress, bacterial stationary phase cultures grown in growth-limiting LB were used (Fig. S1B). For this, the above-described procedure was applied to enterococcal overnight cultures grown in LB.

2.3. Density gradient centrifugation (DGC)

DGC was performed in order to fractionate and purify the MVs further. Therefore, the MV sample was mixed with an equal volume of ice-cold 60% OptiPrep (Sigma-Aldrich, Germany; 60% stock) to obtain a 30% solution. The 30% solution was transferred into the bottom of an ultracentrifugation tube (ultraClear™, 5 mL, 13 × 51 mm, Beckman Instruments Inc., USA) and 2 mL of 25% (w/v), 1 mL of 5% (w/v) OptiPrep solution and PBS were added. Centrifugation was carried out at 100000 ×*g* for 3 h at 4 °C (rotor SW 50.1, Beckman Instruments Inc., USA) in slow acceleration and deceleration mode to prevent disturbance in the various density layers and achieve MV ring formation (see example in Fig. S2A). After centrifugation, the sample was divided into 200 μL fractions, which were transferred into clean Eppendorf tubes. Aliquots of the fractions were analyzed by SDS PAGE (see example in Fig. S2B) and electron microscopy.

To remove the OptiPrep solution, the fractions containing MVs were pooled and concentrated by ultrafiltration (10 kDa molecular weight cut-off, Vivaspin 20, Sartorius, Germany) at 4000 ×*g* for 30 min at 4 °C and re-suspended in 200 μL PBS.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiling of isolated MVs from all the four strains of enterococci was determined by SDS-PAGE using standard molecular stain. Briefly, 15 μL of MV sample were mixed with 5.75 μL 4× NuPage LDS sample buffer (NuPage Novex 4–12%; Invitrogen, Life Technologies, USA) as well as 2.3 μL of dithiothreitol (DTT) and heated for 10 min at 70 °C prior to electrophoresis. Samples were separated by 12% NuPage Bis-Tris SDS-PAGE gel, and the gels were fixed in fixation solution (methanol, acetic acid (50/50 v/v)) for 1 h at room temperature under gentle agitation. The gels were then stained with Coomassie solution (50% methanol, 10% acetic acid, 0.05% Coomassie brilliant blue R-250, Thermo Fisher Scientific USA) for 1 h and excess dye was removed through incubation in de-stain solution (H_2O, methanol, acetic acid (50/40/10 v/v/v)) until bands were visible.

2.5. Transmission electron microscopy (TEM) analysis

Five μL of purified MVs were applied to Formvar coated 75 mesh copper grids (Electron Microscopy Science, USA) and incubated for 5 min. The sample was washed four times with double distilled water and negatively stained with 9 parts of 2% methylcellulose and 1 part of 3% uranyl acetate for 2 min on ice. The excess stain was removed and samples were dried at room temperature. The samples were then visualized with a JEOL JEM 1010 transmission electron microscope (JEOL, Japan) operated at 80 kV.

2.6. Atomic force microscopy (AFM) analysis

AFM analysis of *E. faecium* strains DO and E155 cultivated on LB agar were performed as described previously [22]. A loop of bacterial cells was suspended in ultrapure water and placed on a freshly cleaved mica surface, incubated at room temperature and ultimately blotted dry before being placed into a desiccator. Imaging was carried out using a Nanoscope V Atomic Force Microscope (Bruker AXS, Germany).

2.7. Particle size distribution and measurement of zeta potential

The effective diameter and size distribution of the purified MVs were determined by dynamic light scattering (DLS, also known as photon correlation spectroscopy) (Nicomp Submicron Particle Sizer 370, PSS Nicomp Particle Sizing Systems, USA), at an angle of 90° with a 632 nm laser as described in an earlier study [23]. Briefly, MVs were diluted in PBS in a particle-free environment to a count rate of 250–350 kHz at room temperature. Analyses were run in a vesicle mode data calculated as intensity weighted distribution from three measuring cycles (10 min cycle, 3 cycles) The zeta (ζ) potential of vesicles was determined using Zetasizer Nano Z (Malvern Instruments, United Kingdom). Three parallels were determined for each vesicle suspension.

2.8. Measurement of MV-derived protein per CFU

The protein content of isolated and purified MVs from bacterial culture (2 L) was measured by Bradford assay (Bio Rad, USA). For each strain, CFUs per mL were assessed by plating dilution series on blood agar plates and counting CFUs. With these two values, MV-derived protein in attogram (ag) per CFU was calculated from these two values.

2.9. Lipid staining of purified vesicles

The purified membrane vesicles were stained for 1 h with lipid-specific dye (5 μM of DiD, Thermo Fisher Scientific, USA). The stained vesicles were then ultra-centrifuged at a speed of 100,000 × g for 1 h at 4 °C. The pellets were re-suspended in 20 μL PBS mounted on a glass slide and examined using a confocal microscope (Leica Microsystems CMS GmbH, Germany), with an excitation and emission spectra for DiD at λ_{638nm} and λ_{700nm}, respectively.

2.10. Detection of peptidoglycan

ELISA was used to assess the presence of peptidoglycan, which was exposed by incubating the membrane vesicles and bacteria with 25 mM HCl for 1 h at room temperature under shaking. Microtiter plates (Maxisorb, Thermo Fischer Scientific, USA) were coated with membrane vesicles or bacteria in coating buffer (100 mM Sodium carbonate/bicarbonate, pH 9.6) at 4 °C overnight. After 4 washes with PBS-T (PBS with 0.01% Tween), plates were blocked with 0.05% BSA for 2 h at 37 °C and washed. To evaluate the presence of peptidoglycan in the samples, mouse anti-bacterial peptidoglycan monoclonal antibody (IgG, MAB995, Chemicon, Merck Millipore, USA; 1:1000) was added, and the plate incubated for 2 h at 37 °C. Excess primary antibody was removed by additional washing with PBS-T, before it was detected with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Agilent, USA; 1:500) for 20 min at 37 °C. Thereafter, tetramethylbenzidine (3, 3′, 5, 5′-TMB, Sigma Aldrich, Germany) was added as peroxidase substrate, and the reaction was stopped with sulfuric acid upon color change. Absorption was measured at 450 nm.

2.11. MV-associated protein identification by mass spectrometry

The protein concentration within the MVs was quantified, and 20 μg MV proteins were re-suspended in 8 M urea. The sample was reduced by adding 20 mM DTT and alkylated with 40 mM iodoacetamide. Alkylation was quenched by 10 mM DTT. Proteins were digested 4 h by 1:100 (w/w) lysyl endopeptidase (Wako Biochemicals, USA). The sample was diluted to 1 M urea and digested overnight by 1/20 (w/w) trypsin (V511A, Promega, USA). OMIX C18 tips (Varian Inc., USA) were used for sample cleanup and concentration. Peptide mixtures containing 0.1% formic acid were loaded onto an EASY-nLC1000 system (Thermo Fisher Scientific, USA) and EASY-Spray column (C18, 2 μm, 100 Å, 50 μm, 50 cm). Peptides were fractionated using a 2–100% acetonitrile gradient in 0.1% formic acid over 50 min at a flow rate of

250 nL/min. The separated peptides were analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, USA). Data were collected in data-dependent mode using a Top10 method. The raw data were processed using the Proteome Discoverer 2.1 software. The fragmentation spectra were searched against the predicted proteome from the whole genome sequence (WGS) data of the strain itself using the Sequest HT program. Peptide mass tolerances used in the search were 10 ppm, and fragment mass tolerance was 0.02 Da. Peptide ions were filtered using a false discovery rate (FDR) set to 5% for protein identifications.

The MV-associated proteins were identified in 3 biological replicates of MV derived from bacteria in the exponential growth phase in BHI, and are represented as one in the results table without restrictions (Supplementary table S1). For MV derived from bacteria in the stationary growth phase in LB one biological replicate is described in the result table (Supplementary table S1).

2.12. Bioinformatic characterization of identified MV proteins

To evaluate MV-associated proteins, the entire proteomes were classified into core and strain(s)-specific. The core proteome was assigned with the proteins shared by all four strains whereas the strain(s)-specific proteome had proteins found only in one or few strains. All proteins were clustered using CD-HIT with default settings [24]. Homologous clusters possessing at least one protein from each strain were categorized into core proteins, if not as strain(s)-specific proteins.

Overlapping protein content among MVs isolated from four different strains was demonstrated using Venny 2.0 [25]. These results were shown individually for proteomes of MVs isolated after growth in LB to stationary phase and in BHI to mid-exponential phase.

Prediction of signal peptide cleavage sites was performed in SignalP 4.1 [26] and PrediSi [27] on all proteins identified in the MV proteomes. The subcellular localization of identified proteins was predicted using PSORTb version 3.0 [28]. Gene-term enrichment analysis was performed with DAVID Functional Annotation Tool [29] on proteins with an exponentially modified protein abundance index (empai) above 1.

Selected virulence factors and vaccine candidates were searched for using BLAST [30].

Antimicrobial resistance genes were identified within the respective genomes of the bacterial strains using ResFinder 3.0 [31] with an ID threshold of 90% and a minimum length of 60%. Genes with < 100% ID threshold and 100% minimum length are indicated in Table 2. Identified genes are *aph(3′)-III, aac(6′)-aph(2″); vanR-A, vanS-A, vanH-A, van-A, vanX-A, vanY-A, vanZ-A, vanR-B, vanS-B, vanY-B, vanW-B, vanH-B, vanB, vanX-B; ermB, lnuB, msrC, tetM* and *dfrG*. The corresponding proteins were searched for in the MV proteome in order to identify MV-associated antimicrobial resistance determinants.

3. Results

3.1. Clinical E. faecium strains produce MVs

This study explored whether four different clinical *E. faecium* strains, belonging to distinct hospital adapted high-risk STs, were able to release membrane vesicles in vitro. Notably, the growth rates of the examined strains at the measured intervals showed only minor variation (Fig. S1).

MVs were isolated from *E. faecium* strains, which were grown in BHI until mid-exponential phase (OD_{600} approximately 1.5). To ensure purity of the vesicles from cellular debris and protein aggregates, MVs were further purified and fractionated by gradient centrifugation (Fig. S2A). The purified MVs were analyzed by TEM where the presence of vesicle-like circular shape structures was confirmed (Fig. 1A). Similar structures were detected for the other three examined strains (results not shown). AFM was conducted as a complementary approach and revealed release of MVs by the bacteria to the surrounding environment (Fig. 1B). To measure the size distribution of larger populations of MVs from the four *E. faecium* strains, dynamic light scattering (DLS) was employed. While all strains generated MVs of various sizes, the average size of enterococcal MVs ranged from 37 ± 23 nm in the E155 strain to 83 ± 29 nm in the K59–68 strain (Fig. 1C and Table 1). Furthermore, the size measured by DLS corresponded to the results obtained from TEM images (Fig. 1A–C, Table 1).

Based on protein content, the amount of MVs produced by the four strains ranged from 1.2 ag/CFU in strain K59-68 to 14.6 ag/CFU in strain E155 (Fig. 1D).

To gain insight into the interaction of the MVs in the colloidal system and gain a better understanding of their physiochemical properties (aggregation, flocculation or dispersion), the membrane (ζ-) potential, of the MVs was measured. The ζ-potential was calculated upon measuring particles electrophoretic mobility. The *E. faecium* MV surface charge in all examined strains was found to be negative in PBS and reproducible (Table 1) demonstrating a high quality of the MV isolation.

To further characterize the vesicle properties, the purified MVs of strain E155 were stained with a lipophilic fluorescent dye (DiD) for detection of lipids. The confocal microscopy analysis of the stained MVs confirmed the presence of lipids in MVs (Fig. 2A). The presence of peptidoglycan in the isolated MVs from all four strains was evaluated using a competitive ELISA approach. Interestingly, peptidoglycan could be detected in *E. faecium*-derived MVs from all examined strains. *E. faecium* cells were included as a positive peptidoglycan control for this particular assay (Fig. 2B).

3.2. Different cultural conditions and strain backgrounds account for variation in enterococcal MV proteinaceous cargo

To investigate whether the MV protein cargo is strain dependent and influenced by different conditions, the four strains were grown to exponential phase in a nutritious media, BHI, and to stationary phase in a growth-limiting media, LB. MVs were isolated and purified further by gradient centrifugation, and proteomic analysis was performed using an in-solution approach.

Proteomic analyses demonstrated variability in the number of identified proteins among MV-associated proteins isolated from the four selected strains grown in BHI and LB (Supplementary Tables 1–8).

Comparison of shared versus unique proteins revealed that, in exponential growth in BHI, a lower number (19.2%) of MV-associated proteins was shared than in stationary growth in LB (36.9%), as illustrated in Fig. 3. MVs from K60-39 showed the highest number of total MV-associated proteins and consequently also possess most unique MV-associated proteins under both conditions. In contrast, MVs from K59-68 showed a lower number of total vesicle associated proteins and accordingly the lowest number of unique proteins.

Based on the sequence similarity of the entire proteome encoded by all four genomes, homologous proteins present in all four were classified as core and the remaining as accessory. Accordingly, the MV-associated proteins were categorized based on their occurrence either in core or accessory proteome. Notably, it was found that 88–91% of the MV-associated proteins are from the core proteome (Table 1).

The predicted cellular localization of proteins encoded by the whole genome based on WGS data and the origin of MV-associated proteins was predicted by PSORTb. The predicted localization of proteins showed a common pattern among strains and MV-associated proteins originating from two different conditions (Fig. 4). As expected, most of the MV-associated proteins were predicted as either cytoplasmic or part of the cytoplasmic membrane proteins. A minor number of cell wall or extracellular bound proteins were also predicted in the derived MVs.

SignalP and PrediSi were applied to predict the presence of signal peptides. Although the number of identified proteins varied substantially between strains and conditions, the percentage of putative

Table 2
Presence of virulence factors and antibiotic resistance related proteins associated with MVs.

Virulence factor and functional description		WGS				MV							
		Tag in genome				stationary phase MV (LB)				exponential phase MV (BHI)			
		DO	E155	K60–39	K59–68	DO	E155	K60–39	K59–68	DO	E155	K60–39	K59–68
AtlA	Major autolysin, biofilm formation	Q3Y3J6	E155_00939	7_02408	6_02630	✓	✓	✓	✓	✓	✓	✓	✓
Acm	Collagen binding adhesion, MSCRAMM	Q3XXN7	E155_00761	7_02160	6_02383	✓	✓	✓	✓	X	X	X	✓
CapD	Capsular polysaccharide biosynthesis protein, adhesion	Q3XXA5	E155_00870	7_00899	6_00915	✓	✓	✓	X	✓	✓	✓	✓
CcpA	Catabolite control protein A, growth, virulence	I3U3N8	E155_00210	7_01900	6_02068	✓	✓	✓	X	✓	✓	✓	✓
Esp	Enterococcal surface protein, biofilm formation	-	-	-	6_02803	-	-	-	✓	-	-	-	-
Fnm	Fibronectin binding protein, adhesion	Q3XYE6	E155_00278	7_01343	6_01354	X	✓	✓	X	✓	✓	✓	X
PilA2	Pilus subunit protein A	Q3Y0Y4	-	7_02894	6_03276	X	-	✓	X	✓	-	X	X
PrpA	Prolinrich protein A, binding to extracellular matrix proteins	Q3XZP6	-	7_01420	6_01433	✓	-	X	X	✓	-	X	X
PtsD	Enzyme IID subunit of phosphotransferase system, intestinal colonization determinant	Q3Y1Z0	E155_00486	7_00634	6_00710	X	X	X	X	✓	✓	X	X
SagA	Secreted antigen A, biofilm formation	I3U595	E155_00236	7_02462	6_02688	✓	✓	✓	✓	✓	✓	✓	✓
Scm	Collagen adhesion, MSCRAMM	I3U5K9	-	7_02636	6_02858	✓	-	X	✓	✓	-	X	✓
Vaccine candidate													
PsaA	Metal binding lipoprotein	Q3Y2A0	E155_02566	7_00432	6_00512	✓	✓	✓	✓	✓	✓	✓	X
PBP5	Penicillin binding protein	Q3XZN6	E155_02659	7_01431	6_01444	✓	✓	✓	✓	✓	✓	✓	✓
LysM	Peptidoglycan binding protein	Q3Y2A3	E155_01532	7_00435	6_00515	✓	✓	✓	✓	✓	✓	✓	✓
DdcP	D,D-carboxypeptidase	I3U3E0	E155_02369	7_01824	6_01978	✓	✓	✓	✓	✓	✓	✓	✓
PpiC	Peptidyl-prolyl-cis-trans-isomerase	Q3Y2Y5	E155_01118	7_02348	6_02568	✓	✓	✓	✓	✓	✓	✓	✓
Antibiotic resistance related proteins													
Aac6'-Ie-Aph2″-Ia	Aminoglycoside resistance	-	E155_02844	-	6_03206	-	X	-	✓	-	✓	-	X
Aph3′-III	Aminoglycoside resistance	Q3Y248	E155_02752	7_03002	6_03355	✓	✓	X	✓	✓	✓	X	X
VanR-A	Glycopeptide resistance	-	E155_02402	-	-	-	✓	-	-	-	-	-	-
VanS-A	Glycopeptide resistance	-	E155_02403	-	-	-	✓	-	-	-	-	-	-
VanH-A	Glycopeptide resistance	-	E155_02496	-	-	-	✓	-	-	-	-	-	-
VanA	Glycopeptide reistance	-	E155_02495	-	-	-	✓	-	-	-	✓	-	-
VanX-A	Glycopeptide resistance	-	E155_02494	-	-	-	✓	-	-	-	-	-	-
VanY-A	Glycopeptide resistance	-	E155_02850	-	-	-	✓	-	-	-	✓	-	-
VanZ-A	Glycopeptide resistance	-	E155_02851	-	-	-	✓	-	-	-	✓	-	-
VanS-B	Glycopeptide resistance	-	E155_02765 (ID: 96.49%)	-	-	-	✓	-	-	-	-	-	-
ErmB	Macrolide resistance	Q3Y241	E155_02538	7_03003	6_03356	X	✓	✓	X	X	X	X	X

MSCRAMM - Microbial Surface Component Recognizing Adhesive Matrix Molecules, ✓ presence, X absence, - not within genome.

secreted proteins was comparable (Fig. 5). In all strains 4–5% of the ORFs encode secreted proteins in the whole genome proteomes, while 10–22% secreted proteins were predicted in MVs derived from exponential growth phase in BHI, and 5–12% secreted proteins in MVs derived from stationary growth phase in LB. The highest number of secreted (22%) and extracellular (4%) proteins was associated with MV proteinaceous cargo obtained from K59–68 grown in BHI to the mid-exponential phase.

Gene functional enrichment analyses for strain DO identified 10 functional annotation clusters in MV-associated proteins from exponential growth conditions in BHI and 16 functional annotation clusters in MV-associated proteins from stationary growth phase in LB. The difference in cluster number is probably due to the lower protein number in exponential growth phase (for empai > 1 $n_{protein} = 138$) compared to stationary growth phase (for empai > 1 $n_{protein} = 258$). The analysis showed that ribosomal, ATP synthesis, and membrane proteins were enriched in both exponential and stationary growth phase derived MVs in DO. The gene ontology enrichment analysis is illustrated in Fig. 6.

3.3. Presence of virulence factors, vaccine candidates, and antimicrobial resistance-related proteins among the E. faecium MV-associated proteins

Fifteen distinct *E. faecium* genes encoding virulence factors, associated with biofilm formation and adherence, were identified within the genomes of the 4 examined strains: *atlA* [32], *acm* [33, 34], *bepA* [35], *capD* [36], *ccpA* [37, 38], *ecbA* [39], *esp* [40], *fnm* [41], *pilA2* [42], *pilB/ebp$_{fm}$* [43], *ptsD* [44], *prpA* [45], *sagA* [46–48], *scm* [49] and *sgrA* [39]. *gelE* [50] was also searched for but was absent in the selected genomes.

Presence or absence of selected virulence factors associated with the *E. faecium*-derived MVs is indicated in Table 2. Proteomic analysis demonstrated the absence of BepA, EcbA, PilB/Ebp$_{fm}$ and SgrA in the purified MVs. Interestingly, the two biofilm determinants, namely AtlA and SagA, were found to be associated with MVs of all tested strains under both conditions. The adhesins Acm and CapD as well as the catabolite protein CcpA were also associated with purified MVs of all examined strains. Esp, an enterococcal surface protein enhancing biofilm formation, Fnm, a fibronectin-binding protein, PilA2, a pilus protein, PrpA, an extracellular matrix binding protein, PtsD, a phosphotransferase system protein, and the adhesin Scm, were associated with MVs from some of the examined strains.

The search was expanded to further include the enterococcal bacteriocins EntA, EntP, EntB, EntL50 [51], EntQ [52], Bac32 [53] and

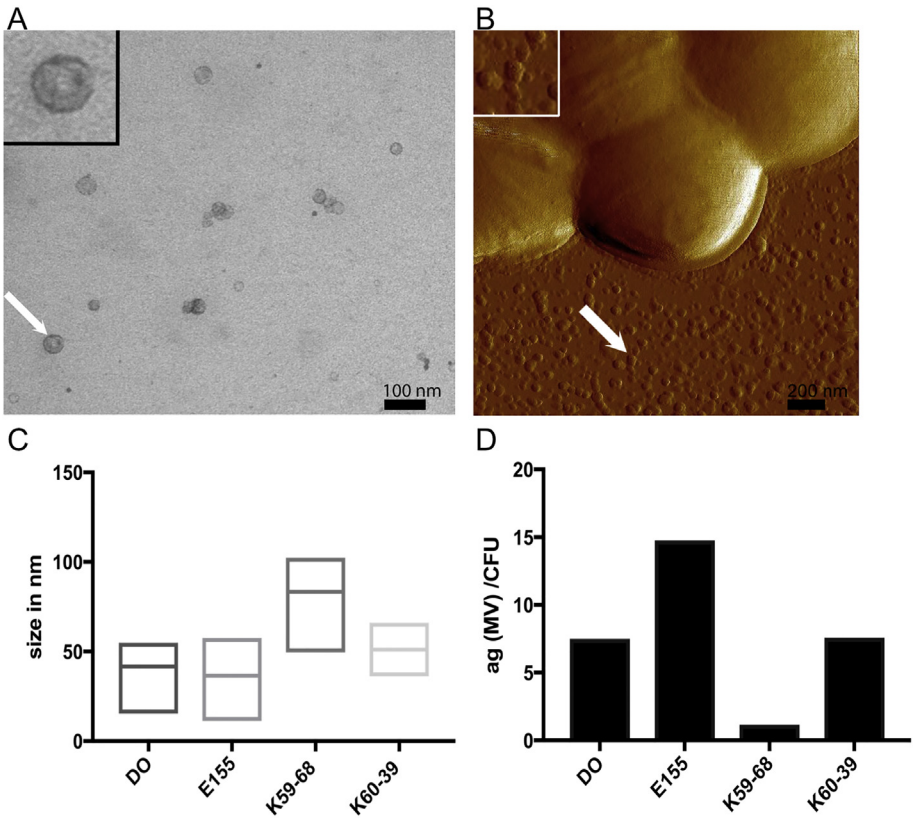


Fig. 1. *E. faecium* releases spherical MVs *in vitro*. A) TEM of MVs isolated from *E. faecium* E155 (the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). B) AFM of *E. faecium* E155 surrounded by MVs (the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). C) Abundance of the various sizes of purified MV from DO, E155, K59-68 and K60-39 grown to exponential phase in BHI as determined by DLS (mean in nm ± SD). D) MV-derived protein per CFU in DO, E155, K59-68 and K60-39 in cultures grown in BHI to OD_{600} approximately 1.5.

Bac43 [54]. Out of those, only the gene *entA* was present in all the four genomes used in this study, but EntA was not found to be associated with the *E. faecium* derived MVs.

As previous studies have identified putative enterococcal vaccine targets, the following candidates were searched for in the vesicular proteome: PsaA, AdcA [55], PBP5, LysM, DdcP, PpiC [56] and SagA [48] (Table 2). PsaA and AdcA are both metal binding lipoproteins [55], and PsaA was found associated with MVs from all four strains. PBP5, LysM, DdcP, and PpiC are putative surface-exposed proteins, which are associated with peptidoglycan [56], and all four were found within all the examined MV samples. Additionally, SagA, which was present in all MV samples, was not only described as a virulence factor [46, 47], but also as the first promising vaccine candidate in *E. faecium*, as it exhibited protective properties in a mouse model [48].

Furthermore, resistance genes were identified in the four genomes using ResFinder [31], and subsequently, the corresponding proteins were searched for in the MV proteomes (Table 2). The protein Aph3′-III, a kanamycin kinase conveying resistance to aminoglycosides, was found in MV samples in both conditions. Aac6′-Ie-Aph2″-Ia, a bifunctional protein conferring broad-spectrum resistance to aminoglycosides such as gentamicin through N- and *O*-acetylation and phosphorylation of hydroxyl groups [57], was associated with DO-, E155- and K59-68-derived MVs, isolated from BHI and/or LB cultures. ErmB, conferring macrolide, such as erythromycin, resistance, was associated with E155- and K60-39-derived MVs grown in LB to the stationary phase.

All proteins encoded by the *vanA*-cluster that contribute to glycopeptide resistance [58] were associated with E155-derived MVs grown in LB to the stationary growth phase: VanR-A, VanS-A, VanH-A, VanA, VanX-A, VanY-A, VanZ-A. A subset of those was also present in MVs isolated from exponential growth phase. Additionally, VanS-B encoded by the *vanB*-cluster was found associated with E155-derived MVs grown to the stationary growth phase. However, the E155 genome also encodes the rest of the genes of the *vanB*-cluster: *vanR-B*, *vanY-B*, *vanW-B*, *vanH-B*, *vanB* and *vanX-B*, but they were absent in the MVs (Table 2).

The macrolide resistance genes *lnuB* (K60-39) and *msrC* (all 4 strains) were present in the respective genomes but absent in the MVs. This also accounts for *tetM* (DO and K59-68) and *dfrG* (E155).

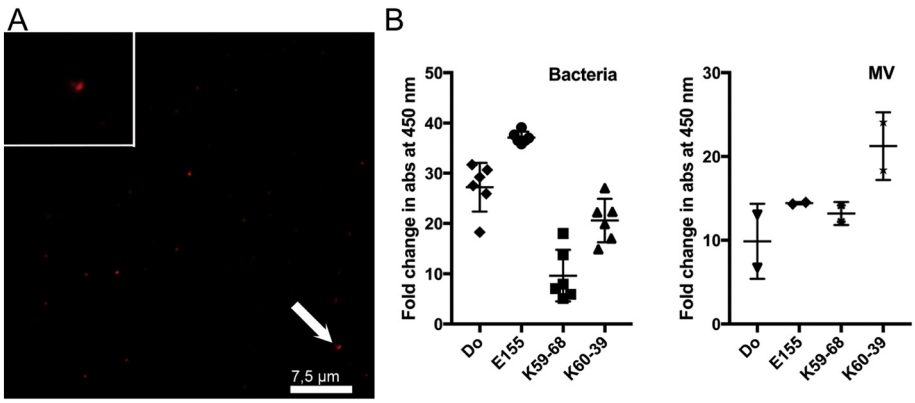


Fig. 2. *E. faecium* MVs contain lipids and peptidoglycan. A) Confocal microscopy of MVs stained with lipid-tracer dye (DiD shown in red, the inset in the upper left corner shows the vesicle indicated by an arrow 2 times magnified). B) Fold changes in absorbance using anti-peptidoglycan antibodies in ELISA to detect peptidoglycan in bacteria and MVs isolated for the four strains at mid-exponential growth phase in BHI compared to the control (uncoated well). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

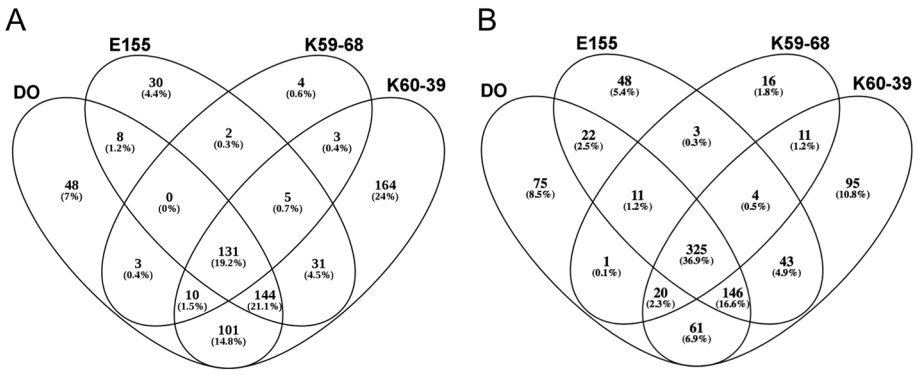


Fig. 3. Four-set Venn diagram representing the number of shared and unique MV-associated proteins of MVs isolated from *E. faecium* cultures. A) Mid-exponential phase growth in BHI, B) Stationary phase growth in LB. Numbers of shared or unique proteins are given in bold, percentage in brackets.

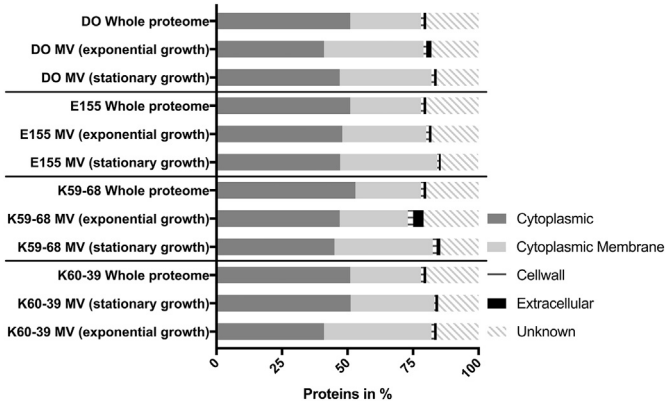


Fig. 4. Localization prediction of proteins in whole genome proteomes and in proteomes of MVs derived from two different conditions. Subcellular localization prediction was done in PsortB and is illustrated in proportional numbers.

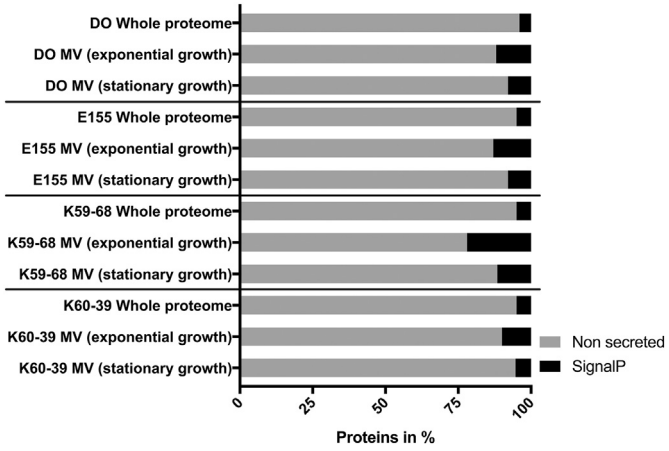


Fig. 5. Proportion of proteins with a signal peptide in *E. faecium* whole genome proteomes and MV associated proteomes from two different conditions. Signal peptide prediction was done by SignalP.

4. Discussion

All cells investigated so far have been found to release vesicles, indicating a universal phenomenon. The release of bacterial membrane vesicles and their functional importance, including both offense and defense, has been widely studied in Gram-negative bacteria [59]. In recent years, vesicle research has also increased in Gram-positive bacteria such as *Listeria* [60], *Bacillus* [16], *Staphylococcus* [61], *Lactobacillus* [62], *Streptococcus* [63] and *Clostridium* [64]. Here we provide the first report describing MVs released by *E. faecium*.

Rivera et al. [16] observed spherically shaped vesicles with the size of 50–300 nm in *B. anthracis*. Similarly, MV isolated from *Listeria*, *Staphylococcus*, *Lactobacillus*, *Streptococcus,* and *Clostridium* ranged from 20 to 400 nm [60, 61, 63, 65]. The morphology of MVs secreted by *E. faecium* was found to be similar to the above-mentioned MVs from Gram-positive bacteria, both in terms of size and appearance (Fig. 1, Table 1). We also quantified the vesicles based on protein measurement through Bradford reagent and found similar MV amounts produced by the four *E. faecium* strains. However, a drawback of these types of measurement approaches is the lack of a universal standard procedure for MV quantification. Until now, vesicular proteins have been quantified using protein-based (SDS-PAGE staining, Bradford reagent or bicinchoninic-acid assay BCA) and lipid-based (lipid probes such as FM4–64) approaches. However, all these methods have both strengths and weaknesses [66]. Moreover, for the proteomic analysis assessment of the purity of the MV sample is utmost important. Contaminating artifacts may co-pellet in the ultracentrifugation step of MV isolation. However, density gradient centrifugation was included in our study, which improves the purity of the MV sample [66, 67].

As previously described in Gram-negative bacteria [68] and the Gram-positive bacterium *Clostridium perfringens* [64], peptidoglycan was confirmed to be associated with enterococcal vesicles, through use of an antibody with specificity for the three-dimensional polymer complex structure of peptidoglycan. In addition, the MVs from all four strains exhibited negative surface charge, based on ζ-potential. The surface charge of *Pseudomonas aeruginosa* and *B. anthracis* derived MVs was also reported to be negative [16, 69] and in *P. aeruginosa* the ζ-potential was more negative in stationary growth phase derived vesicles compared to exponential growth phase [69]. Gram-positive and Gram-negative bacterial cells are negatively charged, too. It has been suggested that the charge is critical to understand the interaction between MVs and the bacterial cells [69] and the selectivity of this interaction since it was found that MVs are more likely to interact with bacterial cells of the parent species than of other genera [70].

Besides the morphological characterization, a mass spectrometry based approach was used to gain insight into the proteomic profile of the isolated MVs. The overall proteomic pattern within MVs was similar regarding the percentage of proteins that derived from the core genome, their origin of localization, and whether they had a signal peptide or not (Figs. 3–5). However, different numbers of MV-associated proteins identified by proteomics were found among the four examined strains and under varying conditions (Table 1, Fig. 3). Among Gram-positive bacteria substantially varying numbers of MV-associated proteins have been described previously, from 431 MV-associated proteins in *C. perfringens* grown in Trypticase-peptone-glucose broth [65] to 36 MV-associated proteins in *B. anthracis* 34F2 in BHI [16]. The growth medium affects the gene expression in bacteria which subsequently alters the amount and/or content of vesicles released into the

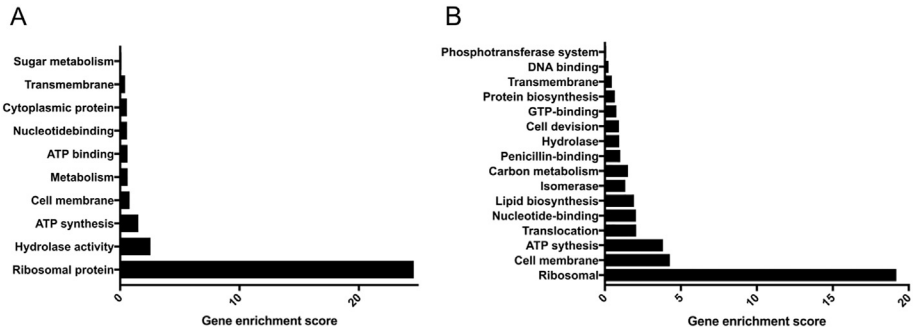


Fig. 6. Distribution of DO MV-associated proteins from two different conditions compared to the whole genome proteome of DO in gene ontology enrichment analysis. Proteome of MVs derived in exponential growth in BHI is shown in A) and stationary growth in LB in B).

growth medium [71]. Similarly, Bager et al. found significant changes in OMV production and protein composition upon altering medium composition in *Gallibacterium anatis* [72]. Moreover, a recent study on the proteome of *Pseudomonas putida* KT2440 by Choi et al. demonstrated that OMV production in LB media was three-fold higher than in two different minimal media containing succinate and benzoate [73]. Other previous studies identified a higher number of vesicular proteins in *P. aeruginosa* in LB compared to a more nutrient rich media [74–76]. Likewise, in our study, we observed a higher number of different proteins in MVs derived from *E. faecium* grown to stationary growth phase in nutrient-limiting LB compared to exponential growth in nutrient rich BHI. Under nutrient limitation and stationary growth, we expect the bacteria suffer more stress, which might explain more different vesicular proteins.

In the presented study we found a high abundance of cytoplasmic proteins, especially ribosomal proteins, which is similar to the number of cytoplasmic proteins encoded by the genome (Figs. 4, 6). This is in agreement with previous studies of MVs derived from Gram-positive bacteria [15, 19, 61]. This implicates, that the MV content represents basically the composition of the parent cell, even though final conclusions would require protein quantification rather than identification. We furthermore found, that high numbers of MV-associated proteins lack a secretion signal, which is in line with a previous study on *S. aureus* [21] as well as *Campylobacter jejuni* [77] and suggests that MVs may play an important role in the delivery of effector molecules, which lack a secretion signal.

As previous studies have shown that MVs of Gram-positive bacteria contain virulence factors [15, 16, 60, 78], serve as vaccine candidates [79] and are contributing to antimicrobial resistance [80], in silico methods were used to assess whether such factors are also associated with *E. faecium* MVs. Indeed, we identified 11 known virulence factors among the MV-associated proteins, including adhesins (CapD and PrpA), collagen-binding proteins (Acm and Scm), and fibronectin-binding protein (Fnm). These virulence factors are of particular importance for enterococcal adhesion, colonization and tissue invasion [34, 36, 41, 45, 49]. The association of collagen-binding proteins, as well as the fibronectin-binding protein and adhesins with MVs may suggest a role of the vesicles in enterococcal adhesion and colonization.

E. faecium utilizes biofilm formation as a survival strategy, which enables protection and persistence [81, 82]. Previous studies demonstrated that OMVs contribute to biofilm formation and stability [83, 84]. The virulence factors AtlA, Esp and SagA that contribute to enterococcal biofilm formation [32, 40, 46–48] were found to be MV-associated. This may suggest that *E. faecium* MVs can contribute to biofilm formation.

As highly stable, non-infectious, non-replicative particles vesicles are interesting vaccine candidates. Furthermore, they contain major immunogenic proteins of the MV producing bacterium that can act as antigens and thus can elicit responses in both arms of the immune system. Additionally, they display adjuvant activity (reviewed in [79, 85]). The first OMV-based vaccine to be licensed for human use was the meningococcal serogroup B vaccine 4CMenB against the Gram-negative *Neisseria meningitidis* [86]. Also in the Gram-positive *Streptococcus pneumoniae* [87] and *S. aureus* [21, 88], the protective effect of immunization with MVs was recently shown. For enterococci, there are to date no available vaccines, but there has been research towards identifying components for a vaccine to prevent *E. faecium* infection [48, 55, 56]. Here we found several previously described vaccine candidates to be associated with MVs (Table 2). However, whether enterococcal MVs can be used as vaccines remains to be investigated.

Special interest has been drawn to *E. faecium* due to their multidrug resistance and high genomic plasticity, which allows them to steadily acquire new resistance determinants. In the Gram-negative *E. coli* it was shown that OMVs not only have a protective role against membrane-active antimicrobials [80], but also that certain antimicrobials increase the production of OMVs and OMV-associated Shiga toxin [89, 90]. Also resistance-genes were found to be transferred via OMVs [91]. Furthermore, OMVs of β-lactamase resistant *Haemophilus influenzae* and *Moraxella catarrhalis* were shown to protect *Streptococcus pyogenes* against amoxicillin [92]. Interestingly, our study demonstrates that both the ribosome methylase ErmB, the aminoglycoside modifying enzymes Aac6′-Ie-Aph2″-Ia and Aph3′-III as well as all proteins of the *vanA* vancomycin resistance cluster can be MV-associated in *E. faecium*. All these proteins mainly function inside the enterococcal cell. Thus they could potentially be active in creating antimicrobial resistance if they can be delivered to a recipient cell by the MVs. However, low empai values of the *vanA*-cluster encoded proteins, ErmB, Aac6′-Ie-Aph2″-Ia and Aph3′-III under the conditions studied here, suggest that these MVs do not have a great potential to cause antimicrobial resistance. Still, MV production and thus resistance-conferring proteins could be more abundant upon induction through antimicrobial stress as seen in MVs from *Stenotrophomonas maltophilia* [93] and *E. coli* [89] and then be presented in high enough concentrations to a recipient cell to confer an antimicrobial effect. This implicates that enterococcal MVs may play a role in antimicrobial resistance, not only for *E. faecium* itself but also for the bacterial community.

The mechanism of vesiculogenesis has not been conclusively clarified in Gram-positive bacteria. Recently, prophage-triggered MV formation was described and it has been postulated that phage endolysins may confer MV escape by locally digesting the cell wall [94]. Interestingly, by using PHASTER tool [95], we detected phage genes within the genomes of DO, K59–68 and K60-39, but not in E155 (results not shown). Thus, prophages may play a role in the release of MV from certain strains. Apart from prophage-triggered MV formation, it has been suggested, that the vesicles passage the peptidoglycan layer with the aid of cell wall modifying enzymes such as peptidoglycan muramidases/ hydrolases, which are part of the Gram-positive type 4 secretion system (T4SS) [6, 15]. In the genomes of the four strains, we found T4SS using T346Hunter [96] (data not shown), suggesting that these strains might use components of this system for vesicle release. However, the mechanism for MV release in enterococci remains elusive.

5. Conclusion

A proteomic approach was used to describe the protein cargo of membrane vesicles derived from four different, clinically relevant *E. faecium* strains under varying conditions. It was found that the MV-associated proteome is strain and condition dependent. The most interesting MV-associated proteins were the virulence factors and antimicrobial resistance related proteins. It is thus tempting to speculate that enterococcal MVs play an important role in infection and antimicrobial resistance. Our findings highlight the potential of bacterial MVs for research on bacterial pathogenesis.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2018.05.017.

Acknowledgements

This work was supported by grants from the Northern Norway Regional Health Authority Medical Research Programme (SFP1157-14; Strategisk-HN05-14) and by travel grants from the National Graduate School in Infection Biology and Antimicrobials (Norwegian Research Council project number 249062).

We thank Jack-Ansgar Bruun for valuable support and expertise with the mass spectrometric analyses. We also thank Augusta Hlin Aspar for technical support with the microscopy.

Data availability

The raw mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [97] partner repository with the dataset identifier PXD008801.

Conflict of interest

The authors declare no potential conflict of interest.

References

[1] C.A. Arias, B.E. Murray, The rise of the *Enterococcus*: beyond vancomycin resistance, Nat. Rev. Microbiol. 10 (2012) 266–278, http://dx.doi.org/10.1038/nrmicro2761.
[2] N.I.A. Higuita, M.M. Huycke, Enterococcal Disease, Epidemiology, and Implications for Treatment, Massachusetts Eye and Ear Infirmary, https://www.ncbi.nlm.nih.gov/books/NBK190429/, (2014) , Accessed date: 28 November 2016.
[3] F. Lebreton, W. van Schaik, A.M. McGuire, P. Godfrey, A. Griggs, V. Mazumdar, J. Corander, L. Cheng, S. Saif, S. Young, Q. Zeng, J. Wortman, B. Birren, R.J.L. Willems, A.M. Earl, M.S. Gilmore, Emergence of epidemic multidrug-resistant *Enterococcus faecium* from animal and commensal strains, MBio 4 (2013), http://dx.doi.org/10.1128/mBio.00534-13 (e00534–13).
[4] R.J.L. Willems, J. Top, M. van Santen, D.A. Robinson, T.M. Coque, F. Baquero, H. Grundmann, M.J.M. Bonten, Global spread of vancomycin-resistant *Enterococcus faecium* from distinct nosocomial genetic complex, Emerg. Infect. Dis. 11 (2005) 821–828, http://dx.doi.org/10.3201/eid1106.041204.
[5] N.J. Bitto, M. Kaparakis-Liaskos, The therapeutic benefit of bacterial membrane vesicles, Int. J. Mol. Sci. 18 (2017) 1287, http://dx.doi.org/10.3390/ijms18061287.
[6] L. Brown, J.M. Wolf, R. Prados-Rosales, A. Casadevall, Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi, Nat. Rev. Microbiol. 13 (2015) 620–630, http://dx.doi.org/10.1038/nrmicro3480.
[7] K.W. Knox, M. Vesk, E. Work, Relation between excreted lipopolysaccharide complexes and surface structures of a lysine-limited culture of *Escherichia coli*, J. Bacteriol. 92 (1966) 1206–1217.
[8] E. Work, K.W. Knox, M. Vesk, The chemistry and electron microscopy of an extracellular lipopolysaccharide from *Escherichia coli*, Ann. N. Y. Acad. Sci. 133 (1966) 438–449.
[9] J.L. Kadurugamuwa, T.J. Beveridge, Membrane vesicles derived from *Pseudomonas aeruginosa* and Shigella flexneri can be integrated into the surfaces of other gram-negative bacteria, Microbiol. Read. Engl. 145 (1999) 2051–2060, http://dx.doi.org/10.1099/13500872-145-8-2051.
[10] W. Elhenawy, M. Bording-Jorgensen, E. Valguarnera, M.F. Haurat, E. Wine, M.F. Feldman, LPS remodeling triggers formation of outer membrane vesicles in *Salmonella*, MBio 7 (2016), http://dx.doi.org/10.1128/mBio.00940-16.
[11] D. Chatterjee, K. Chaudhuri, Association of cholera toxin with *Vibrio cholerae* outer membrane vesicles which are internalized by human intestinal epithelial cells, FEBS Lett. 585 (2011) 1357–1362, http://dx.doi.org/10.1016/j.febslet.2011.04.017.
[12] S. Roier, F.G. Zingl, F. Cakar, S. Schild, Bacterial outer membrane vesicle biogenesis: a new mechanism and its implications, Microb. Cell. 3 (2016) 257–259, http://dx.doi.org/10.15698/mic2016.06.508.
[13] S. Roier, F.G. Zingl, F. Cakar, S. Durakovic, P. Kohl, T.O. Eichmann, L. Klug, B. Gadermaier, K. Weinzerl, R. Prassl, A. Lass, G. Daum, J. Reidl, M.F. Feldman, S. Schild, A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria, Nat. Commun. 7 (2016), http://dx.doi.org/10.1038/ncomms10515.
[14] D.W. Dorward, C.F. Garon, DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria, Appl. Environ. Microbiol. 56 (1990) 1960–1962.
[15] E.-Y. Lee, D.-Y. Choi, D.-K. Kim, J.-W. Kim, J.O. Park, S. Kim, S.-H. Kim, D.M. Desiderio, Y.-K. Kim, K.-P. Kim, Y.S. Gho, Gram-positive bacteria produce membrane vesicles: proteomics-based characterization of *Staphylococcus aureus*-derived membrane vesicles, Proteomics 9 (2009) 5425–5436, http://dx.doi.org/10.1002/pmic.200900338.
[16] J. Rivera, R.J.B. Cordero, A.S. Nakouzi, S. Frases, A. Nicola, A. Casadevall, *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 19002–19007, http://dx.doi.org/10.1073/pnas.1008843107.
[17] J. Lee, S.-H. Kim, D.-S. Choi, J.S. Lee, D.-K. Kim, G. Go, S.-M. Park, S.H. Kim, J.H. Shin, C.L. Chang, Y.S. Gho, Proteomic analysis of extracellular vesicles derived from *Mycobacterium tuberculosis*, Proteomics 15 (2015) 3331–3337, http://dx.doi.org/10.1002/pmic.201500037.
[18] S.-W. Hong, M.-R. Kim, E.-Y. Lee, J.H. Kim, Y.-S. Kim, S.G. Jeon, J.-M. Yang, B.-J. Lee, B.-Y. Pyun, Y.S. Gho, Y.-K. Kim, Extracellular vesicles derived from *Staphylococcus aureus* induce atopic dermatitis-like skin inflammation, Allergy 66 (2011) 351–359, http://dx.doi.org/10.1111/j.1398-9995.2010.02483.x.
[19] A. Olaya-Abril, R. Prados-Rosales, M.J. McConnell, R. Martín-Peña, J.A. González-Reyes, I. Jiménez-Munguía, L. Gómez-Gascón, J. Fernández, J.L. Luque-García, C. García-Lidón, H. Estévez, J. Pachón, I. Obando, A. Casadevall, L. Pirofski, M.J. Rodríguez-Ortega, Characterization of protective extracellular membrane-derived vesicles produced by *Streptococcus pneumoniae*, J. Proteome 106 (2014) 46–60, http://dx.doi.org/10.1016/j.jprot.2014.04.023.
[20] R.J.L. Willems, W.P. Hanage, D.E. Bessen, E.J. Feil, Population biology of Gram-positive pathogens: high-risk clones for dissemination of antibiotic resistance, FEMS Microbiol. Rev. 35 (2011) 872–900, http://dx.doi.org/10.1111/j.1574-6976.2011.00284.x.
[21] F.- Askarian, J.J. Lapek, M. Dongre, C.-M. Tsai, M. Kumaraswamy, A. Kousha, J.A. Valderrama, J.A. Ludviksen, P. Cavanagh, S. Uchiyama, T.E. Mollnes, D.J. Gonzalez, S.N. Wai, V. Nizet, M. Johannessen, *Staphylococcus aureus* membrane-derived vesicles promote bacterial virulence and confer protective immunity in murine infection models, Front. Microbiol. 9 (2018), http://dx.doi.org/10.3389/fmicb.2018.00262.
[22] B. Thay, S.N. Wai, J. Oscarsson, *Staphylococcus aureus* α-toxin-dependent induction of host cell death by membrane-derived vesicles, PLoS One 8 (2013) e54661, , http://dx.doi.org/10.1371/journal.pone.0054661.
[23] S. Fulsundar, K. Harms, G.E. Flaten, P.J. Johnsen, B.A. Chopade, K.M. Nielsen, Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation, Appl. Environ. Microbiol. 80 (2014) 3469–3483, http://dx.doi.org/10.1128/AEM.04248-13.
[24] L. Fu, B. Niu, Z. Zhu, S. Wu, W. Li, CD-HIT: accelerated for clustering the next-generation sequencing data, Bioinforma. Oxf. Engl. 28 (2012) 3150–3152, http://dx.doi.org/10.1093/bioinformatics/bts565.
[25] J.C. Oliveros, VENNY. An Interactive Tool for Comparing Lists with Venn Diagrams. BioinfoGP, CNB-CSIC, Venny 21, http://bioinfogp.cnb.csic.es/tools/venny/index.html, (2015) , Accessed date: 1 November 2017.
[26] H. Nielsen, Predicting secretory proteins with SignalP, Methods Mol. Biol. Clifton NJ. 1611 (2017) 59–73, http://dx.doi.org/10.1007/978-1-4939-7015-5_6.
[27] K. Hiller, A. Grote, M. Scheer, R. Münch, D. Jahn, PrediSi: prediction of signal peptides and their cleavage positions, Nucleic Acids Res. 32 (2004) W375–W379, http://dx.doi.org/10.1093/nar/gkh378.
[28] N.Y. Yu, J.R. Wagner, M.R. Laird, G. Melli, S. Rey, R. Lo, P. Dao, S.C. Sahinalp, M. Ester, L.J. Foster, F.S.L. Brinkman, PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes, Bioinformatics 26 (2010) 1608–1615, http://dx.doi.org/10.1093/bioinformatics/btq249.
[29] D.W. Huang, B.T. Sherman, Q. Tan, J.R. Collins, W.G. Alvord, J. Roayaei, R. Stephens, M.W. Baseler, H.C. Lane, R.A. Lempicki, The DAVID gene functional classification tool: a novel biological module-centric algorithm to functionally analyze large gene lists, Genome Biol. 8 (2007) R183, http://dx.doi.org/10.1186/gb-2007-8-9-r183.
[30] BLAST: Basic Local Alignment Search Tool, (n.d.). https://blast.ncbi.nlm.nih.gov/Blast.cgi (accessed April 14, 2018).
[31] E. Zankari, H. Hasman, S. Cosentino, M. Vestergaard, S. Rasmussen, O. Lund, F.M. Aarestrup, M.V. Larsen, Identification of acquired antimicrobial resistance genes, J. Antimicrob. Chemother. 67 (2012) 2640–2644, http://dx.doi.org/10.1093/jac/dks261.
[32] F.L. Paganelli, R.J.L. Willems, P. Jansen, A. Hendrickx, X. Zhang, M.J.M. Bonten, H.L. Leavis, *Enterococcus faecium* biofilm formation: identification of major autolysin AtlAEfm, associated Acm surface localization, and AtlAEfm-independent extracellular DNA release, MBio 4 (2013), http://dx.doi.org/10.1128/mBio.00154-13.
[33] S.R. Nallapareddy, K.V. Singh, B.E. Murray, Construction of improved temperature-sensitive and mobilizable vectors and their use for constructing mutations in the adhesin-encoding *acm* gene of poorly transformable clinical *Enterococcus faecium* strains, Appl. Environ. Microbiol. 72 (2006) 334–345, http://dx.doi.org/10.1128/

AEM.72.1.334-345.2006.
[34] S.R. Nallapareddy, G.M. Weinstock, B.E. Murray, Clinical isolates of *Enterococcus faecium* exhibit strain-specific collagen binding mediated by Acm, a new member of the MSCRAMM family, Mol. Microbiol. 47 (2003) 1733–1747, http://dx.doi.org/10.1046/j.1365-2958.2003.03417.x.
[35] F.L. Paganelli, J. Huebner, K.V. Singh, X. Zhang, W. van Schaik, D. Wobser, J.C. Braat, B.E. Murray, M.J.M. Bonten, R.J.L. Willems, H.L. Leavis, Genome-wide screening identifies phosphotransferase system permease BepA to be involved in *Enterococcus faecium* endocarditis and biofilm formation, J. Infect. Dis. 214 (2016) 189–195, http://dx.doi.org/10.1093/infdis/jiw108.
[36] L. Ali, M. Spiess, D. Wobser, M. Rodriguez, H.E. Blum, T. Sakınç, Identification and functional characterization of the putative polysaccharide biosynthesis protein (CapD) of *Enterococcus faecium* U0317, Infect. Genet. Evol. 37 (2016) 215–224, http://dx.doi.org/10.1016/j.meegid.2015.11.020.
[37] S.R. Somarajan, J.H. Roh, K.V. Singh, G.M. Weinstock, B.E. Murray, CcpA is important for growth and virulence of *Enterococcus faecium*, Infect. Immun. 82 (2014) 3580–3587, http://dx.doi.org/10.1128/IAI.01911-14.
[38] P. Gao, K.L. Pinkston, A. Bourgogne, M.R. Cruz, D.A. Garsin, B.E. Murray, B.R. Harvey, Library screen identifies *Enterococcus faecalis* CcpA, the catabolite control protein A, as an effector of Ace, a collagen adhesion protein linked to virulence, J. Bacteriol. 195 (2013) 4761–4768, http://dx.doi.org/10.1128/JB.00706-13.
[39] A.P.A. Hendrickx, M. van Luit-Asbroek, C.M.E. Schapendonk, W.J.B. van Wamel, J.C. Braat, L.M. Wijnands, M.J.M. Bonten, R.J.L. Willems, SgrA, a nidogen-binding LPXTG surface adhesin implicated in biofilm formation, and EcbA, a collagen binding MSCRAMM, are two novel adhesins of hospital-acquired *Enterococcus faecium*, Infect. Immun. 77 (2009) 5097–5106, http://dx.doi.org/10.1128/IAI.00275-09.
[40] R.J. Willems, W. Homan, J. Top, M. van Santen-Verheuvel, D. Tribe, X. Manzioros, C. Gaillard, C.M. Vandenbroucke-Grauls, E.M. Mascini, E. van Kregten, J.D. van Embden, M.J. Bonten, Variant *esp* gene as a marker of a distinct genetic lineage of vancomycinresistant *Enterococcus faecium* spreading in hospitals, Lancet 357 (2001) 853–855, http://dx.doi.org/10.1016/S0140-6736(00)04205-7.
[41] S.R. Somarajan, S.L. La Rosa, K.V. Singh, J.H. Roh, M. Höök, B.E. Murray, The fibronectin-binding protein Fnm contributes to adherence to extracellular matrix components and virulence of *Enterococcus faecium*, Infect. Immun. 83 (2015) 4653–4661, http://dx.doi.org/10.1128/IAI.00885-15.
[42] A.P.A. Hendrickx, M.J.M. Bonten, M. van Luit-Asbroek, A.H.M. Schapendonk, R.J.L. Willems Kragten, Expression of two distinct types of pili by a hospital-acquired *Enterococcus faecium* isolate, Microbiology 154 (2008) 3212–3223, http://dx.doi.org/10.1099/mic.0.2008/020891-0.
[43] J. Sillanpää, S.R. Nallapareddy, K.V. Singh, V.P. Prakash, T. Fothergill, H. Ton-That, B.E. Murray, Characterization of the ebpfm pilus-encoding operon of *Enterococcus faecium* and its role in biofilm formation and virulence in a murine model of urinary tract infection, Virulence 1 (2010) 236–246.
[44] X. Zhang, J. Top, M. de Been, D. Bierschenk, M. Rogers, M. Leendertse, M.J.M. Bonten, T. van der Poll, R.J.L. Willems, W. van Schaik, Identification of a genetic determinant in clinical *Enterococcus faecium* strains that contributes to intestinal colonization during antibiotic treatment, J. Infect. Dis. 207 (2013) 1780–1786, http://dx.doi.org/10.1093/infdis/jit076.
[45] A.M.G. Prieto, R.T. Urbanus, X. Zhang, D. Bierschenk, C.A. Koekman, M. van Luit-Asbroek, J.P. Ouwerkerk, M. Pape, F.L. Paganelli, D. Wobser, J. Huebner, A.P.A. Hendrickx, M.J.M. Bonten, R.J.L. Willems, W. van Schaik, The N-terminal domain of the thermo-regulated surface protein PrpA of *Enterococcus faecium* binds to fibrinogen, fibronectin and platelets, Sci. Rep. 5 (2015) srep18255, http://dx.doi.org/10.1038/srep18255.
[46] K.J. Rangan, V.A. Pedicord, Y.-C. Wang, B. Kim, Y. Lu, S. Shaham, D. Mucida, H.C. Hang, A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens, Science 353 (2016) 1434–1437, http://dx.doi.org/10.1126/science.aaf3552.
[47] F.L. Paganelli, M. de Been, J.C. Braat, T. Hoogenboezem, C. Vink, J. Bayjanov, M.R.C. Rogers, J. Huebner, M.J.M. Bonten, R.J.L. Willems, H.L. Leavis, Distinct SagA from hospital-associated clade A1 *Enterococcus faecium* strains contributes to biofilm formation, Appl. Environ. Microbiol. 81 (2015) 6873–6882, http://dx.doi.org/10.1128/AEM.01716-15.
[48] A. Kropec, I.G. Sava, C. Vonend, T. Sakinc, E. Grohmann, J. Huebner, Identification of SagA as a novel vaccine target for the prevention of *Enterococcus faecium* infections, Microbiology 157 (2011) 3429–3434, http://dx.doi.org/10.1099/mic.0.053207-0.
[49] J. Sillanpää, S.R. Nallapareddy, V.P. Prakash, X. Qin, M. Hook, G.M. Weinstock, B.E. Murray, Identification and phenotypic characterization of a second collagen adhesin, Scm, and genome-based identification and analysis of 13 other predicted MSCRAMMs, including four distinct pilus loci, in *Enterococcus faecium*, Microbiol. Read. Engl. 154 (2008) 3199–3211, http://dx.doi.org/10.1099/mic.0.2008/017319-0.
[50] T.J. Eaton, M.J. Gasson, Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates, Appl. Environ. Microbiol. 67 (2001) 1628–1635, http://dx.doi.org/10.1128/AEM.67.4.1628-1635.2001.
[51] V. Strompfová, A. Lauková, M. Simonová, M. Marcináková, Occurrence of the structural enterocin A, P, B, L50B genes in enterococci of different origin, Vet. Microbiol. 132 (2008) 293–301, http://dx.doi.org/10.1016/j.vetmic.2008.05.001.
[52] R. Criado, D.B. Diep, Å. Aakra, J. Gutiérrez, I.F. Nes, P.E. Hernández, L.M. Cintas, Complete sequence of the Enterocin Q-encoding plasmid pCIZ2 from the multiple bacteriocin producer *Enterococcus faecium* L50 and genetic characterization of Enterocin Q production and immunity, Appl. Environ. Microbiol. 72 (2006) 6653–6666, http://dx.doi.org/10.1128/AEM.00859-06.
[53] A.R. Freitas, A.P. Tedim, M.V. Francia, L.B. Jensen, C. Novais, L. Peixe, A. Sánchez-Valenzuela, A. Sundsfjord, K. Hegstad, G. Werner, E. Sadowy, A.M. Hammerum, L. Garcia-Migura, R.J. Willems, F. Baquero, T.M. Coque, Multilevel population genetic analysis of *vanA* and *vanB Enterococcus faecium* causing nosocomial outbreaks in 27 countries (1986-2012), J. Antimicrob. Chemother. 71 (2016) 3351–3366, http://dx.doi.org/10.1093/jac/dkw312.
[54] D. Todokoro, H. Tomita, T. Inoue, Y. Ike, Genetic analysis of bacteriocin 43 of vancomycin-resistant *Enterococcus faecium*, Appl. Environ. Microbiol. 72 (2006) 6955–6964, http://dx.doi.org/10.1128/AEM.00934-06.
[55] F. Romero-Saavedra, D. Laverde, A. Budin-Verneuil, C. Muller, B. Bernay, A. Benachour, A. Hartke, J. Huebner, Characterization of two metal binding lipoproteins as vaccine candidates for enterococcal infections, PLoS One 10 (2015) e0136625, , http://dx.doi.org/10.1371/journal.pone.0136625.
[56] F. Romero-Saavedra, D. Laverde, D. Wobser, C. Michaux, A. Budin-Verneuil, B. Bernay, A. Benachour, A. Hartke, J. Huebner, Identification of peptidoglycan-associated proteins as vaccine candidates for enterococcal infections, PLoS One 9 (2014), http://dx.doi.org/10.1371/journal.pone.0111880.
[57] D.M. Daigle, D.W. Hughes, G.D. Wright, Prodigious substrate specificity of AAC(6′)-APH(2″), an aminoglycoside antibiotic resistance determinant in enterococci and staphylococci, Chem. Biol. 6 (1999) 99–110, http://dx.doi.org/10.1016/S1074-5521(99)80006-4.
[58] M. Arthur, F. Depardieu, C. Molinas, P. Reynolds, P. Courvalin, The *vanZ* gene of Tn*1546* from *Enterococcus faecium* BM4147 confers resistance to teicoplanin, Gene 154 (1995) 87–92, http://dx.doi.org/10.1016/0378-1119(94)00851-I.
[59] M.J. Kuehn, N.C. Kesty, Bacterial outer membrane vesicles and the host–pathogen interaction, Genes Dev. 19 (2005) 2645–2655, http://dx.doi.org/10.1101/gad.1299905.
[60] S. Vdovikova, M. Luhr, P. Szalai, L. Nygård Skalman, M.K. Francis, R. Lundmark, N. Engedal, J. Johansson, S.N. Wai, A novel role of *Listeria monocytogenes* membrane vesicles in inhibition of autophagy and cell death, Front. Cell. Infect. Microbiol. 7 (2017) 154, , http://dx.doi.org/10.3389/fcimb.2017.00154.
[61] M. Gurung, D.C. Moon, C.W. Choi, J.H. Lee, Y.C. Bae, J. Kim, Y.C. Lee, S.Y. Seol, D.T. Cho, S.I. Kim, J.C. Lee, *Staphylococcus aureus* produces membrane-derived vesicles that induce host cell death, PLoS One 6 (2011) e27958, , http://dx.doi.org/10.1371/journal.pone.0027958.
[62] R. Grande, C. Celia, G. Mincione, A. Stringaro, L. Di Marzio, M. Colone, M.C. Di Marcantonio, L. Savino, V. Puca, R. Santoliquido, M. Locatelli, R. Muraro, L. Hall-Stoodley, P. Stoodley, Detection and physicochemical characterization of membrane vesicles (MVs) of *Lactobacillus reuteri* DSM 17938, Front. Microbiol. 8 (2017) 1040, , http://dx.doi.org/10.3389/fmicb.2017.01040.
[63] M.V. Surve, A. Anil, K.G. Kamath, S. Bhutda, L.K. Sthanam, A. Pradhan, R. Srivastava, B. Basu, S. Dutta, S. Sen, D. Modi, A. Banerjee, Membrane vesicles of group B Streptococcus disrupt Feto-maternal barrier leading to preterm birth, PLoS Pathog. 12 (2016) e1005816, , http://dx.doi.org/10.1371/journal.ppat.1005816.
[64] N. Obana, R. Nakao, K. Nagayama, K. Nakamura, H. Senpuku, N. Nomura, Immunoactive clostridial membrane vesicle production is regulated by a sporulation factor, Infect. Immun. 85 (2017) e00096–17, , http://dx.doi.org/10.1128/IAI.00096-17.
[65] Y. Jiang, Q. Kong, K.L. Roland, R. Curtiss, Membrane vesicles of *Clostridium perfringens* type a strains induce innate and adaptive immunity, Int. J. Med. Microbiol. IJMM. 304 (2014) 431–443, http://dx.doi.org/10.1016/j.ijmm.2014.02.006.
[66] P. Dauros Singorenko, V. Chang, A. Whitcombe, D. Simonov, J. Hong, A. Phillips, S. Swift, C. Blenkiron, Isolation of membrane vesicles from prokaryotes: a technical and biological comparison reveals heterogeneity, J. Extracell. Vesicles. 6 (2017) 1324731, , http://dx.doi.org/10.1080/20013078.2017.1324731.
[67] J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from Gram-negative bacteria, Microbiol. Res. 170 (2015) 1–9, http://dx.doi.org/10.1016/j.micres.2014.09.006.
[68] M. Kaparakis, L. Turnbull, L. Carneiro, S. Firth, H.A. Coleman, H.C. Parkington, L. Le Bourhis, A. Karrar, J. Viala, J. Mak, M.L. Hutton, J.K. Davies, P.J. Crack, P.J. Hertzog, D.J. Philpott, S.E. Girardin, C.B. Whitchurch, R.L. Ferrero, Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells, Cell. Microbiol. 12 (2010) 372–385, http://dx.doi.org/10.1111/j.1462-5822.2009.01404.x.
[69] Y. Tashiro, S. Ichikawa, M. Shimizu, M. Toyofuku, N. Takaya, T. Nakajima-Kambe, H. Uchiyama, N. Nomura, Variation of physiochemical properties and cell association activity of membrane vesicles with growth phase in *Pseudomonas aeruginosa*, Appl. Environ. Microbiol. 76 (2010) 3732–3739, http://dx.doi.org/10.1128/AEM.02794-09.
[70] Y. Tashiro, Y. Hasegawa, M. Shintani, K. Takaki, M. Ohkuma, K. Kimbara, H. Futamata, Interaction of bacterial membrane vesicles with specific species and their potential for delivery to target cells, Front. Microbiol. 8 (2017), http://dx.doi.org/10.3389/fmicb.2017.00571.
[71] E.-Y. Lee, J.Y. Bang, G.W. Park, D.-S. Choi, J.S. Kang, H.-J. Kim, K.-S. Park, J.-O. Lee, Y.-K. Kim, K.-H. Kwon, K.-P. Kim, Y.S. Gho, Global proteomic profiling of native outer membrane vesicles derived from *Escherichia coli*, Proteomics 7 (2007) 3143–3153, http://dx.doi.org/10.1002/pmic.200700196.
[72] R.J. Bager, G. Persson, B. Nesta, M. Soriani, L. Serino, M. Jeppsson, T.K. Nielsen, A.M. Bojesen, Outer membrane vesicles reflect environmental cues in *Gallibacterium anatis*, Vet. Microbiol. 167 (2013) 565–572, http://dx.doi.org/10.1016/j.vetmic.2013.09.005.
[73] C.-W. Choi, E.C. Park, S.H. Yun, S.-Y. Lee, Y.G. Lee, Y. Hong, K.R. Park, S.-H. Kim, G.-H. Kim, S.I. Kim, Proteomic characterization of the outer membrane vesicle of *Pseudomonas putida* KT2440, J. Proteome Res. 13 (2014) 4298–4309, http://dx.doi.org/10.1021/pr500411d.

[74] N. Orench-Rivera, M. Kuehn, Environmentally-controlled bacterial vesicle-mediated export, Cell. Microbiol. 18 (2016) 1525–1536, http://dx.doi.org/10.1111/cmi.12676.
[75] A.J. Park, K. Murphy, M.D. Surette, C. Bandoro, J.R. Krieger, P. Taylor, C.M. Khursigara, Tracking the dynamic relationship between cellular systems and extracellular subproteomes in *Pseudomonas aeruginosa* biofilms, J. Proteome Res. 14 (2015) 4524–4537, http://dx.doi.org/10.1021/acs.jproteome.5b00262.
[76] M. Toyofuku, B. Roschitzki, K. Riedel, L. Eberl, Identification of proteins associated with the *Pseudomonas aeruginosa* biofilm extracellular matrix, J. Proteome Res. 11 (2012) 4906–4915, http://dx.doi.org/10.1021/pr300395j.
[77] K.-S. Jang, M.J. Sweredoski, R.L.J. Graham, S. Hess, W.M. Clemons, Comprehensive proteomic profiling of outer membrane vesicles from *Campylobacter jejuni*, J. Proteomics 98 (2014) 90–98, http://dx.doi.org/10.1016/j.jprot.2013.12.014.
[78] E.D. Avila-Calderón, M.G. Araiza-Villanueva, J.C. Cancino-Diaz, E.O. López-Villegas, N. Sriranganathan, S.M. Boyle, A. Contreras-Rodríguez, Roles of bacterial membrane vesicles, Arch. Microbiol. 197 (2015) 1–10, http://dx.doi.org/10.1007/s00203-014-1042-7.
[79] L. van der Pol, M. Stork, P. van der Ley, Outer membrane vesicles as platform vaccine technology, Biotechnol. J. 10 (2015) 1689–1706, http://dx.doi.org/10.1002/biot.201400395.
[80] H.M. Kulkarni, R. Nagaraj, M.V. Jagannadham, Protective role of *E. coli* outer membrane vesicles against antibiotics, Microbiol. Res. 181 (2015) 1–7, http://dx.doi.org/10.1016/j.micres.2015.07.008.
[81] J.A. Mohamed, D.B. Huang, Biofilm formation by enterococci, J. Med. Microbiol. 56 (2007) 1581–1588, http://dx.doi.org/10.1099/jmm.0.47331-0.
[82] G.M. Dunny, L.E. Hancock, N. Shankar, Enterococcal biofilm structure and role in colonization and disease, in: M.S. Gilmore, D.B. Clewell, Y. Ike, N. Shankar (Eds.), Enterococci Commensals Lead. Causes Drug Resist. Infect., Massachusetts Eye and Ear Infirmary, Boston, 2014 http://www.ncbi.nlm.nih.gov/books/NBK190433/.
[83] S.R. Schooling, T.J. Beveridge, Membrane vesicles: an overlooked component of the matrices of biofilms, J. Bacteriol. 188 (2006) 5945–5957, http://dx.doi.org/10.1128/JB.00257-06.
[84] W. Wang, W. Chanda, M. Zhong, The relationship between biofilm and outer membrane vesicles: a novel therapy overview, FEMS Microbiol. Lett. 362 (2015) fnv117, , http://dx.doi.org/10.1093/femsle/fnv117.
[85] M. Kaparakis-Liaskos, R.L. Ferrero, Immune modulation by bacterial outer membrane vesicles, Nat. Rev. Immunol. 15 (2015) 375–387, http://dx.doi.org/10.1038/nri3837.
[86] T. Vesikari, S. Esposito, R. Prymula, E. Ypma, I. Kohl, D. Toneatto, P. Dull, A. Kimura, EU meningococcal B infant vaccine study group, immunogenicity and safety of an investigational multicomponent, recombinant, meningococcal serogroup B vaccine (4CMenB) administered concomitantly with routine infant and child vaccinations: results of two randomised trials, Lancet Lond. Engl. 381 (2013) 825–835, http://dx.doi.org/10.1016/S0140-6736(12)61961-8.
[87] C.-W. Choi, E.C. Park, S.H. Yun, S.-Y. Lee, S.I. Kim, G.-H. Kim, Potential usefulness of *Streptococcus pneumoniae* extracellular membrane vesicles as antibacterial vaccines, J. Immunol. Res. 2017 (2017), http://dx.doi.org/10.1155/2017/7931982 (7931982).
[88] S.J. Choi, M.-H. Kim, J. Jeon, O.Y. Kim, Y. Choi, J. Seo, S.-W. Hong, W.-H. Lee, S.G. Jeon, Y.S. Gho, Y.-K. Jee, Y.-K. Kim, Active immunization with extracellular vesicles derived from Staphylococcus aureus effectively protects against staphylococcal lung infections, mainly via Th1 cell-mediated immunity, PLoS One 10 (2015) e0136021, , http://dx.doi.org/10.1371/journal.pone.0136021.
[89] A. Bauwens, L. Kunsmann, H. Karch, A. Mellmann, M. Bielaszewska, Antibiotic-mediated modulations of outer membrane vesicles in enterohemorrhagic *Escherichia coli* O104:H4 and O157:H7, Antimicrob. Agents Chemother. 61 (2017), http://dx.doi.org/10.1128/AAC.00937-17.
[90] K.W. Chan, C. Shone, J.R. Hesp, Antibiotics and iron-limiting conditions and their effect on the production and composition of outer membrane vesicles secreted from clinical isolates of extraintestinal pathogenic *E. coli*, Proteomics Clin. Appl. 11 (2017), http://dx.doi.org/10.1002/prca.201600091.
[91] C. Rumbo, E. Fernández-Moreira, M. Merino, M. Poza, J.A. Mendez, N.C. Soares, A. Mosquera, F. Chaves, G. Bou, Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*, Antimicrob. Agents Chemother. 55 (2011) 3084–3090, http://dx.doi.org/10.1128/AAC.00929-10.
[92] V. Schaar, I. Uddbäck, T. Nordström, K. Riesbeck, Group A streptococci are protected from amoxicillin-mediated killing by vesicles containing β-lactamase derived from *Haemophilus influenzae*, J. Antimicrob. Chemother. 69 (2014) 117–120, http://dx.doi.org/10.1093/jac/dkt307.
[93] S. Devos, S. Stremersch, K. Raemdonck, K. Braeckmans, B. Devreese, Intra- and interspecies effects of outer membrane vesicles from *Stenotrophomonas maltophilia* on β-lactam resistance, Antimicrob. Agents Chemother. 60 (2016) 2516–2518, http://dx.doi.org/10.1128/AAC.02171-15.
[94] M. Toyofuku, G. Cárcamo-Oyarce, T. Yamamoto, F. Eisenstein, C.-C. Hsiao, M. Kurosawa, K. Gademann, M. Pilhofer, N. Nomura, L. Eberl, Prophage-triggered membrane vesicle formation through peptidoglycan damage in Bacillus subtilis, Nat. Commun. 8 (2017), http://dx.doi.org/10.1038/s41467-017-00492-w.
[95] D. Arndt, J.R. Grant, A. Marcu, T. Sajed, A. Pon, Y. Liang, D.S. Wishart, PHASTER: a better, faster version of the PHAST phage search tool, Nucleic Acids Res. 44 (2016) W16–W21, http://dx.doi.org/10.1093/nar/gkw387.
[96] P.M. Martínez-García, C. Ramos, P. Rodríguez-Palenzuela, T346Hunter: a novel web-based tool for the prediction of type III, type IV and type VI secretion systems in bacterial genomes, PLoS One 10 (2015), http://dx.doi.org/10.1371/journal.pone.0119317.
[97] J.A. Vizcaíno, A. Csordas, N. del-Toro, J.A. Dianes, J. Griss, I. Lavidas, G. Mayer, Y. Perez-Riverol, F. Reisinger, T. Ternent, Q.-W. Xu, R. Wang, H. Hermjakob, 2016 update of the PRIDE database and its related tools, Nucleic Acids Res. 44 (2016) D447–D456, http://dx.doi.org/10.1093/nar/gkv1145.
[98] R.C. Arduino, K. Jacques-Palaz, B.E. Murray, R.M. Rakita, Resistance of *Enterococcus faecium* to neutrophil-mediated phagocytosis, Infect. Immun. 62 (1994) 5587–5594.
[99] M. de Been, W. van Schaik, L. Cheng, J. Corander, R.J. Willems, Recent recombination events in the core genome are associated with adaptive evolution in *Enterococcus faecium*, Genome Biol. Evol. 5 (2013) 1524–1535, http://dx.doi.org/10.1093/gbe/evt111.
[100] T.C.S. Rosvoll, B.L. Lindstad, T.M. Lunde, K. Hegstad, B. Aasnæs, A.M. Hammerum, C.H. Lester, G.S. Simonsen, A. Sundsfjord, T. Pedersen, Increased high-level gentamicin resistance in invasive *Enterococcus faecium* is associated with aac(6′)Ie-aph(2″)Ia-encoding transferable megaplasmids hosted by major hospital-adapted lineages, FEMS Immunol. Med. Microbiol 66 (2012) 166–176, http://dx.doi.org/10.1111/j.1574-695X.2012.00997.x.

PAPER II

Transcriptome profiling of *Staphylococcus aureus* associated Extracellular Vesicles reveals presence of small RNA-cargo

Joshi B[1], Singh B[1], Nadeem A[2, 3], Askarian F[1, 4], Wai SN[2, 3], Johannessen M[1#*], Hegstad K[1, 5#*]

[1]Research Group for Host-Microbe Interactions, Department of Medical Biology, UiT - The Arctic University of Norway, Tromsø, Norway
[2]Umeå Centre for Microbial Research (UCMR), Umeå University, Umeå, Sweden
[3]Department of Molecular Biology, Umeå University, Umeå, Sweden
[4]Faculty of Chemistry, Biotechnology and Food Science, The Norwegian University of Life Sciences (NMBU), Ås, Norway
[5]Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, Department of Microbiology and Infection Control, University Hospital of North-Norway, Tromsø, Norway

[#]These authors contributed equally
* **Correspondence:**
Mona Johannessen Mona.johannessen@uit.no
Kristin Hegstad Kristin.hegstad@uit.no

Abstract
Bacterial extracellular vesicles (EVs) have a vital role in bacterial pathogenesis. However, to date, the small RNA-cargo of extracellular vesicles released by the opportunistic pathogen *Staphylococcus aureus* has not been characterized. Here, we shed light on the association of small RNAs with EVs secreted by *S. aureus* MSSA476 cultured in iron-depleted bacteriologic media supplemented with a subinhibitory dosage of vancomycin to mimic infection condition. Confocal microscopy analysis on intact RNase treated EVs indicated that RNA is associated with EVs. Transcriptomic and *in silico* analysis of EV- associated RNA revealed the presence of potential gene regulatory small RNAs and high levels of tRNAs. Among the enriched small RNAs were SsrA, RsaC and RNAIII RNAs. Our finding invites new insights into the potential role of EV associated RNA as a modulator of host-pathogen interaction.

Key words: *Staphylococcus aureus*, Extracellular vesicles, transcriptomic analysis, small RNAs, tRNA

INTRODUCTION

Staphylococcus aureus, a Gram-positive bacterium, is a frequent colonizer of anterior nares of the healthy human population. These bacteria can cause various infections ranging from minor superficial skin infections to severe life-threatening infections such as osteomyelitis, pneumonia, endocarditis, bacteremia and sepsis (Wertheim et al., 2005; McCaig et al., 2006; Foster et al., 2014). The adaptation of diverse lifestyles and the ability to cause diseases is due to the fact that bacteria harbors arsenals of virulence factors involved in adhesion, invasion and dissemination (Novick, 2003).

Small RNA (sRNA) are heterogeneous small-sized transcripts (50–500 nucleotides) expressed under stressful environmental conditions which play an important role in growth processes, metabolism, stress adaptation and virulence (Tomasini et al., 2014; Westermann, 2018). Prokaryotic sRNAs are often non-coding and mainly originate from intergenic regions (Wagner and Vogel, 2003). They generally form secondary structures such as hairpins and stem-loops (Wagner and Romby, 2015). There are varieties of techniques (*in silico*, laboratory-based) to identify and characterize sRNAs (Lagos-Quintana et al., 2001; Wang et al., 2009; Li et al., 2012). Mizuno and colleagues first reported sRNAs with regulatory functions in *Escherichia coli* in 1980's, and a decade later Novick *et al.* reported regulatory sRNAs in *S. aureus* (Mizuno et al., 1984; Novick et al., 1989). Currently, there are about 250 sRNAs discovered in various strains of *S. aureus* grown under different experimental conditions, and the biological functions of most of them are yet to be determined (Guillet et al., 2013; Hermansen et al., 2018). Still, novel sRNA transcripts are reported from *S. aureus* strains, and the number is increasing with the advancement of high-throughput sequencing technology as well as robust *in silico* computational methods (Liu et al., 2018a; Westermann, 2018).

Extracellular Vesicles (EVs) are nanosized-proteolipids, with a spherical shape that are heterogeneous in size ranging from 50-500 nm (Askarian et al., 2018). Sometimes fusion of vesicles have resulted in formation of filamentous structures, also known as nanopods or nanotubes (Dongre et al., 2011; Dubey and Ben-Yehuda, 2011; Gill et al., 2018). EVs may contain virulence factors (Devos et al., 2015; Askarian et al., 2018; Wagner et al., 2018; Nadeem et al., 2020), such as toxins (Rivera et al., 2010; Coelho et al., 2019) as well as other enzymes (Smalley and Birss, 1987; Elhenawy et al., 2014), quorum sensing molecules (Mashburn and Whiteley, 2005; Brameyer et al., 2018; Morinaga et al., 2018) and nucleic acids such as DNA (Hagemann et al., 2014; Bitto et al., 2017; Langlete et al., 2019) and RNA (Sjöström et al., 2015; Koeppen et al., 2016; Choi et al., 2017b). Their content may vary depending on species and growth conditions (Bager et al., 2013; Ghosal et al., 2015; Koeppen et al., 2016). EVs might act as a decoy against antimicrobial peptides and phages (Manning and Kuehn, 2011), and are also involved in co-operation and/or competition with other pathogens (Lynch and Alegado, 2017; Choi et al., 2020). EVs can also influence biofilm formation and modulate host-immune responses (Manning and Kuehn, 2013; Schwechheimer and Kuehn, 2015; Liu et al., 2018b).

EVs from Gram-negative bacteria harbor sRNA involved in intra-species (microbe-microbe) (Whitworth, 2018) and inter-kingdom (microbe-host) interactions (Koeppen et al., 2016; Frantz et al., 2019) as well as pathogenicity (Song and Wai, 2009). However, scant functional and analytical data exist to support these claims in Gram-positive bacteria (Ghosal et al., 2015; Sjöström et al., 2015; Koeppen et al., 2016; Choi et al., 2018; Malabirade et al., 2018).

During infection, the availability of iron is strictly controlled by the host, and in order to survive pathogens must adapt their transcriptomic and metabolic pathways accordingly (Wilderman et al., 2004; Oglesby-Sherrouse and Murphy, 2013; Mäder et al., 2016). Nutrient limitation and antibiotics is furthermore known to increase vesiculation (Toyofuku et al., 2019). Subinhibitory concentrations of the last resort anti-staphylococcal antibiotic, vancomycin, has

been shown to influence physiology, growth and toxin production by *S. aureus* (Hsu et.al, 2011; Cafiso et al., 2012; He et al., 2017), and has been found to increase EV production in another Gram-positive species, *Enterococcus faecium* (Kim et al., 2019). Hence, in this study, we used iron-chelated media supplemented with vancomycin to evaluate whether the EVs produced by *S. aureus* MSSA476 are associated with sRNAs when grown in conditions that mimic an infection that is being treated with an antibiotic.

MATERIALS AND METHODS

Strain and growth conditions

S. aureus subsp. *aureus* Rosenbach MSSA476 was purchased from LGC standard AB (ATCC-BAA-1721) (Sweden). The bacteria were grown at 37°C on BHI agar plate, BHI broth, or in trace metal-depleted BHI broth containing 0.5 µg/mL of vancomycin. The trace metals including divalent cations such as iron were lowered by treating the BHI broth with 2 g/L of chelex-100 resin (Bio-Rad, Californa, USA). The medium was subsequently filtered according to the manufacturer's instructions.

Isolation of bacterial extracellular vesicles

The bacterial EVs were isolated by a procedure described earlier (Askarian et al., 2018; Wagner et al., 2018), with slight modifications. A fresh overnight culture of the methicillin-susceptible *S. aureus* MSSA476 (1:100 dilution) was inoculated into 500 mL BHI or Chelex-treated BHI broth containing 0.5 µg/mL of vancomycin at least two to five different days (Table S1). Since the RNA isolation was limited from each preparation, the experiment was done in triplicates. The cultures were grown with shaking at 37°C for 16 h. The cultures were then centrifuged at 6000 × g for 30 min. Bacterial pellets were discarded, and the supernatants was filtered through 0.2 µm filters (Millipore Express™ Plus, USA) and ultra-centrifuged at 100,000 × g for 3 h at 4°C in a 45 Ti rotor (Beckman, USA). EV pellets from each isolation were re-suspended in 500 µL RNAlater (Thermo Fisher Scientific, Massachusetts, USA) and kept at -80°C until further use. Prior to RNA isolation, EVs were thawed, pooled and concentrated using ultrafiltration (10 kDa Vivaspin 20, Sartorius, Germany). The vesicles were resuspended in Phosphate-buffered saline (PBS) if EVs were to be used for microscopy or Nanoparticle Tracking Analysis.

Transmission Electron Microscopy (TEM)

TEM was performed as described previously (Cavanagh et al., 2018;Wagner et al., 2018). Briefly, 5 µL of purified EVs were applied to Formvar coated 75 mesh hexagonal copper grids (Electron Microscopy Science, Pennsylvania, USA) and incubated for 5 min. Grids were washed with MQ water, and negatively stained with 2% methylcellulose and 3 % uranyl acetate in a ratio of 9:1. The excess of stain was blotted away, and grids were then left to dry at room temperature. The samples were then visualized with a JEOL JEM 1010 transmission electron microscope (JEOL, Tokyo, Japan) operated at 80 kV.

Atomic Force Microscopy (AFM)

The EVs were imaged by AFM, as described previously (Lindmark et al., 2009;Ahmad et al., 2019). Briefly, EVs were deposited onto a freshly cleaved mica surface (Goodfellow Cambridge Ltd., Cambridge, UK). Prior to imaging, EVs on mica were dried in a desiccator for about 2 h. Images were recorded on a Multimode 8 Nanoscope AFM equipment (Bruker AXS GmbH, Karlsruhe, Germany) using TappingMode. Images were gathered by NanoScope

software using ScanAsyst in air with ScanAsyst cantilevers, at a scan rate of ~0.8–1.5 Hz. The final images were plane fitted in both axes and presented in a surface plot of the height mode.

Nanoparticle Tracking Analysis (NTA)

The size distribution of EVs were determined using NanoSight NS300 (Malvern Instruments Ltd., Worcestershire, UK) equipped with CMOS camera and a blue laser module (488 nm, LM12 version C) (Jamaly et al., 2018). Briefly, EV samples were thawed and diluted (500×) in PBS to obtain a concentration within the recommended measurement range (1-10 × 10^8 particles/mL). Using a 1 mL syringe, the sample was injected into the instrument and videos were captured in triplicate for 30 s. The mean values for size and concentration were analyzed using the NanoSight (NTA software, version 3.0).

Labeling of extracellular vesicles

The EVs were stained using a previously described protocol with slight modifications (Nicola et al., 2009;Vdovikova et al., 2017).The vesicles were first treated with RNase (Roche diagnostics, Basel, Switzerland) to remove extracellular RNA then stained with lipid-specific dye, PKH2 or DiD (Sigma Aldrich) and subsequently with RNA specific dye SYTO RNASelect Green (Thermo Fisher Scientific, Massachusetts, USA). The stained vesicles were then ultra-centrifuged at a speed of 100,000 × g for 1 h at 4°C. The stained EVs were resuspended in PBS. Samples were mounted on a glass slide and examined by Leica SP8 inverted confocal system (Leica Microsystems) equipped with a HC PL APO 63x/1.40 oil immersion lens. Images were captured and processed using LasX (Leica Microsystems). Fluorescence intensity profiles were generated using the plot profile command in ImageJ-FIJI distribution (Schindelin et al., 2012) For quantification, EVs from 8 randomly selected fields (180 μm^2) were counted. Results were pooled from two independent experiments and data are expressed as percentage.

Bacterial growth curve and viability assay

A single colony of MSSA476 was inoculated into two 5 mL of BHI broth and grown overnight with shaking at 37°C. The 5 mL cultures were used to inoculate 500 mL BHI (normal) and 500 mL iron depleted BHI containing antibiotics (stressed). The cultures were incubated with shaking at 37°C, and optical density was measured every 30 min for 16 h. For the viability assay, 500 µL of each culture of bacteria grown under normal and stressed conditions for 16 h, were harvested. Viable plate count was carried out by plating 20 µL of 10-fold serial dilutions (from 10^{-5}-10^{-10}) on blood agar plates, which were incubated for 24 h at 37°C. Dilutions containing 10–100 colonies were counted, and the concentration was calculated as CFU/mL.

Live and dead count

Bacterial cultures grown for 16 h in BHI (normal) and iron depleted BHI containing antibiotics (stressed) were analyzed for live and dead cells using LIVE/DEAD BacLight bacterial Viability and Counting Kit (Thermo Fisher Scientific, Waltham, USA) and BD LSRFortessa flow cytometer. Each bacterial culture was diluted 1000-fold in 1 mL 0.85% filtered NaCl, which contain 0.5 µL SYTO 9, 2.5 µL Propidium iodide (PI), and 10 µL beads of size 6 µm. Beads had a concentration of $1x10^8$/mL and were diluted 100-fold. Cells were stained for 10-15 min at room temperature. Stained bacteria were analyzed using BD LSRFortessa flow cytometer using a voltage of 600, 250, 400 and 800 for forward scatter (FSC), side scatter (SCC), AF488 and PI, respectively. All scales were set to logarithmic amplification with gain voltages of 300, 250 and 200 for FSC, SSC and AF488, respectively. Data were recorded for 1000 bead events. Total events were recorded and density of bacterial culture in terms of bacteria/mL was calculated as (numbers of events in bacterial region)×(dilution factor)/(numbers of events in the bead region×10^{-6}).

Extraction of RNAs

The crude collection EVs was stored in RNAlater; which is a preservative compatible with RNA isolation and downstream applications such as RNA sequencing and Reverse transcription. The EVs were centrifuged using Vivaspin® ultrafiltration spin columns (cutoff 10000MWCO) at 5000 RPM for 15 min. The concentrated EVs were treated with RNaseA (50µg/mL) for 30 min at room temperature to degrade all forms of extracellular RNA. Thereafter, to stop the RNase activity, EVs were treated with 5 µL of RNase inhibitor (Applied Biosystems, Massachusetts, USA) for 15 min at 37°C. Then the small RNA from *S. aureus* EVs were isolated by miRNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. Trizol and Chloroform used during RNA isolation are sufficient to remove traces of RNAlater. The concentration of RNA was measured by Qubit HS kit, which quantify sample concentration ranging from 250 pg/µL to 100 ng/µL (Thermo Fisher Scientific, Waltham, USA), and the quality of RNA was assessed by Nanodrop (Thermo Fisher Scientific, Waltham, USA, USA). A 260/280 ratio of 1.8 or higher was considered optimal. In our RNA prep it was found to be 1.85. In order to evaluate whether the isolated RNA was extravesicular or associated with EVs, RNA concentration was measured on crude EVs, RNase treated EVs and finally in RNA isolated from the RNase-treated EVs.

rRNA depletion, library preparation and sequencing

The isolated EV associated RNA was treated with Ribo-zero rRNA removal kit (Illumina, Munich, Germany) according to the manufacturer's instructions to reduce ribosomal RNA (rRNA). Thereafter, the concentration of RNA was measured using Experion RNA HighSense Chips (Bio-Rad Laboratories, Inc, USA). The depleted RNA was cleaned and concentrated using RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) and RNA clean & concentrator-5 kit (Zymo Research, California, USA). The rRNA-depleted RNA was fragmented, and reverse transcribed into cDNA using high capacity cDNA reverse transcription kit (Applied Biosystems, California, USA), and sequenced on an Illumina NextSeq550 platform.

RNA preparation, cDNA synthesis and qPCR

qPCR was used to confirm the presence of the three enriched sRNAs (SsrA, RSaC and RNAIII). The EV associated RNA was isolated as described above and treated with DNase (ArcticZymes, Tromsø, Norway) before RNA integrity and quantity were measured both by NanoDrop and Qubit. cDNA was prepared by reverse transcription kit (Applied Biosystems) using 100 ng RNA. qPCR reactions were performed in technical duplicates for pooled EV samples using SYBR Green master mix (Applied Biosystem) with the following primer pairs: SsrA-F/R: CACTCTGCATCGCCTAACAG/ GCGTCCAGAGGTCCTGATAC, RsaC-F/R: CAAAGGAAAGGGGCATACAA/ ACGCCATTCCCTACACACTC, RNAIII-F/R: AGTTTCCTTGGACTCAGTGCT/ GGGGCTCACGACCATACTTA. To perform qPCR, briefly, 2 µL of cDNA was used as a template for each 20 µL reaction, which was carried out with 100 nM of primers. Cycling conditions were as follows: initial denaturation 10 min at 95°C, 40 cycles of 15 s at 95°C and 60°C for 1 min as annealing temperature. The data were treated and analyzed with the Applied Biosystems (7300 Real-Time PCR System) to determine the Ct.

PCR and Sanger sequencing

RT-PCR was carried out in a Thermal Cycler (Applied Biosystems, Foster City, CA) in order to verify the three PCR amplicons by agarose gel and DNA sequencing. The PCR was performed in a 20 µL reaction, containing gene-specific primers mentioned above and DreamTaq Green PCR Master Mix (Thermo Fischer Scientific, USA) according to the

manufacturer's instruction. One μL of cDNA was used as the template. The cycling conditions were performed as follows: after an initial denaturation step of 2 min at 95 °C, 40 cycles were performed for 30s at 95 °C, 60 s at 60 °C, and 1 min at 72 °C. A final extension step for 10 min at 72 °C was used. PCR products were further separated on a 1% agarose gel, stained with GelRed and visualized using Syngen Gel Imaging (Bio-Rad Laboratories Inc, USA). The PCR product was cleaned using PCR Clean-Up Kit (Promega, Norway). Sequencing reactions were performed in using a BigDye Terminator version 3.1 kit (Applied Biosystems) according to the manufacturer's instructions with the same primers as for the real-time PCR assay. Sequencing was performed on an Applied Biosystems 3130xl genetic analyzer.

Bioinformatics analysis

The fastQ files obtained after paired-end sequencing was checked for quality using the Galaxy webserver (https://galaxy-uit.bioinfo.no). Bcl2fastq program supplied by Illumina was used to convert bcl files to fastQ files, which automatically trims the adapters and generates clean reads. The clean reads were aligned with the reference genome (MSSA476; GenBank accession no. NC_002953.3) using Bowtie 2 (Langmead and Salzberg, 2012). Mapping of the EV reads to the reference genome resulted in a Sequence Alignment Map file that was converted to a Binary Alignment Map (BAM) file. The BAM and its associated annotation files of the reference genome were loaded into Artemis where manual searches for sRNAs were performed. Visualization and manual inspection of reading coverage were conducted using Artemis version 1.0 (Rutherford et al., 2000). All the sRNAs are listed based on genomic coordinates provided from the bacterial small regulatory RNA repository BSRD (http://kwanlab.bio.cuhk.edu.hk/BSRD). The sRNAs identified from Artemis were run separately for Rfam search in Artemis to gather information about RNA families and RNA elements, including accession numbers. In addition, the Rockhopper tool (Tjaden, 2019) was used to identify transcripts and operons and to elucidate bacterial transcriptomes. Transcripts from Rockhopper were visualized (.wig files) in the Integrative Genomics Viewer (IGV) (Robinson et al., 2011).

RESULTS

RNA is associated with *S. aureus* derived EVs

The MSSA476 bacterial growth in BHI (normal condition) or trace metal-depleted BHI supplemented with a subinhibitory concentration of vancomycin mimicking infection (stress condition) were compared and found to be similar at 16 h (**Figure S1**). The viability of bacteria after 16 h in both media was evaluated by flow cytometry and colony-forming units (CFU) enumeration and showed similar viability which was above 99.6% (**Table 1, Figure S2**).

Then, EVs were isolated from *S. aureus* grown for 16 h under normal or stressed conditions. EVs were obtained from bacteria grown under both conditions. However, the number of particles, as well as protein concentration, was increased when bacteria were stressed (**Figure S3 A, B**). Unfortunately, the yield of sRNA obtained from EVs isolated from unstressed bacteria was too low for RNAseq. Therefore, we focused the study on EVs isolated from stressed bacteria. The morphology of EVs was evaluated by AFM and TEM. Aligned with other studies on MSSA476 (Gurung et al., 2011;Askarian et al., 2018), EVs were spherical in shape, though minor fusions were observed (**Figure 1A, B, Figure S4**). In addition, the size distribution of vesicles was measured using NTA which revealed that the sizes ranged from 20 nm to 200 nm, although the majority of vesicles are between 100-150 nm. The analysis also showed some particles with sizes above 200 nm (**Figure 1C**), which might be due to fusion of EVs.

Next, we wanted to evaluate whether RNA is associated with the EVs. The EVs were treated with RNase to remove external RNAs, and thereafter stained with RNA specific dye. EVs are known to contain lipids (Ghosal et al., 2015), and were therefore stained with a lipid-specific dye. The RNA and lipid-stained particles, which we assumed to be aggregated EVs, were then analyzed by confocal microscopy. As seen in (**Figure 2A, 2B and Figure S5)**, RNA and lipid stain co-localized in the majority of EVs. To confirm the sensitivity of the method, the fluorescence intensity **(Figure 2C**) was determined in co-stained and only lipid stained EVs indicated with dotted line in **Figure 2B**. Finally, we quantified co-localization of RNA and lipid particles by counting 8 random microscopic fields. We observed 73% co-localization of RNA and lipid stained particles, while approximately 21% percent of lipid stained particles were without any RNA stain. A minor population of about 6% RNA stained particles were not associated with lipid stained particles (**Figure 2D**). The later population might represent either non-specific aggregations of RNA dye, or a leakage of RNA from broken EVs during sample preparation, or the presence of small amounts of extra-vesicle RNA even after RNase treatment.

Having found that RNA was associated with RNase treated EVs, we isolated small RNA which was analyzed further by bioanalyser. A smear of RNA in size range 50-200 bp was seen (**Figure 3A**). The obtained RNA was treated using the rRNA depletion kit, which reduced the average concentration of RNA from 77 to 40 ng/µL. Further analysis of the rRNA-depleted samples using bioanalyser revealed appearance of four peaks (**Figure 3B, black arrows)** at 24-28 seconds, known to be typical peak for less than 200 bp sRNA. Of note, no strong peaks appeared for ribosomal RNA (16S and 23S), indicating efficiency of sRNA enrichment by the miRNA kit and subsequent depletion of rRNAs. Hence, our data demonstrated that sRNA are associated with *S. aureus* EVs.

tRNAs and sRNAs were enriched in *S. aureus* derived EVs

The transcriptome profiling of the sRNA content in EVs was performed using RNA-seq analysis. Paired-end sequencing resulted in approximately 458,000 reads of varying length (35-151 nucleotides), of which 87% were aligned to the reference genome of *S. aureus* strain MSSA476 (GenBank accession no. NC_002953.3) (**Table S2-** mapping statistics**)** and were well distributed over the reference genome (**Figure S6**).

The reads corresponded to sRNAs with size distribution from 20 to 500 nt (**Figure S7**). Nearly 3.5% consisted of tRNAs and 0.3% consisted of sRNAs and the rest were protein encoding RNAs or fragments of rRNA (**Figure S8**). A total of 62 RNAs, 276 5′ untranslated regions (5′ UTRs) and 276 3′ untranslated regions (3′ UTRs) were identified. Similarly, further aligning of the reads against *S. aureus* MSSA476 plasmid pSAS resulted in detection of five RNAs, seven 5' UTRs, and 11 3' UTRs (**Table S2** - Summary Rockhopper output). Next, operons in the *S. aureus* genome were defined as regions with continuous coverage of whole transcript reads by RNA-seq. This resulted in identification of 486 multi gene operons consisting of 2-18 genes (for a total of 1415 genes) (**Table S2** - operon Rockhopper output). Phage RNAs, e. g, transcripts encoding terminase subunits, tail proteins and portal protein, were detected among the protein encoding RNAs (**Table S2** - Rockhopper transcript output). Since our focus is on sRNAs, we chose to further describe only the tRNA and sRNA content.

Coverage of tRNA upstream of a regulatory region is shown in **Figure S9.** The read counts of tRNAs in EVs varied from 4-984, with cove scores from 36.67 to 101.60 (**Table S2-** tRNAs in EVs**)**. The most enriched tRNAs includes tRNA for Met, Asp, Leu, Tyr, Ser, Thr, Gly and Phe (**Table 2**).

The 67 sRNAs predicted by Rockhopper software were manually checked with Artemis and 49 sRNAs were validated using the Rfam database. The MSSA476-derived EVs carried several sRNAs with read counts varying from 1 to 80 (**Table S2** - small RNA in EVs). 6S RNA and

SsrA showed the highest read counts of 80 and 65 respectively (**Table 3**, read density of SsrA is shown in **Figure S10).**

Validation of *S. aureus* SrrA, RsaC and RNAIII RNA associated with EVs

Among the enriched sRNA were SsrA, RsaC, and RNAIII (**Table 3**). SsrA and RsaC RNAs are involved in antibiotic resistance through their modulation of RNA fate and protein activity (Lalaouna et al., 2014), while RNAIII, not only have regulatory function but also encode 26 amino acid long δ-toxin (Novick et al., 1993; Caldelari et al., 2013). To validate our results obtained from transcriptomic analyses, we performed qPCR on RNA obtained from EVs using primers targeting SsrA, RsaC and RNAIII. The results presented in boxplot (**Figure 4A**) are based on three biological repeats (**Table S3)** which confirmed presence of these three transcripts associated with EVs. The presence was finally confirmed by PCR of cDNA yielding DNA fragments of expected sizes (Figure 4B), and by Sanger sequencing which confirmed the identity of *ssrA, RsaC* and *RNAIII* (**Figure S11**).

DISCUSSION

S. aureus harbors a multitude of virulence factors that are tightly regulated during infection. Several studies have shown that exposure to sub-MIC antibiotic concentrations enhances *S. aureus* ability to adapt to physiological changes, survive and persist in human hosts (Kaplan et al., 2012;Howden et al., 2013). One of the mechanisms modulating virulence and pathogenicity of *S. aureus* is via the release of EVs (Gurung et al., 2011; Thay et al., 2013; Askarian et al., 2018; Schlatterer et al., 2018; Andreoni et al., 2019). Virulence factors such as hemolysin, loaded as vesicular cargo, are delivered to host cells via fusion of vesicles with the host cholesterol-rich membrane. In addition, *S. aureus* derived EVs also release lipoproteins, which play a significant role in modulating TLR2 activation and are involved in pathogenesis. In general, various pathophysiological functions ranging from cellular inflammation to host cell death could be mediated by *S. aureus* EVs (Hong et al., 2011; Kim et al., 2012).

The first report of RNA associated with EVs was published in 1989 in *Neisseria gonorrhoeae* (Dorward et al., 1989). Henceforth, many intensive studies have been conducted to characterize RNAs and their functions (Scanlan, 2014; Sjöström et al., 2015; Blenkiron et al., 2016; Koeppen et al., 2016; Choi et al., 2018; Malabirade et al., 2018). In Gram-negative organisms, EVs harbor not only virulence factors but also sRNAs that enhance the pathogenicity, which allow bacteria to persist longer in the host cells (Koeppen et al., 2016). To our knowledge, there have not been any studies to characterize sRNA associated with the vesicles of *S. aureus*. Hence, we aimed to perform high-throughput transcriptome sequencing to identify sRNAs associated with *S. aureus* EVs.

A crude EV pellet was used as the source material to enable isolation of sufficient small RNA for sequencing. The crude EV pellet was further concentrated using ultrafiltration columns with cutoff of 10kD, which remove lipoprotein aggregates (Ramirez et. al., 2018). However, TEM images show the presence of other materials in addition to EVs (**Figure 1**). The crude EVs were RNase treated to remove any RNA that is not associated with EVs, before sRNA was isolated for high-throughput RNA sequencing. Adequately replicated RNA quantification isolated from EVs and RNase treated EVs confirmed a reduction in RNA from RNase treatment (**Table S1**). Since staining of RNase treated EVs with RNA and lipid dyes showed co-localisation of RNA and lipid particles assumed to be EVs (**Figure 2**, **Figure S5**), and we could isolate sRNA from RNase-treated EV, we concluded that sRNA is associated with EVs. RNA seq data revealed that SsrA, RsaC and RNAIII were among the most enriched sRNA associated with the EVs. To validate our findings, we repeated isolation of sRNA in

triplicates from from RNase treated EVs and confirmed presence SsrA, RsaC and RNAIII by qPCR, conventional PCR and Sanger sequencing of the PCR products (**Figure 4, Table S1**).

We also identified phage-like sequences in EVs RNA-seq (**Table S2**). The presence of these might be due to vancomycin-induced activation of one or two of the prophages harbored by *S. aureus* MSSA476 (Holden et al., 2004), which then pelleted with the vesicles. This agrees with others, who also obtained phage or phage tail particles in EVs when bacteria were exposed to antibiotics (Kharina et al., 2015;Devos et al., 2017;Andreoni et al., 2019). One of the limitations of using the crude pellet as the source of EVs for isolation of RNA is that it not only contains EVs, but also other nanoscale contaminants (e.g. the filaments and bacteriophage). We assumed that the source of RNA, in our study, is predominantly RNase treated EVs, but it is conceivable that some sequences are from other nanoscale contaminants pelleted along with the EVs, in a form that is protected from RNase, and at a concentration or of a size that is not visible by the fluorescence microscopy.

In bacteria, 16S and 23S rRNA are the most abundant RNAs that accounts for greater than 90% of the total RNA biotype (Petrova et al., 2017). The abundance of rRNA reduces the sequencing depth for other RNA classes, thus an rRNA depletion strategy was implemented to ensure sufficient coverage of the transcriptome from bacterial RNA-seq data. Although in our study, fragments of mRNA were most abundant, we focused on characterizing sRNA, given they play important roles in EV biogenesis and virulence (Diallo and Provost, 2020; Lécrivain and Beckmann, 2020).

The observed EV sizes agreed with other studies from *S. aureus* (Gurung et al., 2011; Askarian et al., 2018; Wang et al., 2018; He et al., 2019). Antibiotics and other stressful conditions are considered as trigger factors for EV formation (Maredia et al., 2012; Prados-Rosales et al., 2014; Andreoni et al., 2019). Moreover, vancomycin was found to increase EV production in another Gram-positive bacterium, *E. faecium* (Kim et al., 2019). In agreement with this, we observed a higher yield of EVs when the bacteria were grown in iron limited media supplemented with vancomycin compared to typical bacteriologic media (**Figure S3**). Since, we inoculated iron-chelated BHI media with an overnight grown inoculum in 1:100 dilutions, there is the possibility of transfer of trace amounts of iron from overnight inoculum to iron-chelated media. However, it has been shown that *S. aureus* utilizes a large proportion of iron within 6 h of aerobic growth in tryptic soy broth media (Ledala et. al., 2014) and an overnight culture would have further depleted in iron contents. Iron utilization by *S. aureus* in BHI might be similar. In addition, the traces of iron transferred during inoculum would have been utilized by growing *S. aureus*, leaving media chelated for iron after a 16 hour incubation (Ledala et. al., 2010 and Ledala et. al, 2014). It will, however, be interesting in the future to explore other culture media, such as RPMI 1640, which may better reflect infection conditions (Dauros-Singorenko et al., 2017).

Although there are multiple studies in Gram-positive bacteria showing altered sRNA expression due to antibiotic treatment (Felden and Cattoir, 2018;Gao et al., 2020), few studies have evaluated RNA content associated with EVs upon antibiotics exposure. Exposure to antimicrobials such as ciprofloxacin, tetracycline and melittin treatment of *Acholeplasma laidlawii* resulted in very variable numbers of sequence reads of predominantly 14-60 nt RNAs associated with EVs. In addition, tRNA fragments (mainly tRNA-Leu, tRNA-Arg, tRNA-Asn, and tRNA-Met) were predominant which is in line with our study (Chernov et al., 2018). There are also few reports in eukaryotic exosomes and protozoal EVs confirming that exosome RNA levels altered by cellular stress (Bayer-Santos et al., 2014).

In general, RNAs are unstable and prone to degradation by RNase present in the extracellular milieu. However, recent reports indicate that RNAs encapsulated in EVs are protected from degradation by the exogenous RNase (Weber et al., 2010;Dauros-Singorenko et al., 2018), which support our results where we could isolate RNA from the RNase-treated EVs. Our

RNase treatment of EVs would also facilitated degradation of eventual contaminating RNA that might passively have been released from the 0.4% dead cells in the culture used for vesicle isolation. Nevertheless, some of the RNA-protein complex sticking to the EVs might still have been protected from RNase treatment (Ramirez et al., 2018).

Transcriptome analysis of the EVs revealed the presence of tRNAs and sRNAs (**Table 2, 3 and Table S2**). Based on the read counts, the tRNA fragments were found to be most abundant. Abundant reads of tRNA fragments have previously been found in EVs released by bacteria (Ghosal et al., 2015;Koeppen et al., 2016), fungi (*Paracoccidiodes brasiliensis*, *Histoplasma capsulatum*) (Da Silva et al., 2015; Alves et al., 2019) and protists (*Trypanosoma cruzi*, *Leishmania* species) (Garcia-Silva et al., 2014; Lambertz et al., 2015). Interestingly, EVs of *Pseudomonas aeruginosa* also contained tRNA-Met fragments which entered the host cell and inhibited IL-8 secretion, which are considered as a chemoattractant of neutrophils (Koeppen et al., 2016).

There has been discussed whether RNAs associated with EVs are "intact", fragmented or specifically processed products. EVs have been found to be associated with various fragments derived from mRNAs, rRNA and tRNA (Mateescu et al., 2017), which is in agreement with our study. Due to the small size of vesicles (20-200 nm), we might speculate that inside EVs, mostly smaller fragmented RNAs should be enriched. However, we cannot exclude the possibility of having full length RNA, given Buck and colleagues reported full length YRNAs exclusively inside Nematode-derived EVs (Buck et al., 2014). In our case, we see fragment lengths of 35-150 nt.

It has been reported that sRNA present in vibrio and other Gram-negative bacteria play a role in vesicle biogenesis (Song et al., 2008;Choi et al., 2017a). MicA from *E. coli* induce EV biogenesis. Likewise, Song and collaborators also identified VrrA, a homolog of *E. coli* MicA in *Vibrio cholera* that controls EV formation and contributes to bacterial fitness in certain stressful environments (Song et al., 2008). Besides sRNAs, Sle1, an autolysin, has been shown to facilitate vesicle biogenesis (Wang et al., 2018).

The presence of EV associated sRNA (SsrA, RsaC, and RNAIII) was confirmed by RNA-seq, qPCR, RT-PCR and sequencing of obtained replicons. Among these, SsrA is involved in defective mRNAs decay, rescue of stalled ribosomes, support of phage growth, and modulation of the activity of DNA binding proteins (Karzai et al., 2000; Janssen and Hayes, 2012). Earlier reports have shown an increase of SsrA RNA in *Streptococcus pyogenes* and *Helicobacter pylori* in the presence of antibiotics (Steiner and Malke, 2001; Thibonnier et al., 2008). In our study, the high coverage of SsrA RNA associated with *S. aureus* EVs might be due to the use of vancomycin stress prior to vesicle isolation. 6S RNA plays an important role in cell survival and persistence during the stationary phase (Wassarman and Storz, 2000; Trotochaud and Wassarman, 2004) and was also found associated with the EVs. Interestingly, RNAIII (**Table 3**), which has major roles in virulence and pathogenicity (Boisset et al., 2007; Toledo-Arana et al., 2007), was also associated with EVs of *S. aureus*. The validation of RNAIII associated with EVs of *S. aureus* opens further study on the possibility of sRNA-mediated interspecies communication, as RNAIII has already been proved to be involved in the regulation of quorum sensing communication systems to coordinate the expression of virulence factors (Diallo and Provost, 2020; Lécrivain and Beckmann, 2020). Indeed, EVs could be used as communication vehicles only if they could transfer associated RNA into host cells and have a functional effect. Another possibility is that bacteria utilize EVs to eliminate unwanted RNAs, including sRNA and tRNA fragments (Groot and Lee, 2020).

Importantly, the role of EVs influence *S. aureus* virulence over the course of systemic infection (Askarian et al., 2018). Recently, RNAs (circulatory/and or EV-associated) were considered as virulence factors due to their role in the infection process via multifaceted signaling pathways. The signaling pathways involved depend upon the delivery of bacterial

RNA into the host cells. It has been shown that bacterial RNA can be delivered to human cytosol (Vanaja et al., 2014) and the phagosomal compartment (Cervantes et al., 2013) and EV associated RNA has been localized in the human cell nucleus of human bladder carcinoma cells (Blenkiron et al., 2016). The sRNAs associated with EVs found in this study has been shown to be involved in quorum sensing (Novick and Geisinger, 2008), oxidative stress (Lalaouna et al., 2019), antibiotic resistance and metabolism (Lalaouna et al., 2014). All these processes are important for virulence and modulation of bacterial pathogenicity. EVs have some striking similarities with exosomes that are secreted from most mammalian cell types. Exosomes are involved in transport of mRNAs and miRNAs from donor to recipient cells to modulate gene expression (Zhang et al., 2015;Lu et al., 2019). They have similar size (around 50-200 nm in diameter) and carry payloads of proteins, lipids, and genetic materials such as the bacterial membrane vesicles. Both types can deliver functional molecules to distant extracellular compartments and tissues.

Recently, it was described that eukaryotic sRNA profiles of serum exosomes derived from individuals with tuberculosis can facilitate the development of potential molecular targets for detection/diagnosis of latent and active tuberculosis (Lvu et al., 2019). In addition to eukaryotic sRNA in exosomes, circulating sRNA (ASdes) from *Mycobacterium tuberculosis* was found in patients suffering from active tuberculosis, implicating their role as diagnostic biomarkers ((Fu et al., 2018). This makes us hypothesize that some of the sRNA we have validated in EVs (RNAIII, SsrA) have a potential to be used as biomarkers for bloodstream infections (Bordeau et al., 2016), joint infections (osteomyelitis) (Deng et al., 2020), tissues infections (e.g. chronic biofilm infections) and/or bacterial persistence (Romilly et al., 2014; Schoenfelder et al., 2019). Identifying sRNAs as biomarkers should not be limited to pathogenic strains but also to nasal and other commensal strains.

A previous study compared RNA contents of group A streptococcal cells versus their EVs, and found that some RNA species were differentially abundant (Resch et al., 2016). For future studies, it would be interesting to do similar studies in *S. aureus*, and also to compare whether media or antibiotics influence the EV cargo. Further investigation is also needed to address whether RNAs found inside the vesicles are entrapped during vesicle biogenesis or if there are some sorting of RNA into the vesicles

In conclusion, to our knowledge, this is the first study describing sRNA associated with *S. aureus* extracellular vesicles. Various tRNAs and sRNAs associated fragments have been identified with several biological or regulatory functions were associated with the EVs. This study opens further questions concerning sorting mechanisms by which RNA can be packed inside EVs and their roles in host-microbe as well as microbe-microbe interactions. Targeting those sRNA may open avenues towards a novel anti-virulence strategy to treat intractable bacterial infections.

DATA AVAILABILITY

This article contains previously unpublished data. The raw sequence libraries are deposited at NCBI under the BioProject ID PRJNA632444.

CONFLICT OF INTEREST

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.

CONTRIBUTION TO THE FIELD

Staphylococcus aureus is an opportunistic pathogen that can cause infections ranging from minor superficial skin infections to severe life-threatening infections such as endocarditis, bacteremia and sepsis. *S. aureus* releases extracellular vesicles and several groups have characterized their protein cargo. However, whether *S. aureus* extracellular vesicles can carry RNAs, including regulatory RNA, remained elusive. In this study, we imaged RNA in RNase-treated extracellular vesicles, suggesting association of RNA with vesicles that is protected against extra-vesicular environment. RNA was isolated and transcriptomic and qPCR analysis revealed the presence of potential gene regulatory small RNAs and high levels of transfer RNAs.

FUNDING

This work was supported by grants from joint "Miljøstøtte" financed by Strategisk-HN05–14 (Helse Nord RFH) and Faculty of Health Sciences A20389 (2014–2017), and by travel grants from the National Graduate School in Infection Biology and Antimicrobials (grant number 249062). The publication charges for this article have been funded by a grant from the publication fund of UiT-The Arctic University of Norway. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

ABBREVIATIONS

ATCC: American type culture collection; AFM: Atomic Force Microscopy; KEGG: Kyoto Encyclopedia of Genes and Genomes; IGV: Integrative Genomics Viewer; EVs: Extracellular vesicles; NTA: Nanoparticle Tracking Analysis; EVs: Extracellular Vesicles; rRNA: Ribosomal RNA; RNA-Seq: RNA Sequencing; sRNA: Small RNA; TEM: Transmission Electron Microscopy; tRNA: transfer RNA, UTRs: Untranslated regions.

AUTHOR CONTRIBUTIONS

BJ, MJ, KH designed the experiments and prepared the manuscript. BJ performed the majority of the lab experiment. AN assisted in confocal microscopy and image analysis. KH assisted in bioinformatics analysis, while BS did flow cytometry and assisted in qPCR experiments. FA and BS contributed in writing of the results-section. BJ, BS, FA, AN, SW, MJ and KH gave intellectual input. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

We are grateful for excellent technical assistance from Ahmed Mekhlif, Kjersti Julin and Hagar Taman. We thank Christopher G. Fenton, Endre Anderssen and Jessin Janice for technical support in bioinformatics work. We are grateful to Kyaw Min Aung and Si Lhyam Myint at Umeå University for technical support with atomic force microscopy and Augusta Hlin Aspar with electron microscopy. We acknowledge the Biochemical Imaging Center (BICU) at Umeå University and the National Microscopy Infrastructure (NMI) for providing assistance in confocal microscopy. We are thankful to Deanna Lynn Wolfson, Department of Physics and Technology at UiT the Arctic University of Norway, for co-localization analysis and image processing of confocal data. We are grateful to Line Wilsgård for NTA particle analysis.

REFERENCES

1. Ahmad, I., Karah, N., Nadeem, A., Wai, S.N., and Uhlin, B.E. (2019). Analysis of colony phase variation switch in *Acinetobacter baumannii* clinical isolates. PLoS One. 14(1). doi: 10.1371/journal.pone.0210082
2. Alves, L.R., Da Silva, R.P., Sanchez, D.A., Zamith-Miranda, D., Rodrigues, M.L., Goldenberg, S., Puccia, R., and Nosanchuk, J.D. (2019). Extracellular vesicle-mediated RNA release in *Histoplasma capsulatum*. mSphere. 4(2). doi: 10.1128/mSphere.00176-19
3. Andreoni, F., Toyofuku, M., Menzi, C., Kalawong, R., Shambat, S.M., François, P., Zinkernagel, A.S., and Eberl, L. (2019). Antibiotics stimulate formation of vesicles in *Staphylococcus aureus* in both phage-dependent and-independent fashions and via different routes. Antimicrob Agents Chemother. 63(2).doi: 10.1128/AAC.01439-18
4. Askarian, F., Lapek Jr, J.D., Dongre, M., Tsai, C.-M., Kumaraswamy, M., Kousha, A., Valderrama, J.A., Ludviksen, J.A., Cavanagh, J.P., and Uchiyama, S. (2018). *Staphylococcus aureus* Membrane-Derived Vesicles Promote Bacterial Virulence and Confer Protective Immunity in Murine Infection Models. Front Microbiol. 20;9:262. doi: 10.3389/fmicb.2018.00262
5. Bager, R.J., Persson, G., Nesta, B., Soriani, M., Serino, L., Jeppsson, M., Nielsen, T.K., and Bojesen, A.M. (2013). Outer membrane vesicles reflect environmental cues in *Gallibacterium anatis*. Vet Microbiol. 167**,** 565-572. doi: 10.1016/j.vetmic.2013.09.005.
6. Bayer-Santos, E., Lima, F.M., Ruiz, J.C., Almeida, I.C., and Da Silveira, J.F. (2014). Characterization of the small RNA content of *Trypanosoma cruzi* extracellular vesicles. Mol Biochem Parasitol. doi: 10.1016/j.molbiopara.2014.02.004..
7. Bitto, N.J., Chapman, R., Pidot, S., Costin, A., Lo, C., Choi, J., D'cruze, T., Reynolds, E.C., Dashper, S.G., and Turnbull, L. (2017). Bacterial membrane vesicles transport their DNA cargo into host cells. Sci Rep. 7(1):7072. doi: 10.1038/s41598-017-07288-4
8. Blenkiron, C., Simonov, D., Muthukaruppan, A., Tsai, P., Dauros, P., Green, S., Hong, J., Print, C.G., Swift, S., and Phillips, A.R. (2016). Uropathogenic *Escherichia coli* releases extracellular vesicles that are associated with RNA. PLoS One. 11(8). doi: 10.1371/journal.pone.0160440
9. Blount, K.F., Wang, J.X., Lim, J., Sudarsan, N., and Breaker, R.R. (2007). Antibacterial lysine analogs that target lysine riboswitches. Nat Chem Biol. 3(1):44-9
10. Boisset, S., Geissmann, T., Huntzinger, E., Fechter, P., Bendridi, N., Possedko, M., Chevalier, C., Helfer, A.C., Benito, Y., and Jacquier, A. (2007). *Staphylococcus aureus* RNAIII coordinately represses the synthesis of virulence factors and the transcription regulator Rot by an antisense mechanism. Genes Dev. 21(11): 1353–1366. doi: 10.1101/gad.423507
11. Bordeau, V., Cady, A., Revest, M., Rostan, O., Sassi, M., Tattevin, P., Donnio, P.-Y., and Felden, B. (2016). *Staphylococcus aureus* regulatory RNAs as potential biomarkers for bloodstream infections. Emerg Infect Dis.22(9): 1570–1578. doi: 10.3201/eid2209.151801
12. Brameyer, S., Plener, L., Müller, A., Klingl, A., Wanner, G., and Jung, K. (2018). Outer membrane vesicles facilitate trafficking of the hydrophobic signaling molecule CAI-1 between *Vibrio harveyi* cells. J Bacteriol. 2018. 200(15) doi: 10.1128/JB.00740-17.
13. Buck, A.H., Coakley, G., Simbari, F., Mcsorley, H.J., Quintana, J.F., Le Bihan, T., Kumar, S., Abreu-Goodger, C., Lear, M., and Harcus, Y. (2014). Exosomes secreted by nematode parasites transfer small RNAs to mammalian cells and modulate innate immunity. Nat Commun. 2014. 5:5488.doi: 10.1038/ncomms6488.
14. Caldelari, I., Chao, Y., Romby, P., and Vogel, J. (2013). RNA-mediated regulation in pathogenic bacteria. Cold Spring Harb Perspect Med. 3(9). doi: 10.1101/cshperspect.a010298
15. Cafiso, V., Bertuccio, T., Spina, D., Purrello, S., Campanile, F., Di Pietro, C., Purrello, M., and Stefani, S. (2012). Modulating activity of vancomycin and daptomycin on the

expression of autolysis cell-wall turnover and membrane charge genes in hVISA and VISA strains. PLoS One. 7(1):e29573. doi: 10.1371/journal.pone.0029573
16. Cavanagh, J.P., Pain, M., Askarian, F., Bruun, J.-A., Urbarova, I., Wai, S.N., Schmidt, F., and Johannessen, M. (2018). Comparative exoproteome profiling of an invasive and a commensal *Staphylococcus haemolyticus* isolate. J Proteomics. 197:106-114. doi: 10.1016/j.jprot.2018.11.013
17. Cervantes, J.L., La Vake, C.J., Weinerman, B., Luu, S., O'Connell, C., Verardi, P.H., and Salazar, J.C. (2013). Human TLR8 is activated upon recognition of Borrelia burgdorferi RNA in the phagosome of human monocytes. *Journal of leukocyte biology* 94**,** 1231-1241.
18. Chernov, V.M., Chernova, O.A., Mouzykantov, A.A., Medvedeva, E.S., Baranova, N.B., Malygina, T.Y., Aminov, R.I., and Trushin, M.V. (2018). Antimicrobial resistance in mollicutes: known and newly emerging mechanisms. FEMS Microbiol Lett.365(18).doi: 10.1093/femsle/fny185.
19. Choi, H.-I., Kim, M., Jeon, J., Han, J.K., and Kim, K.-S. (2017a). Overexpression of MicA induces production of OmpC-enriched outer membrane vesicles that protect against Salmonella challenge. *Biochemical and biophysical research communications* 490**,** 991-996.
20. Choi, J.-W., Kim, S.-C., Hong, S.-H., and Lee, H.-J. (2017b). Secretable small RNAs via outer membrane vesicles in periodontal pathogens. J Dent Res. 96(4):458-466. doi: 10.1177/0022034516685071
21. Choi, J.-W., Kwon, T.-Y., Hong, S.-H., and Lee, H.-J. (2018). Isolation and Characterization of a microRNA-size secretable Small RNA in *Streptococcus sanguinis*. Cell Biochem Biophys. 76(1-2):293-301. doi: 10.1007/s12013-016-0770-5
22. Choi, S.Y., Lim, S., Cho, G., Kwon, J., Mun, W., Im, H., and Mitchell, R.J. (2020). *Chromobacterium violaceum* delivers violacein, a hydrophobic antibiotic, to other microbes in membrane vesicles. Environ Microbiol. 22(2):705-713.doi: 10.1111/1462-2920.14888
23. Coelho, C., Brown, L., Maryam, M., Vij, R., Smith, D.F., Burnet, M.C., Kyle, J.E., Heyman, H.M., Ramirez, J., Prados-Rosales, R. and Lauvau, G., 2019. *Listeria monocytogenes* virulence factors, including listeriolysin O, are secreted in biologically active extracellular vesicles. J Biol Chem. 294(4):1202-1217. doi: 10.1074/jbc.RA118.006472
24. Da Silva, R.P., Puccia, R., Rodrigues, M.L., Oliveira, D.L., Joffe, L.S., César, G.V., Nimrichter, L., Goldenberg, S., and Alves, L.R. (2015). Extracellular vesicle-mediated export of fungal RNA. Scientific reports 5, 7763
25. Dauros-Singorenko, P., Blenkiron, C., Phillips, A., and Swift, S. (2018). The functional RNA cargo of bacterial membrane vesicles. FEMS Microbiol Lett. 365(5). doi: 10.1093/femsle/fny02
26. Dauros-Singorenko, P., Chang, V., Whitcombe, A., Simonov D., Hong, J., Phillips, A., Swift, S., and Blenkiron, C. (2017). Isolation of membrane vesicles from prokaryotes: a technical and biological comparison reveals heterogeneity. J Extracell Vesicles. 6(1):1324731. doi: 10.1080/20013078.2017.1324731
27. Devos, S., Van Oudenhove, L., Stremersch, S., Van Putte, W., De Rycke, R., Van Driessche, G., Vitse, J., Raemdonck, K., and Devreese, B. (2015). The effect of imipenem and diffusible signaling factors on the secretion of outer membrane vesicles and associated Ax21 proteins in *Stenotrophomonas maltophilia*. Front Microbiol6:298. doi: 10.3389/fmicb.2015.00298
28. Devos, S., Van Putte, W., Vitse, J., Van Driessche, G., Stremersch, S., Van Den Broek, W., Raemdonck, K., Braeckmans, K., Stahlberg, H., and Kudryashev, M. Savvides, S.N., Devreese, B. (2017). Membrane vesicle secretion and prophage induction in multidrug-resistant *Stenotrophomonas maltophilia* in response to ciprofloxacin stress. Environ Microbiol. 19(10). doi: 10.1111/1462-2920.13793

29. Deng, S., Wang, Y., Liu, S., Chen, T., Hu, Y., Zhang, G., Zhang, X., and Yu, B. (2020). Extracellular Vesicles: A Potential Biomarker for Quick Identification of Infectious Osteomyelitis. Front Cell Infect Microbiol. 10:323. doi: 10.3389/fcimb.2020.00323
30. Diallo, I., and Provost, P. (2020). RNA-Sequencing Analyses of Small Bacterial RNAs and their Emergence as Virulence Factors in Host-Pathogen Interactions. Int J Mol Sci. 21(5). doi: 10.3390/ijms21051627
31. Dongre, M., Uhlin, B.E., and Wai, S.N. (2011). Bacterial nanotubes for intimate sharing. Front. Microbiol. 11;2:108. doi: 10.3389/fmicb.2011.00108
32. Dorward, D.W., and Garon, C. (1989). DNA-binding proteins in cells and membrane blebs of Neisseria gonorrhoeae. J Bacteriol. 171(8): 4196–4201. doi: 10.1128/jb.171.8.4196-4201.1989
33. Dubey, G.P., and Ben-Yehuda, S. (2011). Intercellular nanotubes mediate bacterial communication. Cell.144(4). doi: 10.1016/j.cell.2011.01.015
34. Elhenawy, W., Debelyy, M.O., and Feldman, M.F. (2014). Preferential packing of acidic glycosidases and proteases into Bacteroides outer membrane vesicles. mBio.5(2). doi: 10.1128/mBio.00909-14
35. Felden, B., and Cattoir, V. (2018). Bacterial adaptation to antibiotics through regulatory RNAs. Antimicrob Agents Chemother. 62(5).doi: 10.1128/AAC.02503-17
36. Foster, T.J., Geoghegan, J.A., Ganesh, V.K., and Höök, M. (2014). Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*. Nat Rev Microbiol. 12(1). doi: 10.1038/nrmicro3161
37. Frantz, R., Teubner, L., Schultze, T., La Pietra, L., Müller, C., Gwozdzinski, K., Pillich, H., Hain, T., Weber-Gerlach, M., and Panagiotidis, G.-D. (2019). The secRNome of *Listeria monocytogenes* harbors small noncoding RNAs that are potent inducers of beta interferon. *MBio* 10**,** e01223-01219.
38. Fu, Y., Li, W., Wu, Z., Tao, Y., Wang, X., Wei, J., Jiang, P., Wu, J., Zhang, Z., Zhang, W.,Zhao, J., and Zhang, F. (2018). Detection of mycobacterial small RNA in the bacterial culture supernatant and plasma of patients with active tuberculosis. Biochem Biophys Res Commun. 503(2):490-494. doi: 10.1016/j.bbrc.2018.04.165
39. Gao, W., Guérillot, R., Lin, Y.H., Tree, J., Beaume, M., François, P., Monk, I.R., Seemann, T., Schrenzel, J., and Howden, B.P. (2020). Comparative Transcriptomic and Functional Assessments of Linezolid-Responsive Small RNA Genes in *Staphylococcus aureus*. mSystems. 5(1):e00665-19.doi: 10.1128/mSystems.00665-19.
40. Garcia-Silva, M.R., Das Neves, R.F.C., Cabrera-Cabrera, F., Sanguinetti, J., Medeiros, L.C., Robello, C., Naya, H., Fernandez-Calero, T., Souto-Padron, T., De Souza, W., and Cayota, A. (2014). Extracellular vesicles shed by *Trypanosoma cruzi* are linked to small RNA pathways, life cycle regulation, and susceptibility to infection of mammalian cells. Parasitol Res. 113(1). doi: 10.1007/s00436-013-3655-1
41. Ghosal, A., Upadhyaya, B.B., Fritz, J.V., Heintz-Buschart, A., Desai, M.S., Yusuf, D., Huang, D., Baumuratov, A., Wang, K., Galas, D., and Wilmes, P. (2015). The extracellular RNA complement of *Escherichia coli*. Microbiologyopen. 4(2):252-266. doi: 10.1002/mbo3.235
42. Gill, S., Catchpole, R., and Forterre, P. (2018). Extracellular membrane vesicles (EVs) in the three domains of life and beyond. FEMS Microbiol Rev. 2019 43(3):273-303. doi: 10.1093/femsre/fuy042
43. Groot, M., and Lee, H. (2020). Sorting Mechanisms for MicroRNAs into Extracellular Vesicles and Their Associated Diseases. *Cells* 9(4): 1044. doi: 10.3390/cells9041044
44. Guillet, J., Hallier, M., and Felden, B. (2013). Emerging functions for the *Staphylococcus aureus* RNome. PLoS Pathog. 9(12). doi: 10.1371/journal.ppat.1003767

45. Gurung, M., Moon, D.C., Choi, C.W., Lee, J.H., Bae, Y.C., Kim, J., Lee, Y.C., Seol, S.Y., Cho, D.T., Kim, S.I., and Lee, J.C. (2011). *Staphylococcus aureus* produces membrane-derived vesicles that induce host cell death. PLoS One. 6(11) doi: 10.1371/journal.pone.0027958.
46. Hagemann, S., Stöger, L., Kappelmann, M., Hassl, I., Ellinger, A., and Velimirov, B. (2014). DNA-bearing membrane vesicles produced by *Ahrensia kielensis* and *Pseudoalteromonas marina*. J Basic Microbiol. 54(10):1062-72.doi: 10.1002/jobm.201300376.
47. He, X., Li, S., Yin, Y., Xu, J., Gong, W., Li, G., Yin, Y., He, X., Guo, T., Huang, Y., Lu, F., and Cao, J. (2019). Membrane vesicles are the dominant structural components of ceftazidime-induced biofilm formation in an oxacillin-sensitive MRSA. Front Microbiol. 10: 571. doi: 10.3389/fmicb.2019.00571
48. He, X., Yuan, F., Lu, F., Yin, Y., & Cao, J. (2017). Vancomycin-induced biofilm formation by methicillin-resistant *Staphylococcus aureus* is associated with the secretion of membrane vesicles. Microbial pathogenesis, 110, 225-231
49. Hermansen, G.M., Sazinas, P., Kofod, D., Millard, A., Andersen, P.S., and Jelsbak, L. (2018). Transcriptomic profiling of interacting nasal staphylococci species reveals global changes in gene and non-coding RNA expression. FEMS Microbiol Lett. 365(5). doi: 10.1093/femsle/fny004
50. Holden, M.T., Feil, E.J., Lindsay, J.A., Peacock, S.J., Day, N.P., Enright, M.C., Foster, T.J., Moore, C.E., Hurst, L., Atkin, R., and Barron, A. (2004). Complete genomes of two clinical *Staphylococcus aureus* strains: evidence for the rapid evolution of virulence and drug resistance. Proc Natl Acad Sci U S A.101(26):9786-91
51. Hong, S.W., Kim, M.R., Lee, E.Y., Kim, J., Kim, Y.S., Jeon, S., Yang, J.M., Lee, B.J., Pyun, B.Y., and Gho, Y. (2011). Extracellular vesicles derived from *Staphylococcus aureus* induce atopic dermatitis-like skin inflammation. *Allergy* 66**,** 351-359.
52. Howden, B.P., Beaume, M., Harrison, P.F., Hernandez, D., Schrenzel, J., Seemann, T., Francois, P., and Stinear, T.P. (2013). Analysis of the small RNA transcriptional response in multidrug resistant *Staphylococcus aureus* after antimicrobial exposure. Antimicrob Agents Chemother. 57(8):3864-74. doi: 10.1128/AAC.00263-13
53. Hsu, C. Y., Lin, M. H., Chen, C. C., Chien, S. C., Cheng, Y. H., Su, I. N., & Shu, J. C. (2011). Vancomycin promotes the bacterial autolysis, release of extracellular DNA, and biofilm formation in vancomycin-non-susceptible *Staphylococcus aureus*. FEMS Immunol. Med. Microbiol. 63(2), 236-247. doi: 10.1111/j.1574-695X.2011.00846.x
54. Jamaly, S., Ramberg, C., Olsen, R., Latysheva, N., Webster, P., Sovershaev, T., Brækkan, S.K., and Hansen, J.-B. (2018). Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using Nanoparticle Tracking Analysis. Sci Rep. 8(1):17216. doi: 10.1038/s41598-018-35401-8
55. Janssen, B.D., and Hayes, C.S. (2012). The tmRNA ribosome-rescue system. Adv Protein Chem Struct Biol. 86: 151–191. doi: 10.1016/B978-0-12-386497-0.00005-0
56. Kaplan, J.B., Izano, E.A., Gopal, P., Karwacki, M.T., Kim, S., Bose, J.L., Bayles, K.W., and Horswill, A.R. (2012). Low levels of β-lactam antibiotics induce extracellular DNA release and biofilm formation in *Staphylococcus aureus*. mBio.3(4). doi: 10.1128/mBio.00198-12
57. Karzai, A.W., Roche, E.D., and Sauer, R.T. (2000). The SsrA–SmpB system for protein tagging, directed degradation and ribosome rescue. Nat Struct Biol.7(6):449-55
58. Kharina, A., Podolich, O., Faidiuk, I., Zaika, S., Haidak, A., Kukharenko, O., Zaets, I., Tovkach, F., Reva, O., Kremenskoy, M., and Kozyrovska, N. (2015). Temperate bacteriophages collected by outer membrane vesicles in *Komagataeibacter intermedius*. J Basic Microbiol.55(4):509-13. doi:10.1002/jobm.201400711

59. Kim, M.H., Kim, S.Y., Son, J.H., Kim, S.I., Lee, H., Kim, S., Shin, M., and Lee, J.C. (2019). Production of membrane vesicles by *Enterococcus faecium* cultured with or without subinhibitory concentrations of antibiotics and their pathological effects on epithelial cells. Front Cell Infect Microbiol. 9:295. doi:10.3389/fcimb.2019.0029
60. Kim, M.R., Hong, S.W., Choi, E.B., Lee, W.H., Kim, Y.S., Jeon, S., Jang, M., Gho, Y., and Kim, Y.K. (2012). *Staphylococcus aureus*-derived extracellular vesicles induce neutrophilic pulmonary inflammation via both T h1 and T h17 cell responses. *Allergy* 67**,** 1271-1281.
61. Koeppen, K., Hampton, T.H., Jarek, M., Scharfe, M., Gerber, S.A., Mielcarz, D.W., Demers, E.G., Dolben, E.L., Hammond, J.H., Hogan, D.A., and Stanton, B. A. (2016). A novel mechanism of host-pathogen interaction through sRNA in bacterial outer membrane vesicles. PLoS Pathog. 12(6). doi:10.1371/journal.ppat.1005672
62. Lagos-Quintana, M., Rauhut, R., Lendeckel, W., and Tuschl, T. (2001). Identification of novel genes coding for small expressed RNAs. Science. 294(5543):853-8
63. Lalaouna, D., Baude, J., Wu, Z., Tomasini, A., Chicher, J., Marzi, S., Vandenesch, F., Romby, P., Caldelari, I., and Moreau, K. (2019). RsaC sRNA modulates the oxidative stress response of *Staphylococcus aureus* during manganese starvation. Nucleic Acids Res. 47(18):9871-9887. doi: 10.1093/nar/gkz728
64. Lalaouna, D., Eyraud, A., Chabelskaya, S., Felden, B., and Masse, E. (2014). Regulatory RNAs involved in bacterial antibiotic resistance. PLoS Pathog. 210(8). doi: 10.1371/journal.ppat.1004299
65. Lambertz, U., Ovando, M.E.O., Vasconcelos, E.J., Unrau, P.J., Myler, P.J., and Reiner, N.E. (2015). Small RNAs derived from tRNAs and rRNAs are highly enriched in exosomes from both old and new world Leishmania providing evidence for conserved exosomal RNA Packaging. BMC Genomics. 16:151. doi: 10.1186/s12864-015-1260-7.
66. Langlete, P., Krabberød, A.K., and Winther-Larsen, H.C. (2019). Vesicles from *Vibrio cholerae* contain AT-rich DNA and shorter mRNAs that do not correlate with their protein products. Front. Microbiol. 10:2708 . doi: 10.3389/fmicb.2019.02708
67. Langmead, B., and Salzberg, S.L. (2012). Fast gapped-read alignment with Bowtie 2. Nat Methods.9(4):357-9. doi: 10.1038/nmeth.1923
68. Lécrivain, A.L., and Beckmann, B.M. (2020). Bacterial RNA in extracellular vesicles: A new regulator of host-pathogen interactions? Biochim Biophys Acta Gene Regul Mech.1863(7):194519. doi: 10.1016/j.bbagrm.2020.194519
69. Ledala, N., Zhang, B., Seravalli, J., Powers, R., and Somerville, G.A. (2014). Influence of iron and aeration on *Staphylococcus aureus* growth, metabolism, and transcription. *J Bacteriol.* 196(12): 2178–2189. doi: 10.1128/JB.01475-14
70. Ledala, N., Sengupta, M., Muthaiyan, A., Wilkinson, B.J., and Jayaswal, R.K. (2010). Transcriptomic response of *Listeria monocytogenes* to iron limitation and Fur mutation. *Appl Environ Microbiol. 76(2):406-16. doi: 10.1128/AEM.01389-09*
71. Li, S., Hwang, X.Y., Stav, S., and Breaker, R.R. (2016). The yjdF riboswitch candidate regulates gene expression by binding diverse azaaromatic compounds. RNA. 22(4):530-41. doi: 10.1261/rna.054890.115
72. Li, W., Ying, X., Lu, Q., and Chen, L. (2012). Predicting sRNAs and their targets in bacteria. Genomics Proteomics Bioinformatics. 10(5): 276-284. doi: 10.1016/j.gpb.2012.09.004
73. Lindmark, B., Rompikuntal, P.K., Vaitkevicius, K., Song, T., Mizunoe, Y., Uhlin, B.E., Guerry, P., and Wai, S.N. (2009). Outer membrane vesicle-mediated release of cytolethal distending toxin (CDT) from *Campylobacter jejuni*. BMC Microbiol. 9:220. doi: 10.1186/1471-2180-9-220

74. Liu, W., Rochat, T., Toffano-Nioche, C., Lam, L., Nguyen, T., Bouloc, P., and Morvan, C. (2018a). Assessment of Bona Fide sRNAs in *Staphylococcus aureus*. Front Microbiol. 9: 228. doi: 10.3389/fmicb.2018.00228
75. Liu, Y., Defourny, K.A., Smid, E.J., and Abee, T. (2018). Gram-positive bacterial extracellular vesicles and their impact on health and disease. Front Microbiol.9:1502. doi: 10.3389/fmicb.2018.01502
76. Liu, Y., Wu, N., Dong, J., Gao, Y., Zhang, X., Shao, N., and Yang, G. (2010). SsrA (tmRNA) acts as an antisense RNA to regulate *Staphylococcus aureus* pigment synthesis by base pairing with crtMN mRNA. FEBS Lett. 584(20):4325-9. doi: 10.1016/j.febslet.2010.09.024
77. Lu, K.-C., Zhang, Y., and Song, E. (2019). Extracellular RNA: mechanisms of it's transporting into target cells. ExRNA 1.1 (2019): 22
78. Lynch, J.B., and Alegado, R.A. (2017). Spheres of hope, packets of doom: the good and bad of outer membrane vesicles in interspecies and ecological dynamics. J Bacteriol.199(15):e00012-17.doi: 10.1128/JB.00012-17.
79. Lvu, L., Zhang, X., Li, C., Yang, T., Wang, J., Pan, L., Jia, H., Li, Z., Sun, Q.,Yue, L., Chen, F., and Zhang, Z. (2019). Small RNA profiles of serum exosomes derived from individuals with latent and active tuberculosis. Front Microbiol.10:1174. doi: 10.3389/fmicb.2019.01174
80. Malabirade, A., Habier, J., Heintz-Buschart, A., May, P., Godet, J., Halder, R., Etheridge, A., Galas, D.J., Wilmes, P., and Fritz, J.V. (2018). The RNA complement of outer membrane vesicles from *Salmonella enterica* serovar Typhimurium under distinct culture conditions. Front Microbiol. 9:2015. doi: 10.3389/fmicb.2018.02015
81. Manning, A.J., and Kuehn, M.J. (2011). Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol. 11:258. doi: 10.1186/1471-2180-11-258.
82. Manning, A.J., and Kuehn, M.J. (2013). Functional advantages conferred by extracellular prokaryotic membrane vesicles. J Mol Microbiol Biotechnol. 23(1-2):131-41. doi: 10.1159/000346548
83. Maredia, R., Devineni, N., Lentz, P., Dallo, S.F., Yu, J., Guentzel, N., Chambers, J., Arulanandam, B., Haskins, W.E., and Weitao, T. (2012). Vesiculation from *Pseudomonas aeruginosa* under SOS. Scientific World Journal. 2012: 402919. doi: 10.1100/2012/402919
84. Mashburn, L.M., and Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature. 437(7057):422-5. doi:10.1038/nature03925
85. Mateescu, B., Kowal, E.J., Van Balkom, B.W., Bartel, S., Bhattacharyya, S.N., Buzás, E.I., Buck, A.H., De Candia, P., Chow, F.W., and Das, S. (2017). Obstacles and opportunities in the functional analysis of extracellular vesicle RNA–an ISEV position paper. J Extracell Vesicles. 6(1):1286095.doi: 10.1080/20013078.2017.1286095
86. Mccaig, L.F., Mcdonald, L.C., Mandal, S., and Jernigan, D.B. (2006). *Staphylococcus aureus*–associated skin and soft tissue infections in ambulatory care. Emerg Infect Dis. 12(11):1715-23. doi :10.3201/eid1211.060190
87. Mizuno, T., Chou, M.-Y., and Inouye, M. (1984). A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc Natl Acad Sci U S A. 81(7):1966-70. doi:10.1073/pnas.81.7.1966
88. Morinaga, K., Yamamoto, T., Nomura, N., and Toyofuku, M. (2018). *Paracoccus denitrificans* can utilize various long-chain N-acyl homoserine lactones and sequester them in membrane vesicles Environ Microbiol Rep. 10(6):651-654. doi: 10.1111/1758-2229.12674.
89. Mäder, U., Nicolas, P., Depke, M., Pané-Farré, J., Debarbouille, M., Van Der Kooi-Pol, M.M., Guérin, C., Dérozier, S., Hiron, A., Jarmer, H., and Van Dijl, J.M. (2016).

Staphylococcus aureus transcriptome architecture: from laboratory to infection-mimicking conditions. PLoS Genet. 12(4). doi: 10.1371/journal.pgen.1005962
90. Nadeem, A., Oscarsson, J., and Wai, S.N. (2020). "Delivery of Virulence Factors by Bacterial Membrane Vesicles to Mammalian Host Cells," in Bacterial Membrane Vesicles. Springer), 131-158.
91. Nicola, A.M., Frases, S., and Casadevall, A. (2009). Lipophilic dye staining of *Cryptococcus neoformans* extracellular vesicles and capsule. Eukaryot Cell. 2009 8(9):1373-80. doi: 10.1128/EC.00044-09
92. Novick, R.P. (2003). Autoinduction and signal transduction in the regulation of staphylococcal virulence. Mol Microbiol. 48(6):1429-49. doi:10.1046/j.1365-2958.2003.03526.x
93. Novick, R.P., and Geisinger, E. (2008). Quorum sensing in staphylococci. *Annual review of genetics* 42**,** 541-564.
94. Novick, R.P., Iordanescu, S., Projan, S.J., Kornblum, J., and Edelman, I. (1989). pT181 plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. Cell.59(2):395-404. doi :10.1016/0092-8674(89)90300-0
95. Novick, R.P., Ross, H., Projan, S., Kornblum, J., Kreiswirth, B., and Moghazeh, S. (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. EMBO J. 12(10): 3967–75
96. Oglesby-Sherrouse, A.G., and Murphy, E.R. (2013). Iron-responsive bacterial small RNAs: variations on a theme. *Metallomics* 5**,** 276-286. doi: 10.1039/c3mt20224k
97. Petrova, O.E., Garcia-Alcalde, F., Zampaloni, C., and Sauer, K. (2017). Comparative evaluation of rRNA depletion procedures for the improved analysis of bacterial biofilm and mixed pathogen culture transcriptomes. Sci Rep. 7:41114.doi: 10.1038/srep41114.
98. Prados-Rosales, R., Weinrick, B.C., Piqué, D.G., Jacobs, W.R., Casadevall, A., and Rodriguez, G.M. (2014). Role for *Mycobacterium tuberculosis* membrane vesicles in iron acquisition. J Bacteriol. 196(6):1250-56. doi: 10.1128/JB.01090-13
99. Ramirez, M.I., Amorim, M.G., Gadelha, C., Milic, I., Welsh, J.A., Freitas, V.M., Nawaz, M., Akbar, N., Couch, Y., and Makin, L. (2018). Technical challenges of working with extracellular vesicles. *Nanoscale* 10**,** 881-906. doi: 10.1039/c7nr08360b
100. Resch, U., Tsatsaronis, J.A., Le Rhun, A., Stübiger, G., Rohde, M., Kasvandik, S., Holzmeister, S., Tinnefeld, P., Wai, S.N., and Charpentier, E. (2016). A two-component regulatory system impacts extracellular membrane-derived vesicle production in group A Streptococcus. *MBio* 7**,** e00207-00216.
101. Rivera, J., Cordero, R.J., Nakouzi, A.S., Frases, S., Nicola, A., and Casadevall, A. (2010). *Bacillus anthracis* produces membrane-derived vesicles containing biologically active toxins. *Proceedings of the National Academy of Sciences***,** 201008843.
102. Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat Biotechnol. 29(1):24-6. doi: 10.1038/nbt.1754
103. Romilly, C., Lays, C., Tomasini, A., Caldelari, I., Benito, Y., Hammann, P., Geissmann, T., Boisset, S., Romby, P., and Vandenesch, F. (2014). A non-coding RNA promotes bacterial persistence and decreases virulence by regulating a regulator in *Staphylococcus aureus*. *PLoS Pathog* 10**,** e1003979
104. Romby, P., and Charpentier, E. (2010). An overview of RNAs with regulatory functions in gram-positive bacteria. Cell Mol Life Sci.67(2):217-37. doi: 10.1007/s00018-009-0162-8
105. Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M.-A., and Barrell, B. (2000). Artemis: sequence visualization and annotation. Bioinformatics. 2000 Oct;16(10):944-5

106. Scanlan, D. (2014). Bacterial vesicles in the ocean. Science. 343(6167):143-4. doi: 10.1126/science.1248566
107. Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., and Schmid, B. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods.* 9(7):676-82. doi: 10.1038/nmeth.2019
108. Schlatterer, K., Beck, C., Hanzelmann, D., Lebtig, M., Fehrenbacher, B., Schaller, M., Ebner, P., Nega, M., Otto, M., and Kretschmer, D. (2018). The mechanism behind bacterial lipoprotein release: Phenol-soluble modulins mediate toll-like receptor 2 activation via extracellular vesicle release from *Staphylococcus aureus*. mBio.9(6):e01851-18. doi: 10.1128/mBio.01851-18.
109. Schoenfelder, S.M., Marincola, G., Geiger, T., Goerke, C., Wolz, C., and Ziebuhr, W. (2013). Methionine biosynthesis in *Staphylococcus aureus* is tightly controlled by a hierarchical network involving an initiator tRNA-specific T-box riboswitch. PLoS Pathog. 9(9). doi: 10.1371/journal.ppat.1003606
110. Schoenfelder, S.M., Lange, C., Prakash, S.A., Marincola, G., Lerch, M.F., Wencker, F.D., Förstner, K.U., Sharma, C.M. and Ziebuhr, W., 2019. The small non-coding RNA RsaE influences extracellular matrix composition in *Staphylococcus epidermidis* biofilm communities. PLoS pathogens, 15(3), p.e1007618
111. Schuster, C.F., and Bertram, R. (2016). Toxin-antitoxin systems of *Staphylococcus aureus*. Toxins (Basel). 8(5).doi: 10.3390/toxins8050140
112. Schwechheimer, C., and Kuehn, M.J. (2015). Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol.13(10):605-19. doi: 10.1038/nrmicro3525
113. Sjöström, A.E., Sandblad, L., Uhlin, B.E., and Wai, S.N. (2015). Membrane vesicle-mediated release of bacterial RNA. Sci Rep. 5:15329. doi: 10.1038/srep15329
114. Smalley, J., and Birss, A. (1987). Trypsin-like enzyme activity of the extracellular membrane vesicles of Bacteroides gingivalis W50. *Microbiology* 133**,** 2883-2894.
115. Song, T., Mika, F., Lindmark, B., Liu, Z., Schild, S., Bishop, A., Zhu, J., Camilli, A., Johansson, J., and Vogel, J. (2008). A new *Vibrio cholerae* sRNA modulates colonization and affects release of outer membrane vesicles. *Molecular microbiology* 70**,** 100-111.
116. Song, T., and Wai, S.N. (2009). A novel sRNA that modulates virulence and environmental fitness of Vibrio cholerae. *RNA biology* 6**,** 254-258.
117. Steiner, K., and Malke, H. (2001). relA-independent amino acid starvation response network of Streptococcus pyogenes. J Bacteriol. 183(24):7354-64
118. Sudarsan, N., Cohen-Chalamish, S., Nakamura, S., Emilsson, G.M., and Breaker, R.R. (2005). Thiamine pyrophosphate riboswitches are targets for the antimicrobial compound pyrithiamine. Chem Biol.12(12):1325-35. doi:10.1016/j.chembiol.2005.10.007
119. Szafranska, A.K., Oxley, A.P., Chaves-Moreno, D., Horst, S.A., Roßlenbroich, S., Peters, G., Goldmann, O., Rohde, M., Sinha, B., Pieper, D.H., and Medina, E. (2014). High-resolution transcriptomic analysis of the adaptive response of *Staphylococcus aureus* during acute and chronic phases of osteomyelitis. mBio. 5(6). doi: 10.1128/mBio.01775-14
120. Thay, B., Wai, S.N., and Oscarsson, J. (2013). *Staphylococcus aureus* α-toxin-dependent induction of host cell death by membrane-derived vesicles. PLoS One. 8(1):e54661.doi: 10.1371/journal.pone.0054661
121. Thibonnier, M., Thiberge, J.-M., and De Reuse, H. (2008). Trans-translation in Helicobacter pylori: essentiality of ribosome rescue and requirement of protein tagging for stress resistance and competence. PLoS One. 3(11) doi: 10.1371/journal.pone.0003810
122. Tjaden, B. (2019). A Computational System for Identifying Operons Based on RNA-Seq Data. Methods. 2020 Apr 1;176:62-70. doi: 10.1016/j.ymeth.2019.03.026

123. Toledo-Arana, A., Repoila, F., and Cossart, P. (2007). Small noncoding RNAs controlling pathogenesis. Curr Opin Microbiol. 2007 Apr;10(2):182-8
124. Tomasini, A., François, P., Howden, B.P., Fechter, P., Romby, P., and Caldelari, I. (2014). The importance of regulatory RNAs in *Staphylococcus aureus*. Infect Genet Evol. 2014 Jan;21:616-26. doi: 10.1016/j.meegid.2013.11.016
125. Toyofuku, M., Nomura, N., and Eberl, L. (2019). Types and origins of bacterial membrane vesicles. Nat Rev Microbiol.17(1):13-24. doi: 10.1038/s41579-018-0112-2
126. Trotochaud, A.E., and Wassarman, K.M. (2004). 6S RNA function enhances long-term cell survival. J Bacteriol. 186(15):4978-85. doi:10.1128/JB.186.15.4978-4985.2004
127. Vanaja, S.K., Rathinam, V.A., Atianand, M.K., Kalantari, P., Skehan, B., Fitzgerald, K.A., and Leong, J.M. (2014). Bacterial RNA: DNA hybrids are activators of the NLRP3 inflammasome. Proc Natl Acad Sci .111(21):7765-70. doi: 10.1073/pnas.1400075111.
128. Vdovikova, S., Luhr, M., Szalai, P., Nygård Skalman, L., Francis, M.K., Lundmark, R., Engedal, N., Johansson, J., and Wai, S.N. (2017). A novel role of *Listeria monocytogenes* membrane vesicles in inhibition of autophagy and cell death. Front Cell Infect Microbiol. 7:154. doi: 10.3389/fcimb.2017.00154. eCollection 2017.
129. Wagner, E.G.H., and Romby, P. (2015). "Small RNAs in bacteria and archaea: who they are, what they do, and how they do it," in Advances in genetics. Elsevier), 133-208.
130. Wagner, E.G.H., and Vogel, J. (2003). Noncoding RNAs encoded by bacterial chromosomes. Noncoding RNAs: molecular biology and molecular medicine. Kluwer Academic/Plenum Publishers, New York, 242-258.
131. Wagner, T., Joshi, B., Janice, J., Askarian, F., Škalko-Basnet, N., Hagestad, O., Mekhlif, A., Wai, S., Hegstad, K., and Johannessen, M. (2018). *Enterococcus faecium* produces membrane vesicles containing virulence factors and antimicrobial resistance related proteins. J Proteomics. 187:28-38. doi: 10.1016/j.jprot.2018.05.017
132. Wang, X., Thompson, C.D., Weidenmaier, C., and Lee, J.C. (2018). Release of *Staphylococcus aureus* extracellular vesicles and their application as a vaccine platform. Nat Commun. 9(1):1379. doi: 10.1038/s41467-018-03847-z
133. Wang, Z., Gerstein, M., and Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. Nat Rev Genet. 10(1):57-63. doi: 10.1038/nrg2484
134. Wassarman, K.M., and Storz, G. (2000). 6S RNA regulates *E. coli* RNA polymerase activity. Cell. 101(6):613-23. doi:10.1016/s0092-8674(00)80873-9
135. Weber, J.A., Baxter, D.H., Zhang, S., Huang, D.Y., Huang, K.H., Lee, M.J., Galas, D.J., and Wang, K. (2010). The microRNA spectrum in 12 body fluids. 56(11):1733-41. doi: 10.1373/clinchem.2010.147405
136. Wertheim, H.F., Melles, D.C., Vos, M.C., Van Leeuwen, W., Van Belkum, A., Verbrugh, H.A., and Nouwen, J.L. (2005). The role of nasal carriage in *Staphylococcus aureus* infections. Lancet Infect Dis. 5(12):751-62.
137. Westermann, A.J. (2018). Regulatory RNAs in Virulence and Host-Microbe Interactions. Microbiol Spectr. 6(4). doi: 10.1128/microbiolspec
138. Whitworth, D.E. (2018). Interspecies conflict affects RNA expression. FEMS Microbiol Lett.365(10). doi: 10.1093/femsle/fny096.
139. Wilderman, P.J., Sowa, N.A., Fitzgerald, D.J., Fitzgerald, P.C., Gottesman, S., Ochsner, U.A., and Vasil, M.L. (2004). Identification of tandem duplicate regulatory small RNAs in *Pseudomonas aeruginosa* involved in iron homeostasis. Proc Natl Acad Sci. 101(26):9792-7 doi: 10.1073/pnas.0403423101.
140. Zhang, J., Li, S., Li, L., Li, M., Guo, C., Yao, J., and Mi, S. (2015). Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics. 13(1):17-24. doi: 10.1016/j.gpb.2015.02.001

Table 1. Summary of bacterial counts using flow cytometry and total plate count method

Sample	Flow cytometry data			Plate count (CFU)
	Bacterial count (bacteria/mL)	Live bacteria (%)	Dead bacteria (%)	
S. aureus grown under normal condition	1.8×10^{10}	99.9	0.1	3×10^{10}
S. aureus grown under stress condition	8.8×10^{9}	99.6	0.4	2.3×10^{10}

Table 2. The most enriched MSSA476 EV-associated tRNAs based on read counts

Element	Genomic coordinates	Full name (anticodon)	Cove score	Read count	GC content	Bases of selection
tRNA	1937102..1937175	tRNA Met (CAT)	75.92	984	62.16	CGCGGGATGGAGCAGTTCGGTAGCTCGTCGGGCTCATAACCCGAAG GTCGGTGGTTCAAATCCGCCTCCCGCAA
tRNA	1937017..1937092	tRNA Asp (GTC)	83.32	859	61.84	GGTCTCGTAGTGTAGCGGTTAACACGCCTGCCTGTCACGCAGGAGAT CGCGGGTTCGATTCCCGTCGAGACCGCCA
tRNA	533919..534007	tRNA Leu (TAA)	75.33	607	61.80	GCCGGGGTGGCGGAACTGGCAGACGCACAGGACTTAAAATCCTGCG GTGAGAGATCACCGTACCGGTTCGATTCCGGTCCTCGGCACCA
tRNA	1937971..1938059	tRNA Leu (TAA)	76.44	590	61.80	GCCGGGGTGGCGGAACTGGCAGACGCACAGGACTTAAAATCCTGCG GTGAGTGATCACCGTACCGGTTCGATTCCGGTCCTCGGCACCA
tRNA	1936757..1936837	tRNA Tyr (GTA)	69.29	525	61.73	GGAGGGGTAGCGAAGTGGCTAAACGCGGCGGACTGTAAATCCGCTC CTTCGGGTTCGGCAGTTCGAATCTGCCCCCCTCCA
tRNA	1937190..1937282	tRNA Ser (TGA)	74.09	829	61.29	GGAGGAATACCCAAGTCCGGCTGAAGGGATCGGTCTTGAAAACCGA CAGGGCCTTAACGGGCCGCGGGGGTTCGAATCCCTCTTCCTCCGCCA
tRNA	1937407..1937496	tRNA Ser (TGA)	61.04	657	60.00	GGAGGAATACCCAAGTCCGGCTGAAGGGATCGGTCTTGAAAACCGA CAGGGGCTTAACGGCTCGCGGGGGTTCGAATCCCTCTTCCTCCG
tRNA	1936843..1936918	tRNA Thr (TGT)	92.93	560	55.26	GCCGGCCTAGCTCAATTGGTAGAGCAACTGACTTGTAATCAGTAGGT TGGGGGTTCAAGTCCTCTGGCCGGCACCA
tRNA	533837..533911	tRNA Gly (GCC)	86.82	518	54.67	GCAGAAGTAGTTCAGCGGTAGAATACAACCTTGCCAAGGTTGGGGT CGCGGGTTCGAATCCCGTCTTCTGCTCCA
tRNA	1936926..1936998	tRNA Phe (GAA)	76.42	635	50.68	GGTTCAGTAGCTCAGTTGGTAGAGCAATGGATTGAAGCTCCATGTGT CGGCAGTTCGACTCTGTCCTGAACCA

See Supplementary table S2 for the full list of tRNAs.

Table 3. The most enriched MSSA476EV- associated small RNAs ranked based on read counts[a]

Element	RFAM accession	Description	Functions	Genomic coordinates	Gene length (nt)	Strand[b] (F/R)	Read count[c]	Bit score	GC content (%)
6S RNA	RF00013	protein-binding small RNA	Involved in antibiotic resistance (Lalaouna et al., 2014)	1685656..1685846	197	R	80	97.5	42.41
SsrA RNA	RF00023	protein-binding small RNA	Rescues stalled ribosomes during translation of defective mRNAs and biosynthesis of pigment (Liu et al., 2010;Guillet et al., 2013)	837496..837857	362	F	65	162.6	43.92
RsaC	RF0188	trans-encoded antisense RNA	Oxidative stress and metal-dependent nutritional immunity (Lalaouna et al., 2019)	6736626..674066	441	R	33	555.6	35.37
T-box	RF00230	regulatory elements	Involved in amino acid metabolism (Schoenfelder et al., 2013)	1674489..1674687 385924..386088 386093..386293 1199542..1199714 12486..12696	178 165 201 173 211	R F R F F	32 24 22 16 13	90.55 96.6 114.2 80.94 93.3	34.27 31.52 39.8 34.10 33.65
4.5S RNA	RF00169	trans-encoded antisense RNA	Processing of tRNAs (Szafranska et al., 2014)	485461..485730	270	F	24	69.1	48.52
FMN riboswitch	RF00050	regulatory element	Controls expression of de novo riboflavin	1551305..1551439	135	R	23	121.7	46.67
SAM riboswitch	RF00162	regulatory element	Involved in amino acid (methionine) metabolism	2372869..2372964	96	R	21	78.2	47.92
fstAT	RF01797	trans-encoded antisense RNA	Type I toxin-antitoxin system that interfere with bacterial membrane (Schuster and Bertram, 2016)	1873399..1873493	95	R	19	90.2	42.11
rli28	RF01492	trans-encoded antisense RNA	Role in virulence (Romby and Charpentier, 2010)	2205592..2205772	181	R	19	123.8	34.25

L19_leader	RF00556	regulatory element	NA	1254259..1254301	43	F	18	50.0	41.86
yjdF	RF01764	regulatory element	Regulate gene expression upon binding with heterocyclic aromatic compounds (Li et al., 2016).	423980..424080	101	F	17	92.9	38.61
rli28	RF01492	trans-encoded antisense RNA	Role in virulence (Romby and Charpentier, 2010)	2030445..2030623	179	R	17	85.6	37.43
TPP riboswitch	RF00059	regulatory element	Involved in biosynthesis and transport of thiamine (Sudarsan et al., 2005)	2155664..2155766	103	F	16	70.9	40.78
T-box leader	RF00230	regulatory element	Involved in amino acid metabolism	1791148..1791366	203	R	15	80.52	31.53
RNAIII	RF00503	trans-encoded antisense RNA	Involved in virulence (hemolysins) (Boisset et al., 2007)	2086458..2086973	516	R	14	472.2	28.68
RsaJ	RF01822	trans-encoded antisense RNA	NA	2479454..2479740	287	F	13	332.1	30.66
Lysine riboswitch	RF00168	regulatory element	Regulate expression of lysine biosynthesis and transport genes (Blount et al., 2007)	1732415..1732590	176	F	12	104.6	31.82
fstAT	RF01797	trans-encoded antisense RNA	Type-I toxin-antitoxin systems (Blenkiron et al., 2016)	2483979..2484075	97	F	12	78.12	40.21
L10_Leader	RF00557	regulatory element	NA	566720..566866	127	F	10	86.8	31.21

See Supplementary Table S2 for the full list of sRNAs

[a]The cutoff value was assigned as >10, b. Direction of strand alignment, F= Forward, R= Reverse; c. The total number of sRNA sequence reads.NA=Not available.

Table S1. Description of sample preparation and downstream applications from three independent EVs sample preparation. RNA concentration was measured by Qubit (RNA HS Kit) in crude EVs, EVs treated with RNase and RNA isolated from RNase-treated EVs

	No. of overnight cultures combined for isolation of crude EVs	RNA concentration (ng/µL)				Downstream application
		Crude EVs	RNase treated EVs	RNA isolated from EVs	rRNA depleted RNA from column	
RNA concentration in Biological Replicate (I)	5	NA	NA	77	40	RNA seq, AFM, confocal microscopy, TEM
RNA concentration in Biological Replicate (II)	2	Below detection limit	Below detection limit	2.78	NA	qPCR
RNA concentration in Biological Replicate (III)	2	2.73	Below detection limit	5.16	NA	qPCR, PCR and Sanger seq

NA: Not available

Table S3. Average Ct values of EV associated RNA from three different biological replicates with one-two technical replicates, as detected by real-time PCR

Gene	Biological replicate (I)		Biological replicate (II)	Biological replicate (III)	
	Ct values				
	Technical replicate (I)	Technical replicate (II)	Technical replicate (I)	Technical replicate (I)	Technical replicate (II)
ssrA	27.89±0.34	33.92±0.83	35.52±0.07	38.79±0.3	35.88±0.21
rsaC	23.68±0.09	25.04±0.31	24.19±0.12	26.12±0.2	26.4±0.13
RNAIII	30.81±0.18	34.54±1.47	24.77±0.24	23.95±0.2	24.3±0.23
gyrase	29.25±0.34	30.57±0.3	22.89±0.14		

Figure Legends

Figure 1. *S. aureus* MSSA 476 grown under infection mimicking condition produces spherical EVs of various sizes. (A) Low and (B) high magnification TEM images of crude EVs isolated from bacteria grown in iron-depleted BHI supplemented with subinhibitory concentration of vancomycin (black arrow indicates vesicles) (C) NTA showing size distribution of EVs (mean in nm ± SD) isolated from *S. aureus* MSSA476.

Figure 2. Confocal microscopy of **(A)** enlarged extracellular vesicle particles/aggregates and **(B)** multiple EV particles/aggregates from the field of view. The vesicles were stained with lipid specific dye, PKH2 (red) and RNA-specific dye, SYTO RNASelect (green). Scale bar, 5 μm. **(C)** Line graph showing fluorescence intensity profile of the dotted line across the extracellular vesicles in panel (B). Arrowhead indicates absence of SYTO RNASelect fluorescence in the PKH2 stained extracellular vesicle. **(D)** Quantification of RNA and lipid positive particles. Data points from two different experiments and 8 fields of view (180 μm).

Figure 3. Analysis of the sRNA isolated from *S. aureus* EVs. (A) Virtual gel like image from bioanalyser. L: Ladder and lane 2 shows the RNA of the EVs. (B) Electropherogram displaying sRNA. The first peak in the electropherogram represent lower marker that is used as an alignment to RNA ladder. Other four small peaks appearing at the interval of migration time 24 to 28 sec represent sRNA.

Figure 4. Real Time PCR validation analysis. Boxplot representation of Ct values for SsrA, RsaC and RNAIII isolated from *S. aureus* EVs. Boxes represent interquartile range, central line is median, Whiskers are upper and lower adjacent values and Dots represent number of data points. B) Agarose gel electrophoresis of the RT-PCR products obtained from RNA isolated from RNase treated EVs. The expected amplicon sizes for SsrA, RsaC and RNAIII are 204, 204 and 202 bp, respectively. The left lane shows molecular weight markers.

Figure S1. Growth curves of *S. aureus* grown under BHI (normal condition) and iron depleted BHI media with subinhibitory concentration of vancomycin (stressed condition).

Figure S2. Flow cytometry analysis of S. *aureus* grown in A) BHI (normal condition)B) iron depleted BHI media with subinhibitory concentration of vancomycin (stressed condition) for 16 h. Side scatter is represented on X-axis and AF488 on Y- axis. Gates P1, P3 and P6 were set for beads, live cells and dead cells, respectively.

Figure S3: Measurement of EVs yield obtained from bacteria grown in BHI (normal condition) and iron-depleted BHI with subinhibitory concentration of vancomycin (stressed condition). A) EV number quantified by NTA B) Protein concentration in EVs. The mean ± SD is shown. Results represent three independent experiments. *$P < 0.05$, unpaired t-test.

Figure S4. AFM image of MVs isolated from bacteria grown in iron-depleted BHI supplemented with subinhibitory concentration of vancomycin (White arrow indicates vesicles).

Figure S5. Confocal microscopy on intact RNase treated EV particles/aggregates stained with (A) lipid specific dye, DiD (red), and (B) RNA-specific dye, SYTO RNAselect (green). (C) The image shows the overlay observed under the microscope. The overall percentage of co-localisation in one microscopic field was found to be 78.3% which is in line with the results

using another lipid specific dye in Figure 2. The white rectangular box and lower panel highlights the magnified region in the inset. The scale bar is drawn to 7.5 μm.

Figure S6. Transcriptome landscape of *S. aureus* EVs. RNA sequencing reads were mapped to reference genome (NC_002953) using Rockhopper v 2.0.3. The mapped RNAs, UTRs and, multi-gene operons were visualized in the IGV genome browser. The first red track corresponds to RNA transcripts. The blue track corresponds to UTRs of protein coding genes. The pink track corresponds to multi-gene operons. The final purple track at the bottom of the image corresponds to protein coding genes and RNA genes annotated in RefSeq.

Figure S7. A) Schematic representation of sRNAs identification. B) Size length distribution of sRNAs associated with EVs of *S. aureus*.

Figure S8. Distribution of EV RNAs showing percent of reads mapped to each RNA biotype in *S aureus* chromosomes and plasmid respectively.

Figure S9. Coverage of tRNA downstream of the PerR regulatory region visualized by Artemis genome browser. The pink highlighted region represents the coverage plot. The X-axis represent the position in the genome and the Y-axis represent the numbers of reads mapped (coverage) at that location.

Figure S10. Artemis genome viewer windows showing read density profiles of SsrA (tmRNA). The pink highlighted region represents the coverage plot. X-axis represent the position in the genome and the Y-axis represent the numbers of reads mapped (coverage) at the specific location.

Figure S11. Sanger's sequencing alignment of SsrA, RsaC and RNAIII RNAs. Sequences were aligned using BioEdit alignment tool.

Figure 1

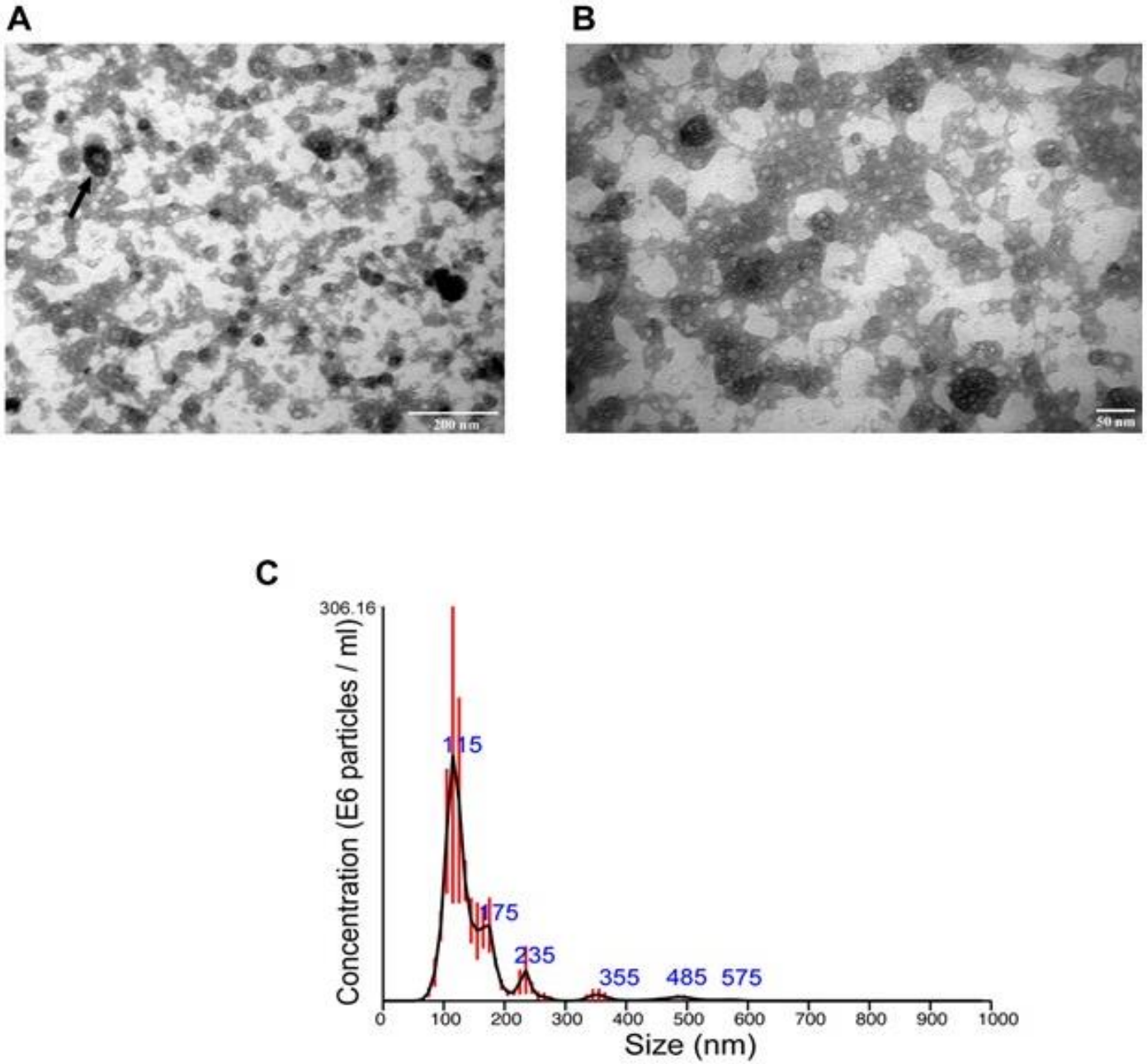

A
200 nm
B
50 nm
C
306.16
Concentration (E6 particles / ml)
115
175
235
355
485
575
0
100
200
300
400
500
600
700
800
900
1000
Size (nm)

Figure 2

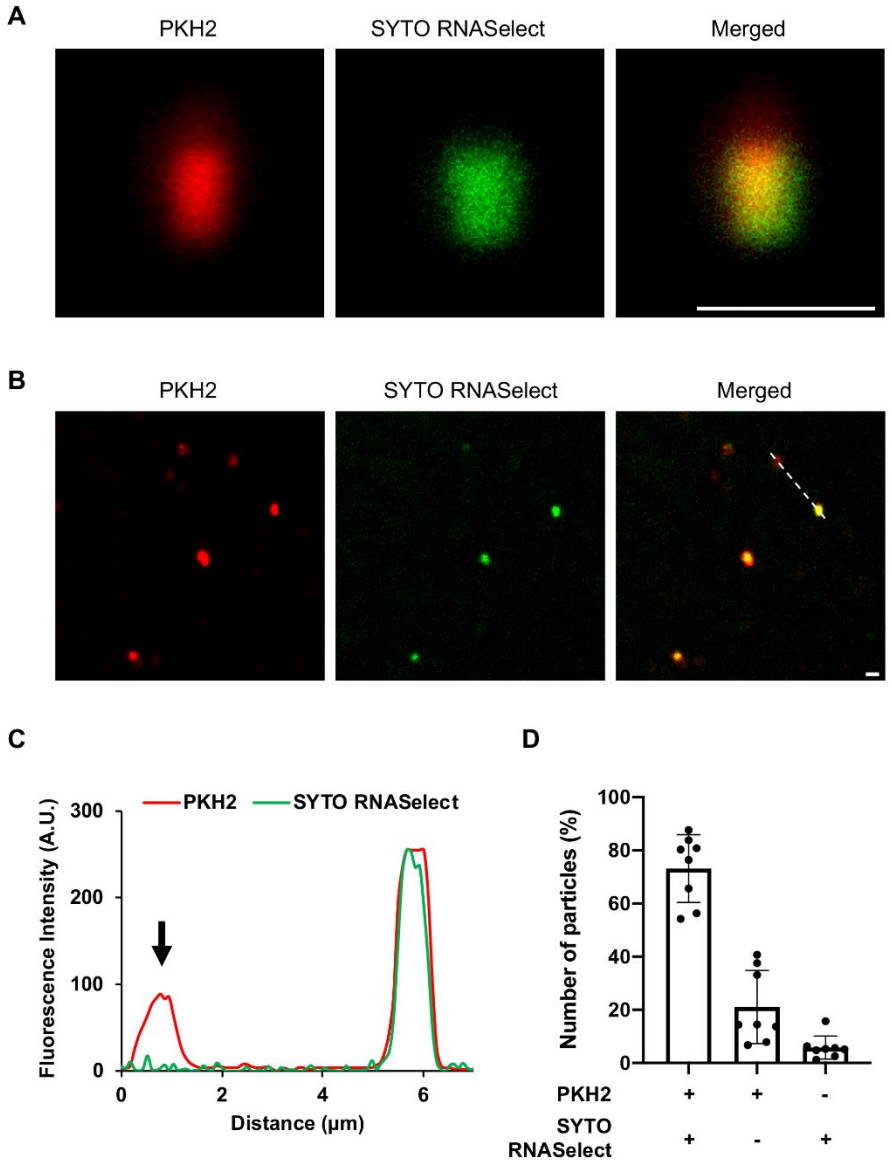

A
PKH2
SYTO RNASelect
Merged
B
PKH2
SYTO RNASelect
Merged
C
PKH2
SYTO RNASelect
Fluorescence Intensity (A.U.)
300
200
100
0
0
2
4
6
Distance (μm)
D
Number of particles (%)
100
80
60
40
20
0
PKH2
+
+
-
SYTO RNASelect
+
-
+

Figure 3

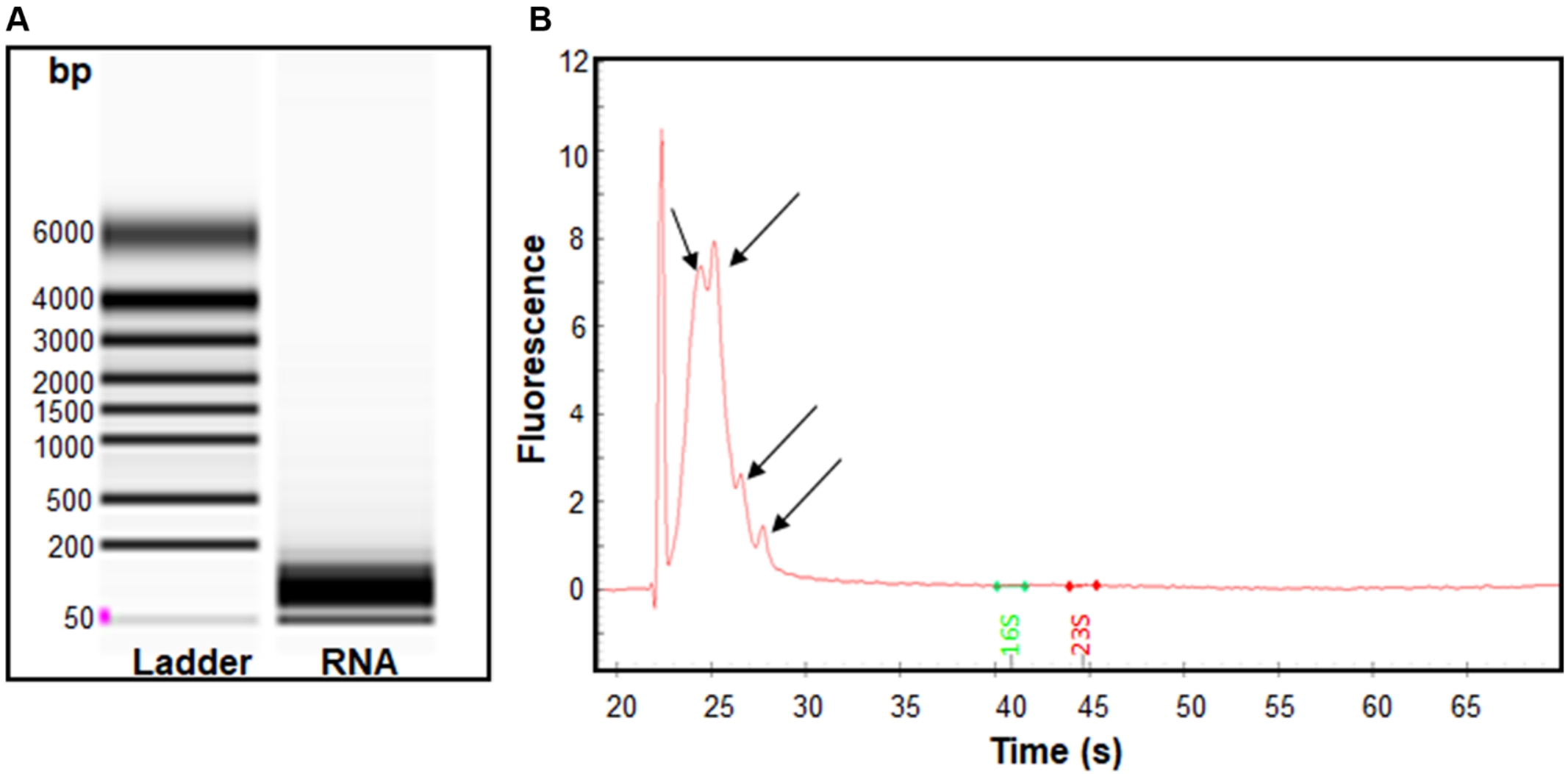

A
bp
6000
4000
3000
2000
1500
1000
500
200
50
Ladder
RNA
B
Fluorescence
12
10
8
6
4
2
0
16S
23S
20
25
30
35
40
45
50
55
60
65
Time (s)

Figure 4

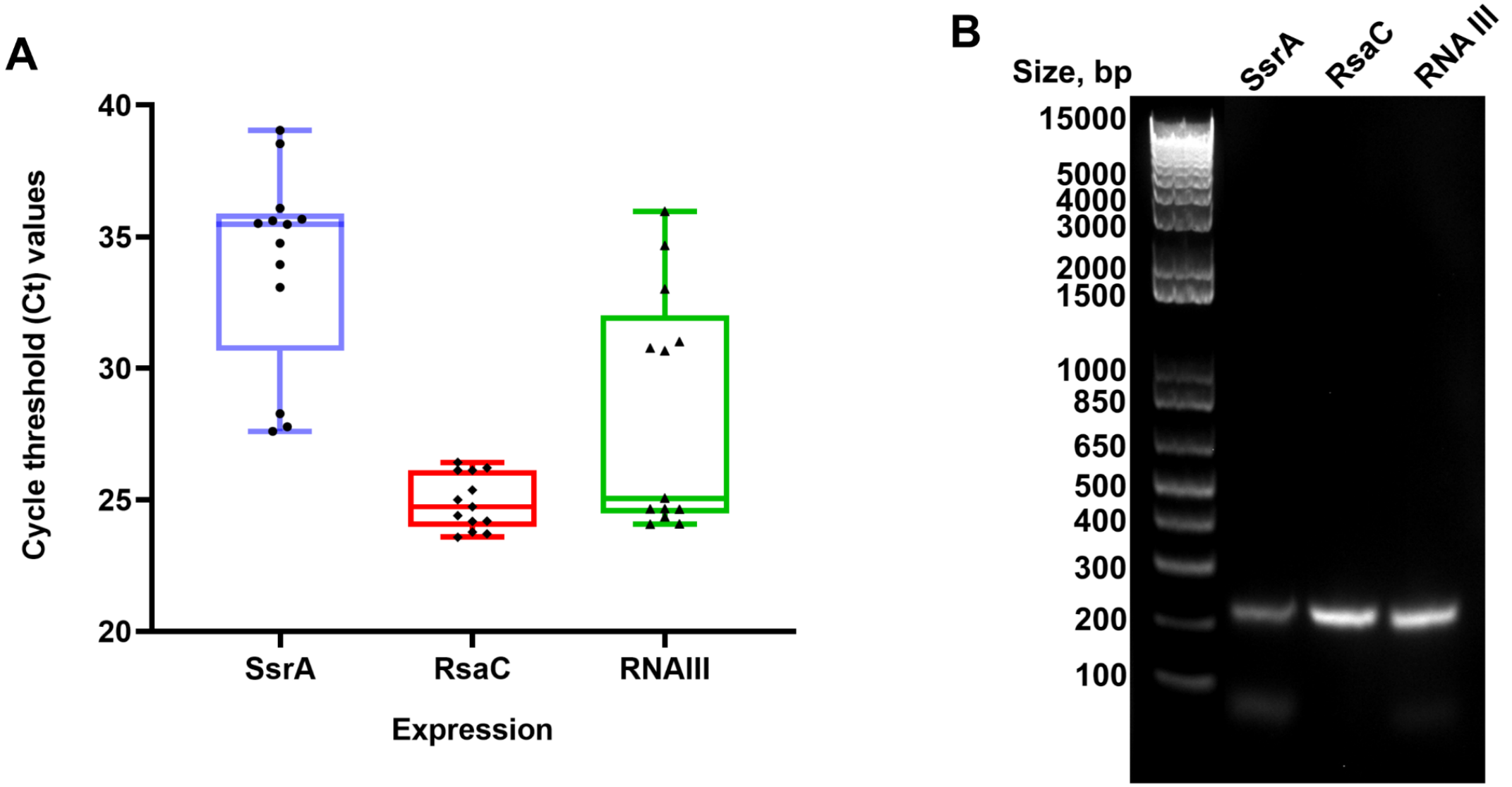

A
Cycle threshold (Ct) values
40
35
30
25
20
SsrA
RsaC
RNAIII
Expression
B
Size, bp
SsrA
RsaC
RNA III
15000
5000
4000
3000
2000
1500
1000
850
650
500
400
300
200
100

Figure S1

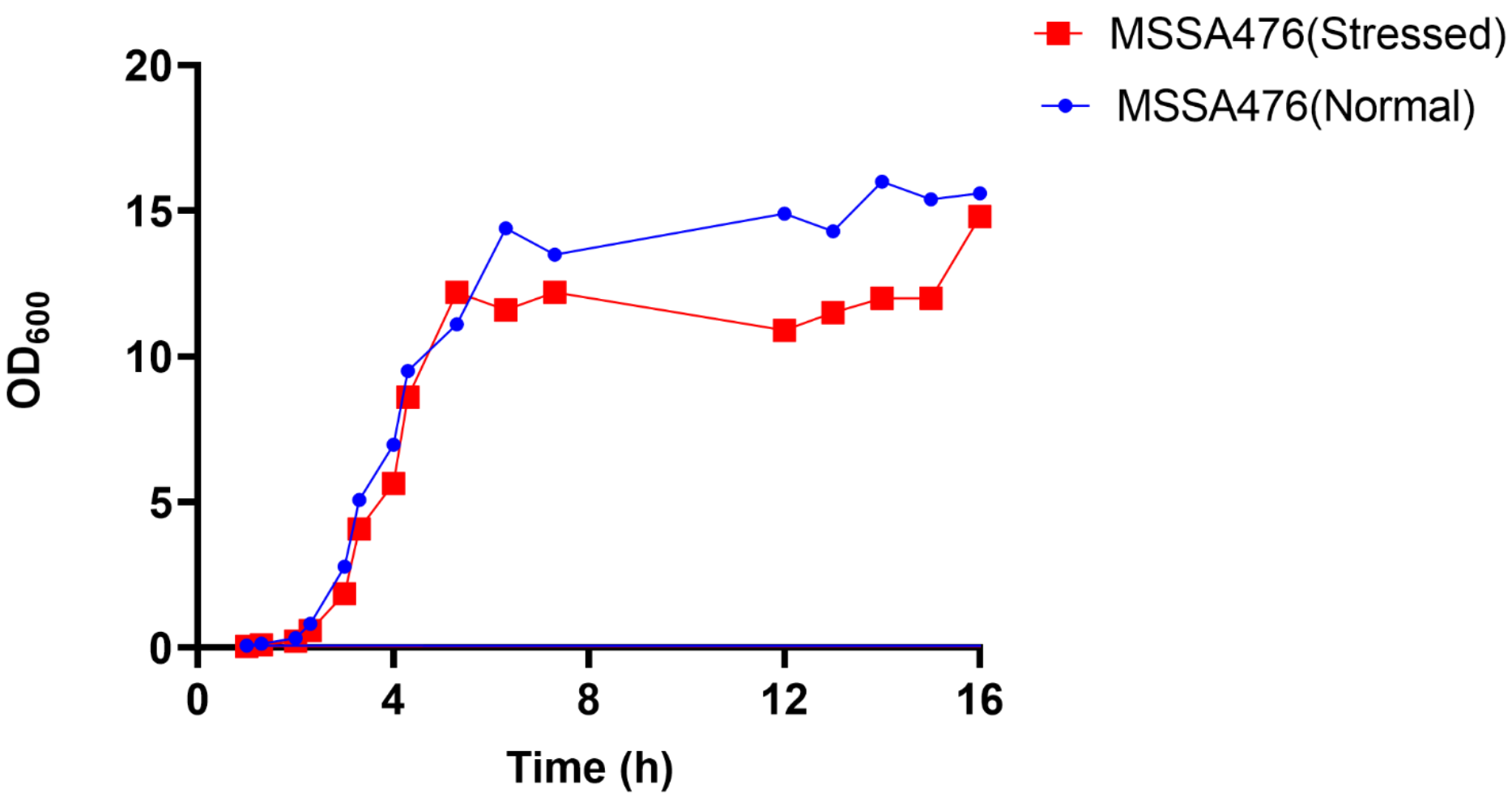
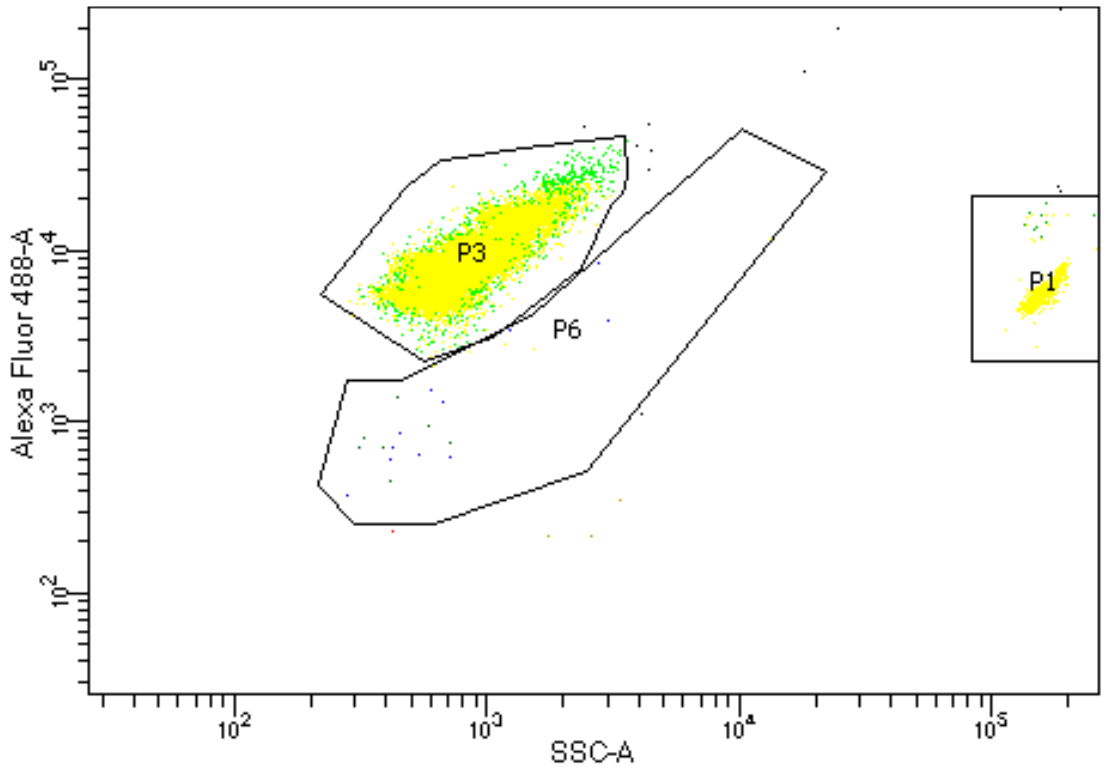

MSSA476(Stressed)
MSSA476(Normal)
OD600
20
15
10
5
0
0
4
8
12
16
Time (h)

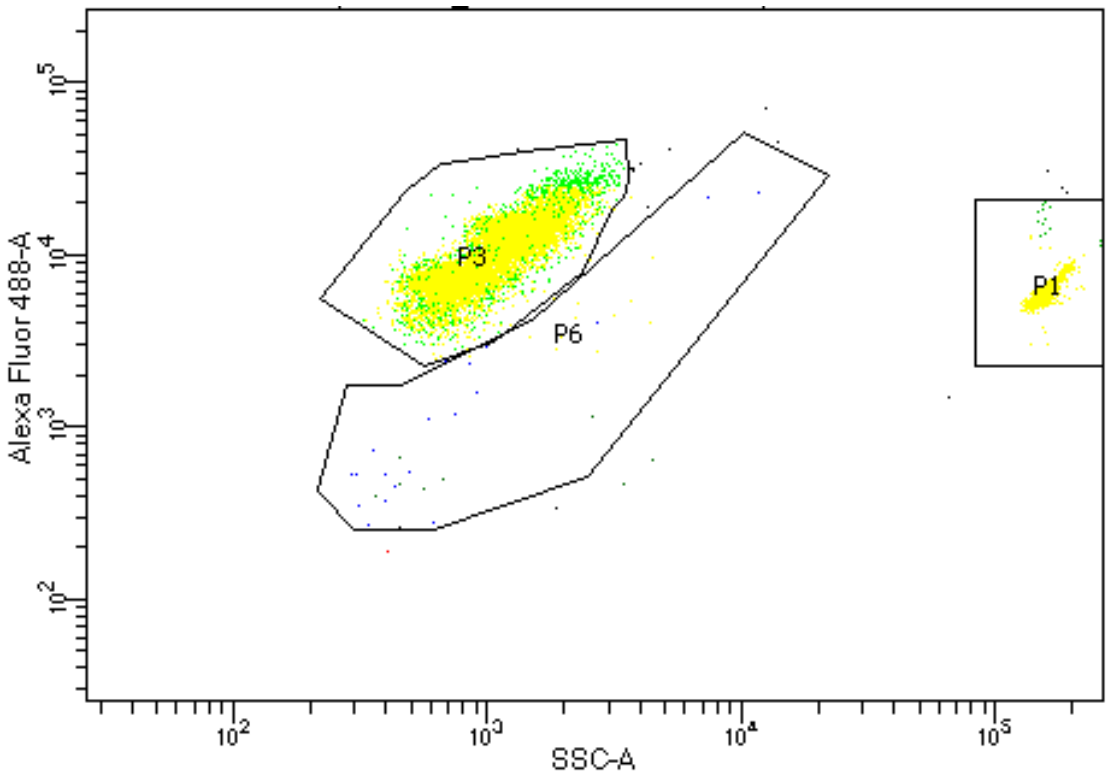
Figure S2

A



S. aureus grown under normal condition

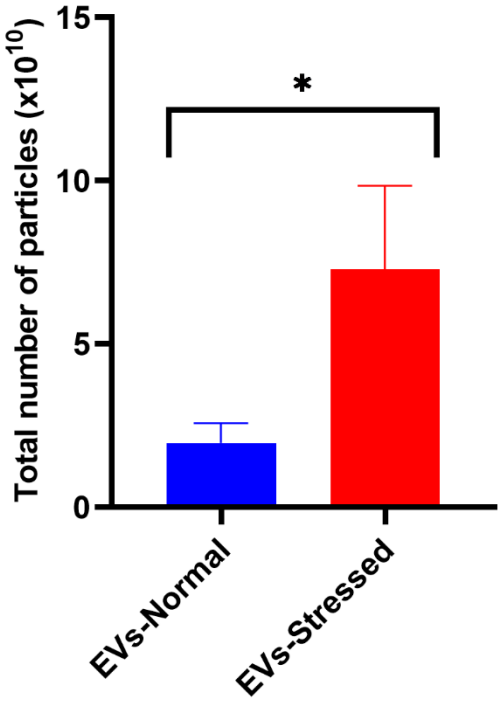
B



S. aureus grown under stressed condition

Figure S3

A


Total number of particles (x10^10)
15
10
5
0
*
EVs-Normal
EVs-Stressed

B

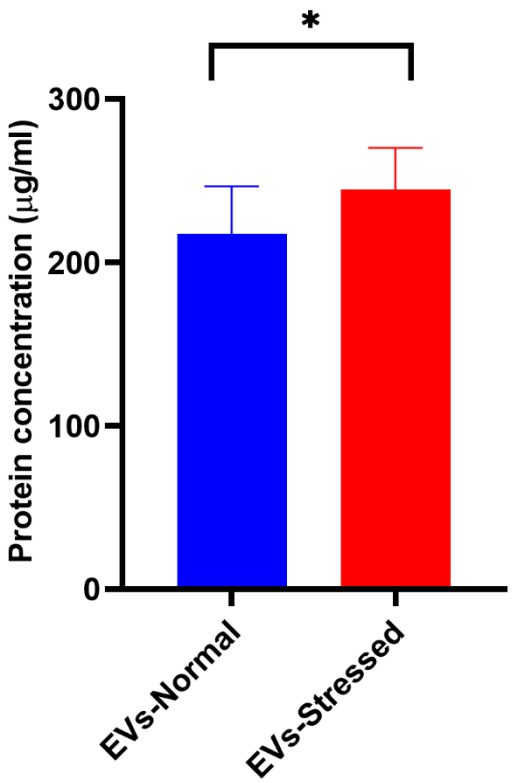

Protein concentration (µg/ml)
300
200
100
0
*
EVs-Normal
EVs-Stressed

Figure S4

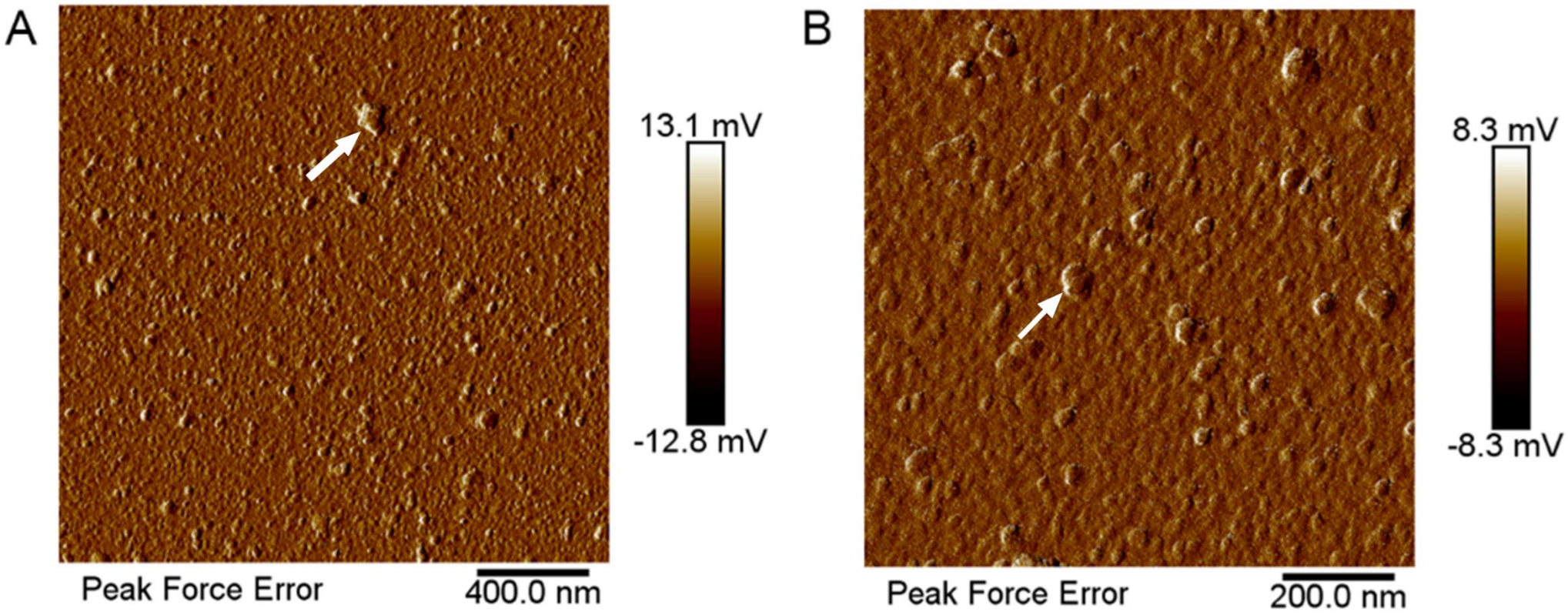

A
13.1 mV
-12.8 mV
Peak Force Error
400.0 nm
B
8.3 mV
-8.3 mV
Peak Force Error
200.0 nm

Figure S5

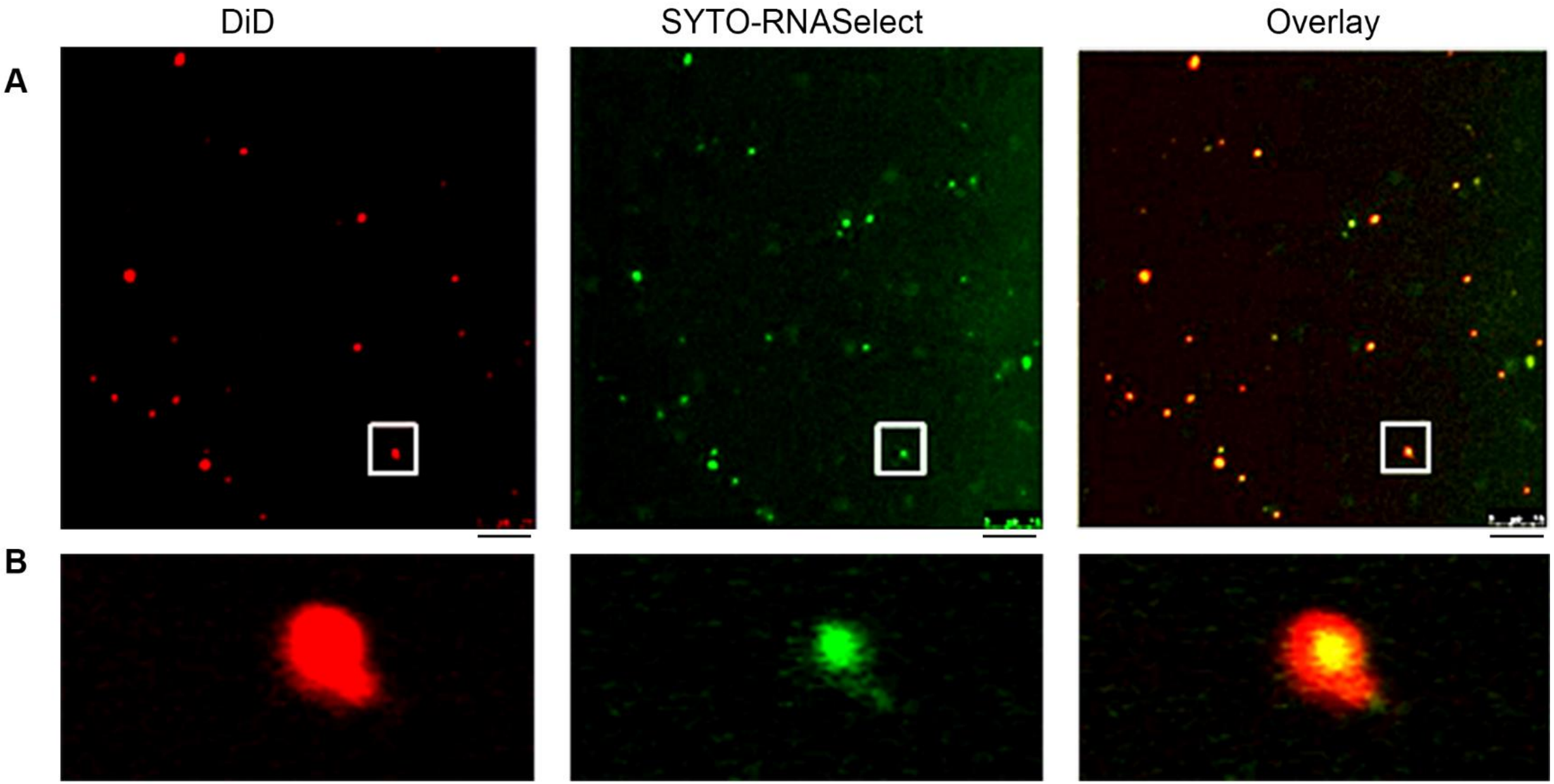


Figure S6

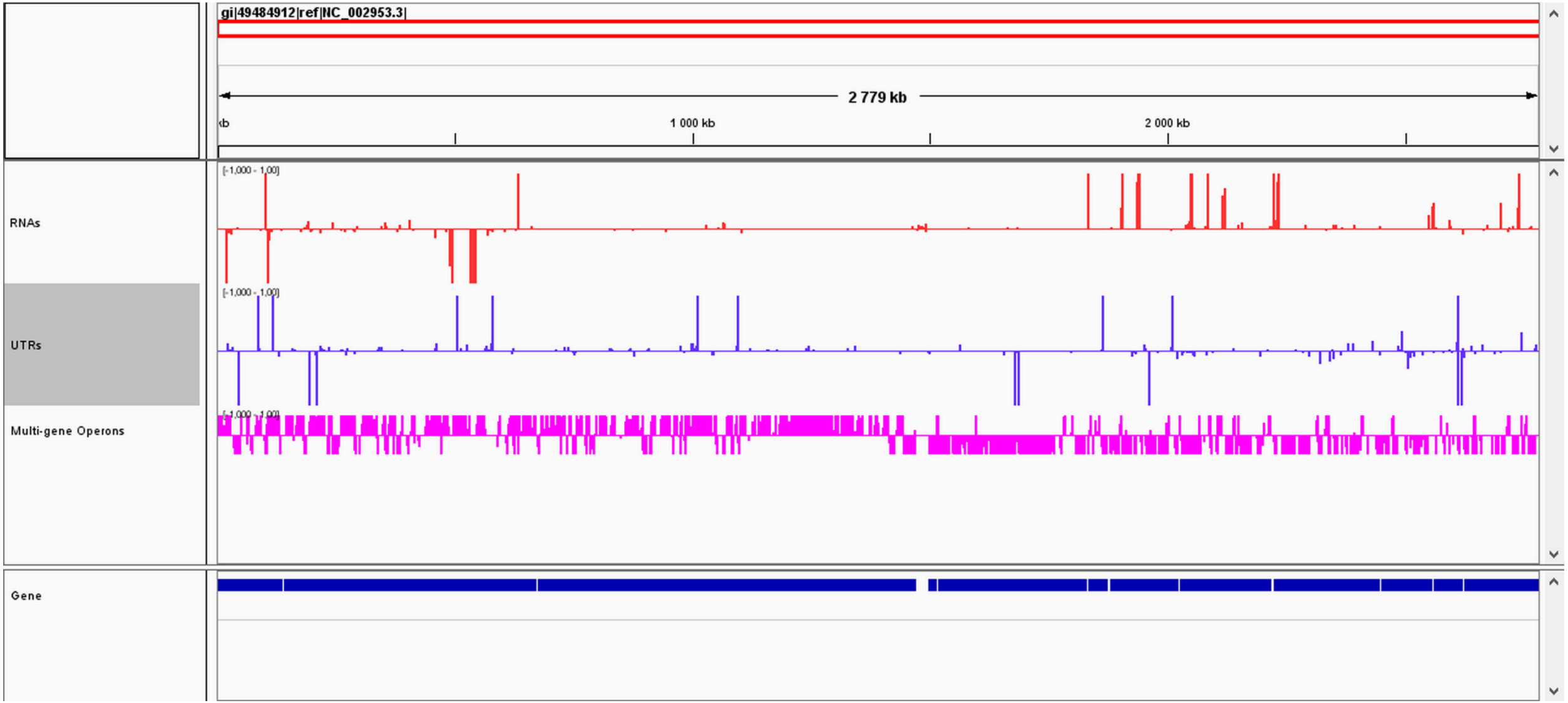

gi|49484912|ref|NC_002953.3|
2 779 kb
kb
1 000 kb
2 000 kb
RNAs
[-1,000 - 1,00]
UTRs
[-1,000 - 1,00]
Multi-gene Operons
[-1,000 - 1,00]
Gene

Figure S7

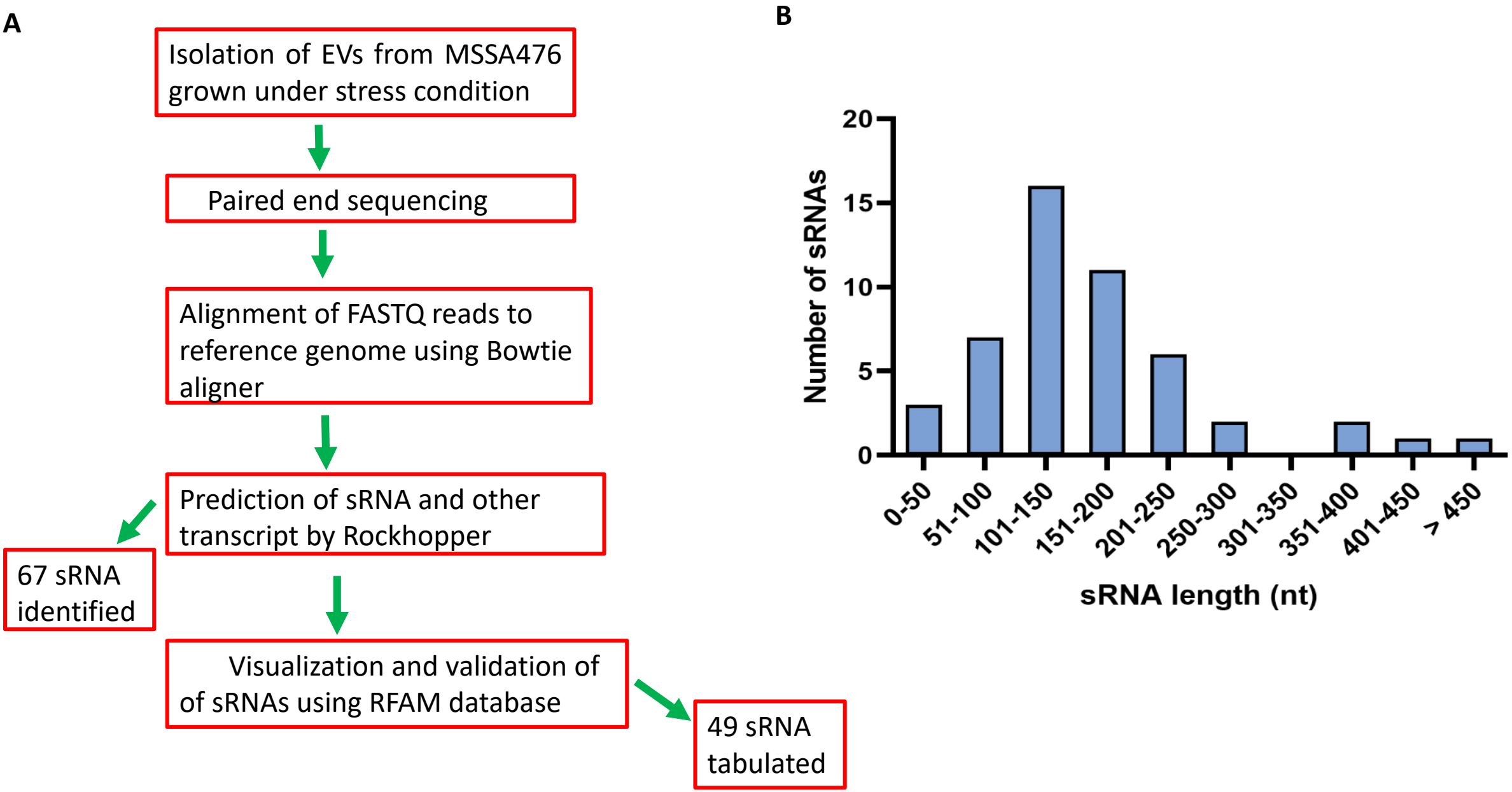
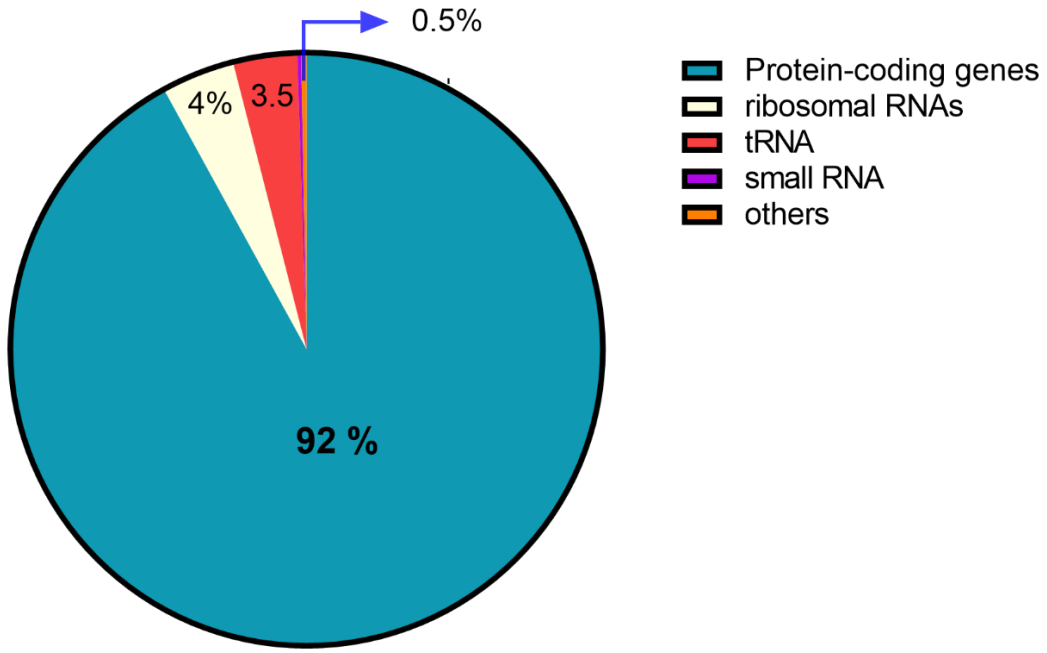

A
Isolation of EVs from MSSA476 grown under stress condition
Paired end sequencing
Alignment of FASTQ reads to reference genome using Bowtie aligner
Prediction of sRNA and other transcript by Rockhopper
67 sRNA identified
Visualization and validation of of sRNAs using RFAM database
49 sRNA tabulated
B
Number of sRNAs
20
15
10
5
0
0-50
51-100
101-150
151-200
201-250
250-300
301-350
351-400
401-450
> 450
sRNA length (nt)

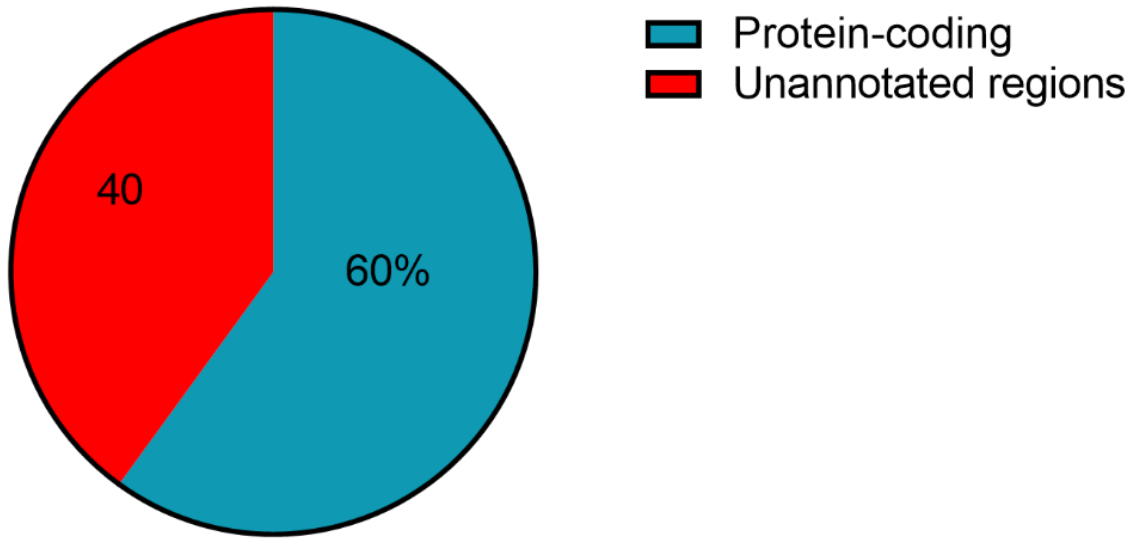
Figure S8

A



% Read mapped to chromosome

B



% Read mapped to plasmid

Figure S9

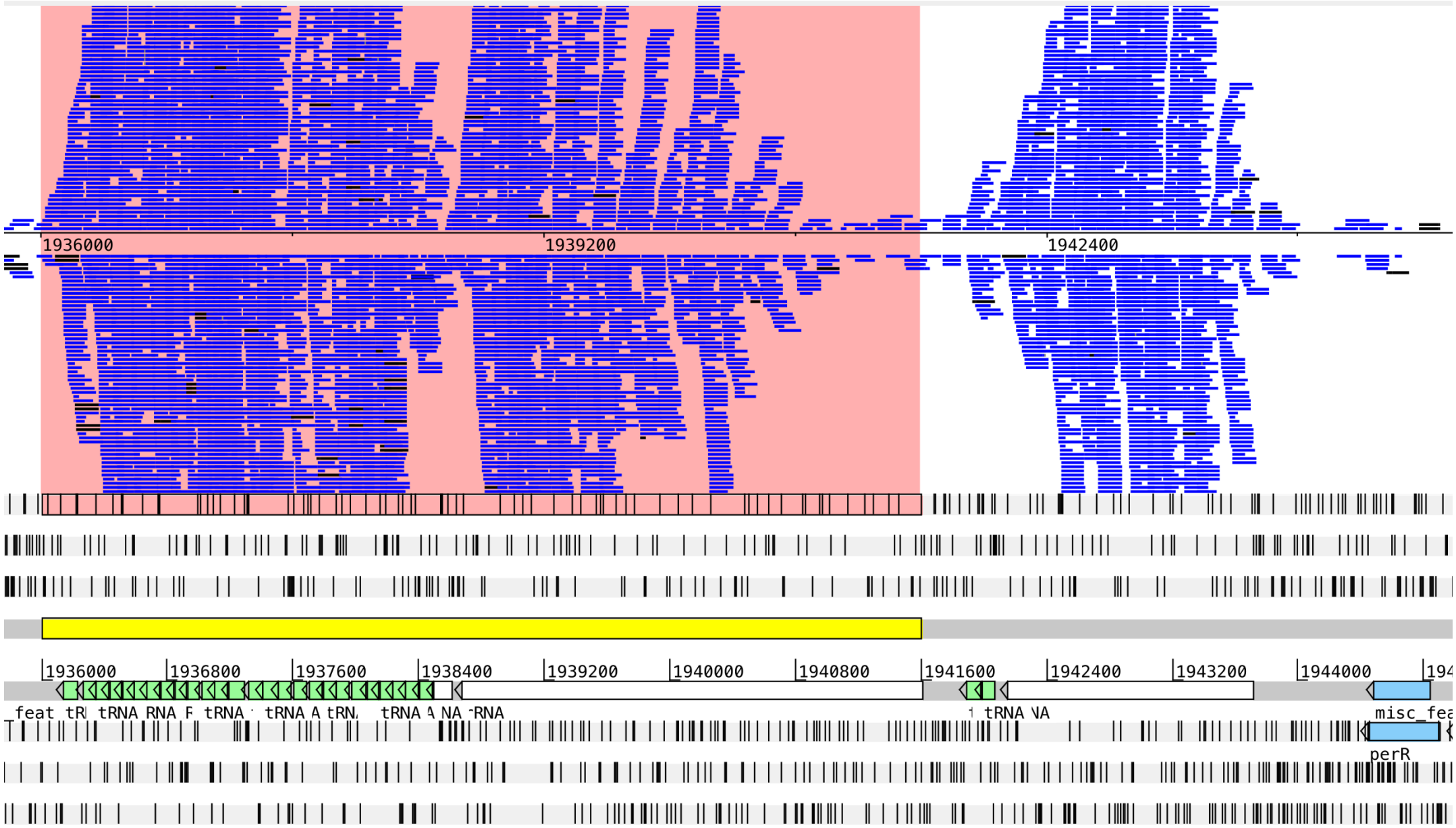

1936000
1939200
1942400
1936000
1936800
1937600
1938400
1939200
1940000
1940800
1941600
1942400
1943200
1944000
misc_fea
perR

Figure S10

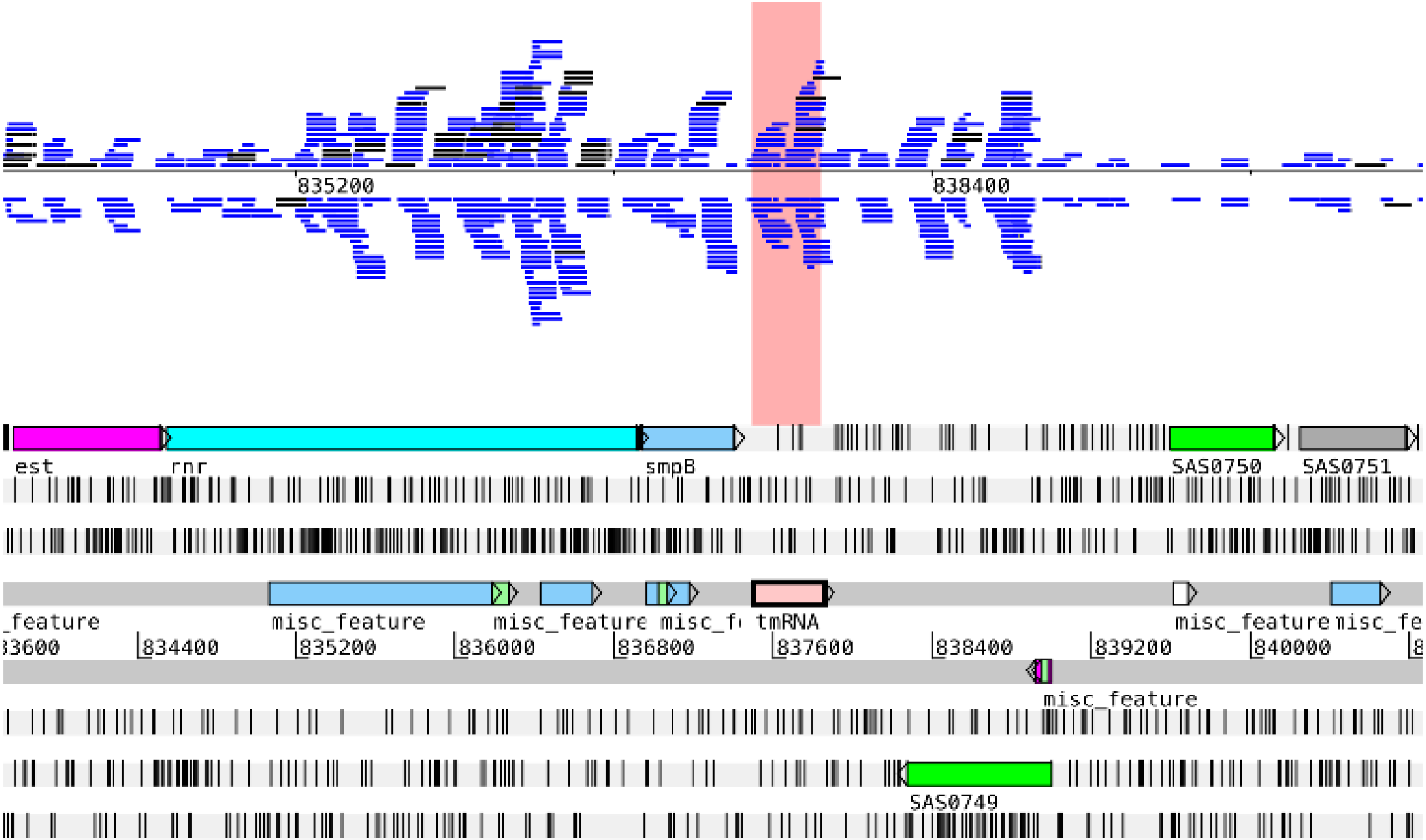
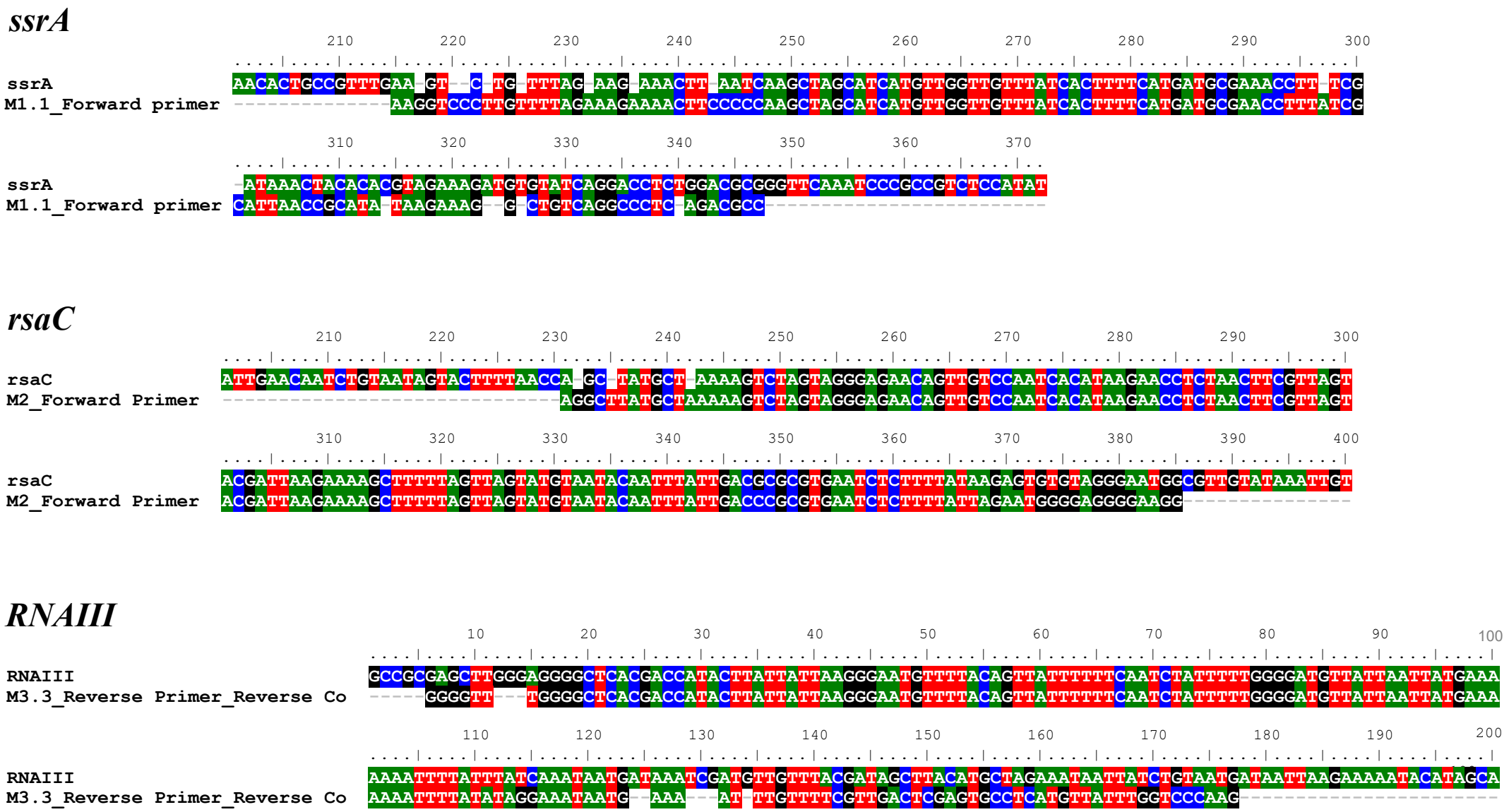

835200
838400
est
rnr
smpB
SAS0750
SAS0751
_feature
misc_feature
misc_feature
misc_f
tmRNA
misc_feature
misc_fe
3600
834400
835200
836000
836800
837600
838400
839200
840000
misc_feature
SAS0749

Figure S11


ssrA
210 220 230 240 250 260 270 280 290 300
ssrA
M1.1_Forward primer
310 320 330 340 350 360 370
ssrA
M1.1_Forward primer
rsaC
210 220 230 240 250 260 270 280 290 300
rsaC
M2_Forward Primer
310 320 330 340 350 360 370 380 390 400
rsaC
M2_Forward Primer
RNAIII
10 20 30 40 50 60 70 80 90 100
RNAIII
M3.3_Reverse Primer_Reverse Co
110 120 130 140 150 160 170 180 190 200
RNAIII
M3.3_Reverse Primer_Reverse Co

Supplementary Tables: Table of Contents

TABLE S2, Sheet 1: Mapping statistics of the RNA-sequencing results

	MVs
Read number	457861
Bowtie aligned read number	358758
Reads with multiple alignments (%)	4
Overall read alignment rates (%)	87

TABLE S2, Sheet 2: *Staphylococcus aureus* subsp. aureus MSSA476 (Summary Rockhopper outout)

Total reads:	463516	
Successfully aligned reads:	390362	84 %
Staphylococcus aureus subsp. *aureus* MSSA476 chromosome	Aligning (sense) to protein-coding genes:	47 %
	Aligning (antisense) to protein-coding genes:	45 %
	Aligning (sense) to ribosomal RNAs:	2 %
	Aligning (antisense) to ribosomal RNAs:	2 %
	Aligning (sense) to transfer RNAs:	0 %
	Aligning (antisense) to transfer RNAs:	0 %
	Aligning (sense) to miscellaneous RNAs:	0 %
	Aligning (antisense) to miscellaneous RNAs:	0 %
	Aligning to unannotated regions:	4 %
Successfully aligned reads:	1922	0 %
Staphylococcus aureus subsp. *aureus* MSSA476 plasmid pSAS	Aligning (sense) to protein-coding genes:	31 %
	Aligning (antisense) to protein-coding genes:	29 %
	Aligning (sense) to ribosomal RNAs:	0 %
	Aligning (antisense) to ribosomal RNAs:	0 %
	Aligning (sense) to transfer RNAs:	0 %
	Aligning (antisense) to transfer RNAs:	0 %
	Aligning (sense) to miscellaneous RNAs:	0 %
	Aligning (antisense) to miscellaneous RNAs:	0 %
	Aligning to unannotated regions:	41 %
Analyzing transcripts		
Staphylococcus aureus subsp. *aureus* MSSA476 chromosome		
Computing transcript boundaries		
	Number of 5'UTRs:	276
	Number of 3'UTRs:	276
	Number of predicted RNAs:	62
	Number of predicted RNAs (not antisense):	7
	Number of predicted RNAs (antisense):	55
Computing likely operons		
	Number of gene-pairs predicted to be part of the same operon:	934
	Number of predicted multi-gene operons:	481
Staphylococcus aureus subsp. *aureus* MSSA476 plasmid pSAS		
Computing transcript boundaries		
	Number of 5'UTRs:	7
	Number of 3'UTRs:	11
	Number of predicted RNAs:	5
	Number of predicted RNAs (not antisense):	1
	Number of predicted RNAs (antisense):	4
Computing likely operons		
	Number of gene-pairs predicted to be part of the same operon:	5
	Number of predicted multi-gene operons:	5

TABLE S2, Sheet 3: *Staphylococcus aureus* subsp. aureus MSSA476 (Operon Rockhopper outout)

Start	Stop	Strand	Number of Genes	Genes
3670	9668	+	4	SAS0003, recF, SAS0005, SAS0006
14724	15745	+	2	SAS0010, SAS0011
17321	22164	+	4	SAS0013, SAS0014, rplI, SAS0016
24924	29586	+	4	SAS0018, SAS0019, SAS0020, SAS0021
34546	40678	-	3	SAS0025, SAS0026, SAS0027
40866	41931	-	3	SAS0028, SAS0029, SAS0030
42831	45827	-	2	*ccrB, ccrA1*
46015	48080	-	2	SAS0034, SAS0035
49912	51482	+	2	SAS0037, SAS0038
62876	63897	-	2	SAS0054, SAS0055
64028	67672	+	3	SAS0056, SAS0057, SAS0058
70254	72024	-	2	SAS0062, SAS0063
82989	85773	+	4	SAS0074, SAS0075, SAS0076, SAS0077
88413	90981	+	2	SAS0079, SAS0080
101255	104253	-	3	SAS0087, SAS0088, SAS0089
104484	115701	+	9	SAS0090, SAS0091, SAS0092, SAS0093, SAS0094, SAS0095, SAS0096, SAS0097, SAS0098
118313	119939	+	2	SAS0102, SAS0103
120149	123954	+	3	SAS0104, SAS0105, SAS0106
127096	129162	+	2	SAS0110, SAS0111
129243	131111	+	2	SAS0112, SAS0113
131242	133629	-	3	SAS0114, SAS0115, SAS0116
144318	158530	+	14	SAS0124, SAS0125, SAS0126, SAS0127, SAS0128, SAS0129, SAS0130, SAS0131, SAS0132, SAS0133, SAS0134, SAS0135, SAS0136, SAS0137
161133	161849	-	2	SAS0140, SAS0141
166897	170427	+	4	SAS0147, SAS0148, SAS0149, SAS0150
174285	182117	+	2	SAS0154, SAS0155
183194	187481	-	4	SAS0157, *argJ*, *argC*, SAS0160
194532	198827	+	4	SAS0165, *murQ*, SAS0167, SAS0168
203176	205783	+	3	SAS0172, SAS0173, SAS0174
205990	207967	+	2	SAS0175, SAS0176
208026	209366	+	3	SAS0177, SAS0178, SAS0179
211796	218108	+	4	SAS0182, SAS0183, SAS0184, SAS0185
221180	225673	+	4	SAS0189, SAS0190, SAS0191, SAS0192
225744	227888	+	2	SAS0193, SAS0194
231737	235009	-	3	SAS0198, SAS0199, SAS0200
235597	240723	+	4	SAS0201, SAS0202, SAS0203, SAS0204
243925	247400	-	2	SAS0207, SAS0208
248910	252003	-	2	SAS0210, SAS0211
256101	257445	-	2	SAS0215, SAS0216
261182	262323	+	2	SAS0219, SAS0219a
262433	265303	+	3	SAS0220, SAS0221, SAS0222
265530	269077	+	4	SAS0223, SAS0224, SAS0225, SAS0226
269605	273055	+	3	*ispD*, SAS0228, SAS0229
275526	280724	+	4	*ispD*, SAS0233, SAS0234, SAS0235
281786	284283	+	2	SAS0237, SAS0238
284396	285533	+	2	SAS0239, SAS0240
286494	288737	+	2	SAS0242, SAS0243
290242	292484	-	3	SAS0245, SAS0246, SAS0247
298545	301301	-	3	SAS0253, SAS0254, SAS0255
304740	318351	+	11	SAS0259, SAS0260, SAS0261, SAS0262, SAS0263, SAS0264, SAS0265, SAS0266, SAS0267, SAS0268, SAS0269

Start	Stop	Strand	Number of Genes	Genes
320202	324267	+	8	SAS0272, SAS0273, SAS0274, SAS0275, SAS0276, SAS0277, SAS0278, SAS0279
330158	331897	+	2	SAS0285, SAS0286
333479	336726	+	3	SAS0288, SAS0289, SAS0290
336832	339285	-	2	SAS0291, SAS0292
348766	352868	+	5	SAS0300, SAS0301, SAS0302, SAS0303, SAS0304
354302	358352	-	4	*ulaA*, SAS0307, SAS0308, SAS0309
362745	365326	+	3	SAS0314, SAS0315, SAS0316
367498	371271	+	3	SAS0319, SAS0320, SAS0321
371363	372296	-	2	SAS0322, SAS0323
373033	375442	+	4	SAS0325, SAS0326, SAS0327, SAS0328
379563	385858	-	4	SAS0332, SAS0333, SAS0334, SAS0335
387524	389747	+	3	SAS0337, SAS0338, SAS0339
390268	391088	+	2	*rpsF*, SAS0342
400727	402835	-	2	SAS0357, SAS0358
409524	414440	+	4	SAS0364, SAS0365, SAS0366, *guaA*
416684	417577	-	2	SAS0370, SAS0371
423051	423741	-	2	SAS0377, SAS0378
426094	427418	-	3	SAS0381a, SAS0382, SAS0383
438971	441779	+	2	SAS0394, SAS0395
442185	444401	+	2	SAS0396, SAS0397
446012	447605	+	2	SAS0400, SAS0401
448633	451416	+	4	SAS0403, SAS0404, SAS0405, SAS0406
454880	459082	+	2	SAS0410, SAS0411
460289	461734	+	2	SAS0414, SAS0415
463744	465784	+	2	SAS0417, SAS0418
466081	468648	+	3	SAS0419, SAS0420, SAS0421
470578	471457	+	2	SAS0424, SAS0425
471626	473523	-	2	SAS0426, SAS0427
474704	480684	+	2	SAS0429, *gltD*
483006	485399	+	2	SAS0432, SAS0433
488422	489342	+	2	SAS0436, *recR*
496066	498379	+	3	SAS0438, *tmk*, SAS0440
498593	500687	+	3	SAS0441, SAS0442, SAS0443
500961	502768	+	3	SAS0444, SAS0445, SAS0446
503053	505830	+	2	SAS0447, SAS0448
505997	507437	+	2	SAS0449, *ksgA*
508110	510193	+	3	*ipk*, SAS0453, SAS0454
514458	521915	+	6	SAS0459, SAS0460, SAS0461, SAS0462, SAS0463, SAS0464
522601	524440	+	2	SAS0466, SAS0467
529227	530846	+	3	SAS0471, SAS0472, SAS0473
546794	548245	+	2	SAS0476, SAS0477
549823	554336	+	4	SAS0479, SAS0480, SAS0481, SAS0482
554820	557282	+	2	SAS0483, SAS0484
559716	563422	+	5	SAS0486, *cysS*, SAS0488, SAS0489, SAS0490
564386	565129	+	2	*secE*, SAS0494
590563	591835	-	2	SAS0514, SAS0515
592519	593975	+	2	SAS0517, SAS0518
609049	610978	-	3	SAS0524, SAS0525, SAS0526
612092	613274	+	2	SAS0528, SAS0529
616187	619609	+	5	SAS0532, SAS0533, SAS0534, SAS0535, SAS0536
621498	622535	+	2	SAS0539, SAS0540
624782	626011	-	2	SAS0543, SAS0544
627480	629305	+	2	*eutD*, SAS0548
629892	632889	+	3	SAS0549, SAS0550, SAS0551
633459	635208	-	2	SAS0553, SAS0554
635833	637872	+	2	SAS0555, SAS0556
643511	644833	+	2	SAS0565, SAS0566
647818	649629	+	2	SAS0570, SAS0571
651952	654038	+	2	SAS0574, *argS*
657356	658859	+	2	SAS0579, SAS0580
664032	664476	-	2	SAS0586, SAS0587

Start	Stop	Strand	Number of Genes	Genes
664635	671049	+	8	SAS0588, SAS0589, SAS0590, SAS0591, SAS0592, SAS0593, SAS0594, SAS0595
674592	677091	-	3	SAS0597, SAS0598, SAS0599
681802	683963	+	2	SAS0605, SAS0606
691210	694060	+	3	SAS0612, SAS0613, SAS0614
695304	696243	+	2	SAS0616, SAS0617
701159	703805	+	3	SAS0623, SAS0624, SAS0625
703949	706589	+	2	SAS0626, SAS0627
707314	708954	+	2	SAS0628, SAS0629
714886	716075	+	2	SAS0634, SAS0635
718393	720098	+	2	SAS0638, SAS0639
723073	724785	-	3	SAS0645, SAS0646, SAS0647
726051	729352	+	2	SAS0649, SAS0650
731178	732408	+	2	SAS0653, SAS0654
735858	736759	-	2	SAS0657, SAS0658
740392	744034	+	3	SAS0663, SAS0664, SAS0665
749341	751530	-	3	SAS0670, SAS0671, SAS0672
753276	755082	-	3	SAS0675, SAS0676, SAS0677
755433	757769	+	3	SAS0678, SAS0679, SAS0680
758129	759827	+	2	SAS0682, SAS0683
762518	766188	+	2	SAS0685, SAS0686
766410	768894	+	2	SAS0687, SAS0688
774742	776128	-	2	SAS0693, SAS0694
776929	779395	+	2	*nrdI*, SAS0696
781192	783864	+	3	SAS0698, SAS0699, SAS0700
785328	786583	-	2	SAS0702, *murB*
790431	792944	-	3	SAS0708, SAS0709, SAS0710
797770	799519	+	2	SAS0715, SAS0716
805944	806827	+	2	SAS0721, SAS0722
807090	811935	+	2	SAS0723, *uvrA*
812542	816259	+	4	SAS0725, SAS0726, SAS0727, SAS0728
818026	819929	+	2	SAS0730, SAS0731
828845	831126	+	2	*tpiA*, SAS0741
833773	837405	+	3	SAS0745, SAS0746, *smpB*
851300	851777	-	2	SAS0761, SAS0762
860286	860961	+	2	SAS0776, SAS0777
861211	863763	+	3	SAS0779, SAS0780, SAS0781
868004	869699	+	2	SAS0786, SAS0787
872256	874377	+	2	SAS0790, SAS0791
874553	877587	+	3	SAS0792, SAS0793, SAS0794
879544	881749	+	3	SAS0798, SAS0799, SAS0800
882161	886423	+	5	SAS0801, SAS0802, SAS0803, SAS0804, SAS0805
888792	889400	+	2	SAS0809, SAS0810
893079	894788	+	2	SAS0813, SAS0814
896247	902011	-	7	SAS0816, SAS0817, SAS0818, SAS0819, SAS0820, SAS0821, SAS0822
909875	912449	-	2	SAS0830, SAS0831
914456	916151	+	3	SAS0833, SAS0834, SAS0835
916311	923441	+	2	SAS0836, SAS0837
934225	937605	+	4	SAS0847, SAS0848, SAS0849, SAS0850
939087	941284	+	2	SAS0853, SAS0854
941953	947652	+	5	SAS0856, SAS0857, SAS0858, SAS0859, SAS0860
949630	953447	+	4	SAS0862, SAS0863, SAS0864, SAS0865
959681	960875	-	2	SAS0871, SAS0872
961758	967694	+	6	SAS0874, SAS0875, *ppnK*, SAS0877, SAS0878, SAS0879
973651	975994	-	2	SAS0885, SAS0886
976427	979714	+	3	SAS0887, SAS0888, *prfC*
983262	984941	-	4	SAS0894, SAS0895, SAS0896, SAS0897
985836	986512	+	3	SAS0901, SAS0902, SAS0903
986905	989287	+	4	SAS0905, SAS0906, SAS0907, SAS0908
989347	995400	+	14	SAS0909, SAS0910, SAS0911, SAS0912, SAS0913, SAS0914, SAS0915, SAS0916, SAS0917, SAS0918, SAS0919, SAS0920, SAS0921, SAS0922
998944	1001041	+	3	SAS0927, SAS0928, SAS0929

Start	Stop	Strand	Number of Genes	Genes
1001648	1006809	+	5	SAS0931, SAS0932, SAS0933, SAS0934, SAS0935
1006878	1008970	+	5	SAS0936, SAS0937, SAS0938, SAS0939, SAS0940
1009967	1022983	+	9	SAS0943, SAS0944, SAS0945, SAS0946, SAS0947, SAS0948, SAS0949, SAS0950, SAS0951
1023464	1025231	+	2	SAS0953, SAS0954
1025807	1029491	+	2	SAS0955, SAS0956
1033891	1034684	+	2	SAS0961, SAS0962
1037934	1038892	+	2	SAS0967, SAS0968
1039611	1041385	+	2	SAS0970, SAS0971
1045611	1050245	+	4	SAS0978, SAS0979, SAS0980, SAS0981
1050483	1052031	-	2	SAS0982, SAS0983
1065266	1069236	-	4	SAS0993, SAS0994, SAS0995, SAS0996
1071969	1083203	+	11	SAS1000, SAS1001, SAS1002, SAS1003, SAS1004, SAS1005, SAS1006, SAS1007, SAS1008, *purH*, SAS1010
1083468	1086257	-	3	SAS1011, SAS1012, SAS1013
1090769	1092756	+	2	SAS1018, SAS1019
1093424	1095801	+	2	SAS1021, SAS1022
1096945	1098860	-	2	SAS1024, SAS1025
1101055	1103148	+	2	SAS1028, SAS1029
1103239	1105941	+	2	SAS1030, SAS1031
1106528	1110852	+	5	SAS1033, SAS1034, SAS1035, SAS1036, SAS1037
1118297	1118948	-	2	SAS1045, SAS1046
1126401	1127798	+	2	SAS1051, SAS1052
1128125	1129612	+	2	SAS1053, SAS1054
1131556	1132582	+	2	SAS1058, *coaD*
1138275	1141887	+	4	SAS1065, SAS1066, SAS1067, SAS1068
1141949	1143169	+	3	SAS1069, SAS1070, SAS1070a
1144530	1147990	+	2	*pheS*, *pheT*
1149533	1150321	+	2	SAS1075, SAS1076
1150394	1154464	+	2	SAS1077, SAS1078
1158046	1160627	+	2	*sdhA*, *sdhB*
1160863	1162758	+	3	SAS1084, SAS1085, SAS1086
1170680	1171007	+	2	SAS1097a, SAS1098
1174258	1176214	+	2	SAS1102, SAS1103
1184723	1188735	+	4	SAS1112, *mraW*, SAS1114, SAS1115
1189027	1192678	+	3	*mraY*, *murD*, SAS1118
1192784	1195401	+	2	SAS1119, SAS1120
1195661	1198006	+	4	SAS1121, SAS1122, SAS1123, SAS1124
1198089	1199536	+	2	SAS1125, SAS1126
1204450	1205858	+	2	*lspA*, SAS1131
1207003	1214779	+	5	SAS1133, *pyrB*, *pyrC*, SAS1136, *carB*
1214886	1216434	+	3	SAS1138, *pyrE*, SAS1140
1219507	1220348	+	2	*gmk*, *rpoZ*
1220564	1224171	+	2	SAS1145, SAS1146
1226292	1232850	+	6	SAS1149, SAS1150, SAS1151, SAS1152, SAS1153, SAS1154
1233078	1235246	+	3	SAS1155, SAS1156, SAS1157
1236258	1238293	+	2	SAS1159, SAS1160
1240761	1243955	+	4	SAS1162, SAS1163, SAS1164, SAS1165
1245552	1252080	+	4	SAS1168, SAS1169, SAS1170, SAS1171
1252978	1254218	+	2	*rimM*, *trmD*
1258147	1259782	+	2	*rbgA*, *rnhB*
1259891	1261987	+	2	*sucC*, SAS1180
1262214	1264604	+	2	SAS1181, SAS1182
1269778	1271216	+	2	SAS1186, SAS1187
1271282	1273483	+	2	*hslU*, SAS1189
1275792	1277087	+	2	*pyrH*, *frr*

Start	Stop	Strand	Number of Genes	Genes
1277460	1279019	+	2	SAS1194, SAS1195
1279231	1282240	+	2	SAS1196, SAS1197
1287104	1291508	+	5	SAS1199, *nusA*, SAS1201, SAS1202, *infB*
1292414	1294317	+	2	SAS1205, SAS1206
1299331	1305704	+	5	SAS1210, SAS1211, SAS1212, SAS1213, SAS1214
1305809	1307659	+	3	SAS1215, SAS1216, SAS1217
1313781	1316408	+	2	SAS1223, SAS1224
1316929	1319357	+	3	SAS1226, SAS1227, SAS1228
1319660	1324848	+	3	SAS1229, *mutL*, SAS1231
1329747	1331862	+	3	SAS1235, *miaA*, SAS1237
1332672	1335167	+	2	SAS1239, SAS1240
1335411	1337138	+	2	SAS1241, SAS1242
1343195	1343599	+	2	SAS1251, SAS1252
1348288	1351590	+	4	SAS1259, SAS1260, SAS1261, SAS1262
1356093	1359356	+	3	SAS1268, SAS1269, SAS1270
1372505	1376659	+	2	SAS1285, SAS1286
1384872	1389271	+	2	SAS1294, SAS1295
1392379	1393675	+	2	SAS1298, SAS1299
1403108	1409425	+	7	SAS1307, SAS1308, *trpD*, *trpC*, SAS1311, SAS1312, *trpA*
1409747	1412287	+	2	SAS1314, SAS1315
1414916	1418179	-	4	SAS1320, SAS1321, SAS1322, SAS1323
1420985	1422484	-	2	SAS1326, SAS1327
1422531	1424376	-	2	SAS1328, SAS1329
1431312	1434655	+	4	SAS1335, *dapA*, SAS1337, SAS1338
1434798	1438294	+	3	SAS1339, SAS1340, SAS1341
1439974	1442059	+	3	SAS1345, SAS1346, SAS1347
1444640	1447331	-	2	SAS1351, SAS1352
1447512	1448553	-	2	SAS1353, SAS1354
1449133	1453213	-	2	SAS1355, *sucA*
1453497	1455508	-	2	SAS1357, SAS1358
1456295	1458517	-	3	SAS1359, *murG*, SAS1361
1461387	1463074	-	4	SAS1364, SAS1365, SAS1366, SAS1367
1463160	1464493	-	2	SAS1368, SAS1369
1466073	1468146	-	4	SAS1371, SAS1372, SAS1373, SAS1374
1468391	1469261	+	2	SAS1375, SAS1375a
1498906	1501299	-	2	SAS1380, SAS1381
1502988	1507326	-	2	SAS1383, SAS1384
1511752	1513016	-	3	SAS1388, SAS1389, SAS1390
1513555	1516361	+	2	*recU*, SAS1393
1516925	1518606	-	3	SAS1394, SAS1395, SAS1396
1520548	1526572	-	4	SAS1398, SAS1399, SAS1400, SAS1401
1528219	1534200	-	6	SAS1404, SAS1405, SAS1406, SAS1407, *aroB*, SAS1409
1535539	1537800	-	3	SAS1411, *ubiE*, SAS1413
1538674	1540999	-	2	*gpsA*, *engA*
1547914	1550236	-	2	SAS1422, SAS1423
1554595	1557677	-	2	SAS1429, SAS1430
1557922	1560397	-	2	SAS1431, SAS1432
1560512	1562532	-	3	SAS1433, SAS1434, SAS1435
1566422	1567444	-	2	SAS1441, SAS1442
1572396	1575080	-	2	SAS1447, SAS1448
1579040	1580471	-	2	SAS1452, SAS1453

Start	Stop	Strand	Number of Genes	Genes
1580822	1585521	-	4	SAS1454, SAS1455, SAS1456, SAS1457
1585672	1587819	-	2	SAS1458, SAS1459
1588251	1591076	-	4	SAS1460, SAS1461, *xseA*, *nusB*
1591136	1593332	-	3	SAS1464, SAS1465, SAS1466
1593806	1595450	-	2	SAS1467, SAS1468
1595555	1596368	+	2	SAS1469, SAS1470
1598171	1602093	-	3	SAS1473, SAS1474, *gcvT*
1603009	1606475	-	6	SAS1477, SAS1478, SAS1479, SAS1480, SAS1481, SAS1482
1606527	1610657	-	6	SAS1483, SAS1484, SAS1485, SAS1486, SAS1487, SAS1488
1614199	1615459	-	2	SAS1492, SAS1493
1616412	1618658	-	2	SAS1495, SAS1496
1618772	1620552	-	2	SAS1497, SAS1498
1627220	1631071	-	6	SAS1504, *era*, SAS1506, SAS1507, SAS1508, SAS1509
1631375	1633804	-	3	SAS1510, SAS1511, SAS1512
1634493	1638681	-	4	SAS1514, SAS1515, *prmA*, SAS1517
1640718	1642353	-	2	SAS1519, SAS1520
1647640	1650307	-	2	SAS1525, SAS1526
1651125	1656792	-	9	SAS1528, SAS1529, SAS1530, SAS1531, SAS1532, SAS1533, SAS1534, SAS1535, SAS1536
1660543	1666096	-	6	SAS1540, SAS1541, SAS1542, SAS1543, SAS1544, SAS1545
1666422	1670393	-	5	*greA*, SAS1547, SAS1548, SAS1549, SAS1550
1670678	1671693	-	3	SAS1551, SAS1552, SAS1553
1674729	1677876	-	2	SAS1555, SAS1556
1678457	1680718	-	2	*mnmA*, SAS1558
1682280	1682648	-	2	SAS1560, SAS1561
1685924	1688968	-	2	*aspS*, *hisS*
1689429	1692975	-	3	SAS1568, SAS1569, SAS1570
1693382	1696195	-	2	SAS1571, SAS1572
1698952	1704832	-	7	SAS1574, *tgt*, *queA*, *ruvB*, *ruvA*, SAS1579, *obgE*
1705317	1706247	-	3	*rpmA*, SAS1582, *rplU*
1706609	1707981	-	2	SAS1584, SAS1585
1709672	1711062	-	2	*radC*, SAS1590
1711332	1715246	-	2	SAS1591, *valS*
1716432	1717628	-	2	SAS1594, SAS1595
1719035	1721628	-	3	SAS1597, SAS1598, *hemC*
1721670	1723853	-	2	SAS1600, *hemA*
1727691	1729247	-	2	SAS1605, SAS1606
1729792	1730548	-	2	*rpmI*, *infC*
1735045	1737836	-	3	SAS1612, SAS1613, *nrdR*
1739242	1743399	-	3	*coaE*, SAS1617, SAS1618
1745679	1748044	-	2	SAS1620, SAS1621
1752929	1755676	-	2	SAS1625, *pfkA*
1755945	1757746	-	2	SAS1627, SAS1628
1759620	1763779	-	2	SAS1630, SAS1631
1774460	1776822	-	2	SAS1642, SAS1643
1781934	1784685	+	2	SAS1649, SAS1650
1799578	1801404	+	2	SAS1660, SAS1661
1806507	1813171	-	4	*murC*, SAS1667, SAS1668, SAS1669
1816558	1818008	-	2	*trmB*, SAS1675
1818559	1820820	-	2	SAS1676, SAS1677
1821513	1824305	-	3	SAS1678, SAS1679, SAS1680

Start	Stop	Strand	Number of Genes	Genes
1832983	1835736	-	2	SAS1683, *leuS*
1837318	1838831	+	2	SAS1686, SAS1687
1842110	1845461	-	4	*ribH*, SAS1692, SAS1693, SAS1694
1847967	1849573	+	2	SAS1696, SAS1697
1855492	1856285	+	2	SAS1706, SAS1707
1862281	1863511	-	2	SAS1713, SAS1714
1863825	1866309	-	2	SAS1716, SAS1717
1871836	1872406	-	2	SAS1725, SAS1726
1879882	1882630	-	2	SAS1731, SAS1732
1888168	1896483	-	7	SAS1739, SAS1740, SAS1741, SAS1742, SAS1743, SAS1744, SAS1745
1897813	1899733	-	2	*hlgB*, *hlgA*
1900630	1901886	+	2	SAS1750, SAS1751
1905219	1907566	-	2	SAS1753, *hemH*
1909551	1911507	-	2	SAS1757, SAS1758
1912205	1912754	+	2	SAS1760, SAS1760a
1915148	1920208	-	3	SAS1763, SAS1764, SAS1765
1923626	1925383	-	2	SAS1769, SAS1770
1928923	1929345	+	2	SAS1774, SAS1775
1929410	1930186	-	2	SAS1776, SAS1777
1930345	1931947	-	2	SAS1778, SAS1779
1932098	1934270	-	2	SAS1780, SAS1781
1944993	1946401	-	2	SAS1784, SAS1785
1954651	1958138	-	4	SAS1792, SAS1793, SAS1794, *recX*
1962608	1964082	-	2	SAS1801, SAS1802
1964214	1964960	+	2	SAS1803, SAS1804
1966561	1969322	-	4	SAS1806, SAS1807, SAS1808, SAS1809
1972943	1974989	-	2	SAS1813, SAS1814
1981852	1985053	-	3	*gatB*, *gatA*, *gatC*
1987047	1993147	-	4	SAS1826, SAS1827, SAS1828, SAS1829
1995886	1997409	+	2	SAS1832, SAS1833
1997701	1998457	-	2	SAS1834, SAS1835
1998728	2001011	-	2	*nadE*, SAS1837
2001195	2003085	+	2	SAS1838, SAS1839
2013965	2015361	-	2	SAS1850, SAS1851
2016695	2020333	-	5	SAS1854, SAS1855, SAS1856, SAS1857, SAS1858
2025851	2026362	+	2	SAS1863a, SAS1864
2029184	2030205	-	2	SAS1869, SAS1870
2032611	2042693	-	4	SAS1875, SAS1876, SAS1877, SAS1878
2043442	2047254	-	7	SAS1881, SAS1882, SAS1883, SAS1884, SAS1885, SAS1886, SAS1887
2047342	2051553	-	5	SAS1888, SAS1889, SAS1890, SAS1891, SAS1892
2051682	2052494	-	2	SAS1893, SAS1894
2052609	2054260	-	5	SAS1895, SAS1896, SAS1897, SAS1898, SAS1899
2054601	2057118	-	5	SAS1901, SAS1902, SAS1903, SAS1904, SAS1905
2057331	2060737	-	4	SAS1906, SAS1907, SAS1908, SAS1909
2060830	2061308	-	2	SAS1910, SAS1911
2062014	2062382	-	2	SAS1912a, SAS1913
2063763	2064265	-	2	SAS1917, SAS1918
2065390	2066870	+	2	SAS1920, SAS1921
2069110	2071200	-	2	SAS1924, SAS1925
2078674	2079476	-	2	SAS1932, SAS1933
2087174	2089933	+	4	SAS1941, SAS1942, SAS1943, SAS1944

Start	Stop	Strand	Number of Genes	Genes
2090302	2092742	-	2	SAS1945, SAS1946
2102039	2104631	-	4	SAS1954, SAS1955, SAS1956, SAS1957
2105109	2108594	+	2	SAS1958, SAS1959
2108985	2115837	+	6	SAS1961, SAS1962, SAS1963, SAS1964, leuD, SAS1966
2122456	2125054	-	2	SAS1967, SAS1968
2125487	2127039	-	3	SAS1969, SAS1970, SAS1971
2128508	2129037	-	2	SAS1973, SAS1974
2130336	2133232	-	4	*acpS*, SAS1977, SAS1978, SAS1979
2133441	2137743	-	3	SAS1980, SAS1981, SAS1982
2138015	2141367	+	2	SAS1983, SAS1984
2143773	2146216	-	2	SAS1986, *ddl*
2148243	2148757	-	2	SAS1990, SAS1991
2148923	2151082	+	2	SAS1992, SAS1993
2152654	2155584	-	4	*thiE*, SAS1996, SAS1997, SAS1998
2158279	2160018	-	2	*fabZ*, SAS2003
2160924	2166269	-	6	*atpC*, SAS2006, SAS2007, SAS2008, SAS2009, SAS2010
2166721	2167823	-	2	SAS2012, SAS2013
2167990	2171587	-	4	SAS2014, *upp*, *glyA*, SAS2017
2171694	2173156	-	2	SAS2018, SAS2019
2173240	2175739	-	3	SAS2020, *prfA*, SAS2022
2189418	2191795	-	2	SAS2035, SAS2036
2192728	2194393	-	2	SAS2038, SAS2039
2200041	2201240	-	2	SAS2045, SAS2046
2202493	2203795	+	2	SAS2048, SAS2049
2211611	2216868	+	4	SAS2057, SAS2058, SAS2059, SAS2060
2224979	2228104	-	3	*glmM*, SAS2064, SAS2065
2240465	2242354	-	2	SAS2071, SAS2072
2245088	2248079	-	3	SAS2076, SAS2077, SAS2078
2248471	2252485	-	3	SAS2079, SAS2080, SAS2081
2255295	2256083	-	2	SAS2084, SAS2085
2261788	2268157	-	7	SAS2090, SAS2091, SAS2092, SAS2093, SAS2094, SAS2095, SAS2096
2276958	2279363	-	2	SAS2105, SAS2106
2280339	2281188	-	2	*rpsI*, *rplM*
2281428	2284698	-	4	*truA*, SAS2111, *cbiO*, *cbiO*
2285236	2286565	-	2	*rplQ*, SAS2115
2286640	2287804	-	4	SAS2116, *rpsM*, *rpmJ*, *infA*
2287997	2296295	-	18	*adk*, *secY*, *rplO*, *rpmD*, *rpsE*, *rplR*, *rplF*, *rpsH*, *rpsN*, *rplE*, *rplX*, *rplN*, *rpsQ*, SAS2133, *rplP*, *rpsC*, *rplV*, *rpsS*
2296362	2299151	-	5	*rplB*, *rplW*, *rplD*, *rplC*, *rpsJ*
2314265	2315909	+	2	SAS2155, SAS2156
2316939	2321027	-	6	SAS2158, *mobA*, SAS2160, SAS2161, SAS2162, SAS2163
2321586	2323127	-	2	SAS2165, SAS2166
2323297	2325370	-	3	SAS2167, SAS2168, SAS2169
2333148	2338196	+	7	*ureA*, *ureB*, *ureC*, *ureE*, SAS2182, SAS2183, SAS2184
2339550	2342275	-	2	SAS2187, SAS2188
2351356	2352405	-	2	SAS2198, SAS2199
2352615	2356042	-	2	SAS2200, SAS2201
2364723	2365261	+	2	SAS2210, SAS2211
2365357	2366194	-	2	SAS2212, SAS2213
2376462	2379361	-	2	SAS2223, SAS2224
2385197	2386572	-	2	SAS2230, SAS2231

Start	Stop	Strand	Number of Genes	Genes
2386790	2388914	-	2	SAS2232, SAS2233
2392365	2394392	-	2	SAS2237, SAS2238
2394489	2395396	+	2	SAS2239, SAS2240
2397516	2400107	-	2	SAS2243, SAS2244
2405957	2407677	-	2	SAS2250, SAS2251
2407813	2409853	+	2	SAS2252, SAS2253
2410016	2410911	+	2	SAS2254, SAS2255
2420982	2422453	-	2	SAS2263, SAS2264
2440294	2448976	-	7	SAS2282, SAS2283, SAS2284, SAS2285, SAS2286, SAS2287, SAS2288
2449284	2452975	-	3	SAS2289, SAS2290, SAS2291
2458576	2459093	-	2	SAS2298, SAS2299
2459366	2460346	-	2	SAS2300, SAS2301
2461948	2464155	-	3	SAS2303, SAS2304, SAS2305
2472070	2473996	+	2	SAS2311, SAS2312
2474048	2479353	-	6	SAS2313, SAS2314, SAS2315, SAS2316, SAS2317, SAS2318
2479806	2483348	-	2	SAS2319, SAS2320
2502112	2505623	-	4	SAS2337, SAS2338, SAS2339, SAS2340
2512013	2513444	-	2	SAS2345, SAS2346
2515124	2515647	-	2	SAS2348, SAS2349
2519513	2525684	-	6	SAS2353, SAS2354, SAS2355, SAS2356, SAS2357, SAS2358
2525824	2528771	-	3	SAS2359, SAS2360, SAS2361
2533848	2535141	-	3	SAS2366, SAS2367, SAS2368
2541264	2544519	-	2	SAS2376, SAS2377
2547569	2548976	-	2	SAS2381, SAS2382
2565005	2567262	-	2	SAS2391, SAS2392
2575508	2576975	+	2	SAS2399, SAS2400
2580839	2582422	-	2	SAS2403, SAS2404
2587844	2589216	+	2	SAS2412, SAS2413
2591598	2594237	-	3	SAS2416, SAS2417, SAS2418
2602604	2603681	-	2	SAS2426, SAS2427
2612090	2614506	-	3	SAS2435, SAS2436, SAS2437
2624315	2626490	-	2	SAS2445, SAS2446
2626917	2632453	-	5	SAS2447, SAS2448, SAS2449, SAS2450, SAS2451
2641488	2643022	-	3	SAS2461, SAS2462, SAS2463
2643154	2644408	+	2	SAS2464, SAS2465
2644506	2646354	+	2	SAS2466, SAS2467
2647488	2649928	-	2	SAS2469, SAS2470
2650223	2651168	-	3	SAS2471, SAS2472, SAS2473
2659104	2661151	-	3	SAS2482, p*anC, panB*
2682280	2684663	-	2	SAS2502, SAS2503
2691619	2694371	-	2	SAS2508, SAS2509
2694479	2696268	-	3	SAS2510, SAS2511, SAS2512
2703620	2706014	-	2	SAS2518, SAS2519
2706099	2708377	-	2	SAS2520, SAS2521
2714927	2717831	+	2	SAS2527, SAS2528
2726137	2736664	-	7	SAS2533, SAS2534, SAS2535, SAS2536, SAS2537, SAS2538, *secY*
2745171	2746404	+	2	SAS2542, SAS2543
2747692	2748671	-	2	SAS2546, SAS2547
2748906	2751041	-	3	SAS2548, SAS2549, SAS2550
2752614	2756029	+	4	SAS2552, SAS2553, SAS2554, SAS2555

Start	Stop	Strand	Number of Genes	Genes
2759610	2766535	-	9	SAS2557, SAS2558, SAS2559, *hisH*, *hisB*, SAS2562, SAS2563, *hisG*, *hisZ*
2768625	2771167	-	2	SAS2568, SAS2569
2771220	2772651	-	2	SAS2570, SAS2571
2788329	2790957	+	2	SAS2585, SAS2586
2794796	2797392	-	2	*gidB*, SAS2593

TABLE S2, Sheet 4: *Staphylococcus aureus* subsp. aureus MSSA476 (Transcript Rockhopper output)

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1878		+	*dnaA*	SAS0001	chromosomal replication initiation protein	67
3289		+	-	SAS0002	DNA polymerase III subunit beta	88
3915		+	-	SAS0003	hypothetical protein	30
5024		+	*recF*	SAS0004	recombination protein F	22
6968	7004	+	-	SAS0005	-	111
9668	9808	+	-	SAS0006	DNA gyrase subunit A	128
9755	9677	-	-	SAS0007	hypothetical protein	306
12407		+	*hutH*	SAS0008	histidine ammonia-lyase	77
	12725	+	-	SASs001	-	3
14072		+	-	SAS0009	seryl-tRNA synthetase	76
15419		+	-	SAS0010	hypothetical protein	127
15745		+	-	SAS0011	hypothetical protein	37
	16047	+	-	SASs002	-	48
17076	17076	+	-	SAS0012	hydrolase	124
18307		+	-	SAS0013	hypothetical protein	65
20289		+	-	SAS0014	hypothetical protein	116
20732	20763	+	*rplI*	SAS0015	50S ribosomal protein L9	172
22164	22164	+	-	SAS0016	replicative DNA helicase	184
23726	23726	+	-	SAS0017	adenylosuccinate synthetase	158
	24230	+	-	SASt001	Glu tRNA	708
	24310	+	-	SASt002	Asp tRNA	1206
	24308	-	-	Predicted RNA	antisense: SASt002	801
25631		+	-	SAS0018	response regulator protein	79
27470		+	-	SAS0019	sensor kinase	139
28797		+	-	SAS0020	hypothetical protein	248
29586		+	-	SAS0021	hypothetical protein	98
30775		+	-	SAS0022	metallo-beta-lactamase superfamily protein	79
33321	33449	+	-	SAS0023	5'-nucleotidase	163
	31389	-	-	Predicted RNA	antisense: SAS0023	650
34168		+	-	SAS0024	rRNA large subunit methyltransferase	95
34546		-	-	SAS0025	type I restriction enzyme protein	76
37588		-	-	SAS0026	type I restriction enzyme specificity protein	10
38891		-	-	SAS0027	type I restriction enzyme methylase	80
40866		-	-	SAS0028	hypothetical protein	78
41101	41098	-	-	SAS0029	hypothetical protein	132
41620		-	-	SAS0030	hypothetical protein	98
42024		-	-	SAS0031	hypothetical protein	93
42831		-	*ccrB*	SAS0032	site-specific recombinase	78
44478		-	*ccrA1*	SAS0033	site-specific recombinase	94
46015		-	-	SAS0034	hypothetical protein	97
47784		-	-	SAS0035	hypothetical protein	94
48253		-	-	SAS0036	hypothetical protein	20
50616		+	-	SAS0037	hypothetical protein	9
51482		+	-	SAS0038	hypothetical protein	23
52234		+	-	SAS0040	hypothetical protein	0
53458		+	-	SAS0043	fusidic acid resistance protein	49
54773		+	-	SAS0044	hypothetical protein	8
55452		-	-	SAS0046	hypothetical protein	32
56889		+	-	SAS0047	hypothetical protein	6
58476		+	-	SAS0048	hypothetical protein	48
60956		+	*seh*	SAS0051	enterotoxin H	38
62325		+	-	SAS0053	acetyltransferase (GNAT) family protein	18

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
62876		-	-	SAS0054	hypothetical protein	41
63631		-	-	SAS0055	hypothetical protein	17
65095		+	-	SAS0056	hypothetical protein	83
66460		+	-	SAS0057	metallo-beta-lactamase superfamily protein	60
67672		+	-	SAS0058	oxidoreductase	59
68339		-	-	SAS0059	hypothetical protein	43
69837		+	-	SAS0060	hypothetical protein	16
70182		+	-	SAS0061	hypothetical protein	10
70254		-	-	SAS0062	hypothetical protein	55
71467		-	-	SAS0063	hypothetical protein	48
72991		+	-	SAS0064	LysR family regulatory protein	35
73346		-	-	SAS0065	hypothetical protein	32
74713		+	-	SAS0066	LysR family regulatory protein	17
75749		+	-	SAS0067	hypothetical protein	14
79168		+	-	SAS0068	hypothetical protein	61
79707		+	-	SAS0069	hypothetical protein	52
81888		+	-	SAS0072	lipoprotein	14
82710		+	-	SAS0073	lipoprotein	7
83753	83753	+	-	SAS0074	lipoprotein	134
85132		+	-	SAS0075	hypothetical protein	60
85458		+	-	SAS0076	hypothetical protein	67
85773		+	-	SAS0077	hypothetical protein	44
88262		+	-	SAS0078	regulatory protein	95
89591	89591	+	-	SAS0079	peptidase	105
90981	90981	+	-	SAS0080	transporter protein	199
91468		-	-	SAS0081	Na+/Pi-cotransporter protein	96
93453		-	-	SAS0082	myosin-cross-reactive antigen	94
96052		+	-	SAS0083	hypothetical protein	58
97906		+	-	SAS0084	L-lactate permease 1	70
98235		-	-	SAS0085	immunoglobulin G binding protein A precursor	74
100134		-	-	SAS0086	regulatory protein	54
101255	101222	-	-	SAS0087	siderophore transport system permease	117
102250		-	-	SAS0088	siderophore transport system permease	178
103261		-	-	SAS0089	lipoprotein	52
105464		+	-	SAS0090	pyridoxal-phosphate dependent protein	161
106471	106471	+	-	SAS0091	ornithine cyclodeaminase	130
108246		+	-	SAS0092	siderophore biosynthesis protein	158
109495		+	-	SAS0093	transport protein	487
	108493	-	-	predicted RNA	antisense: SAS0093	676
111221		+	-	SAS0094	siderophore biosynthesis protein	92
112980		+	-	SAS0095	siderophore biosynthesis protein	229
113731		+	-	SAS0096	aldolase	406
114933	114933	+	-	SAS0097	pyridoxal-dependent decarboxylase	161
115701	115707	+	-	SAS0098	hypothetical protein	346
116304		+	-	SAS0099	hypothetical protein	31

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
116980		+	-	SAS0100	hypothetical protein	45
117968	118155	+	-	SAS0101	acetoin reductase	103
118017	117905	-	-	SAS0101a	hypothetical protein	216
119284		+	-	SAS0102	NAD dependent epimerase/dehydratase family protein	64
119939		+	-	SAS0103	sugar transferase	91
121315		+	-	SAS0104	galactosyl transferase	76
122534		+	-	SAS0105	hypothetical protein	88
123954		+	-	SAS0106	polysaccharide biosynthesis protein	61
124821	124856	+	-	SAS0107	superoxide dismutase	113
125914	126168	+	-	SAS0108	surface anchored protein	112
126105		-	-	SAS0109	GntR family transcriptional regulator	50
127803		+	-	SAS0110	purine nucleoside phosphorylase	83
129162		+	-	SAS0111	transport system protein	63
129905		+	-	SAS0112	deoxyribose-phosphate aldolase	74
131111	131625	+	-	SAS0113	phosphopentomutase	170
131242	130934	-	-	SAS0114	binding-protein-dependent transport systems membrane component	197
132054		-	-	SAS0115	binding-protein-dependent transport system membrane component	131
132856	132856	-	-	SAS0116	ABC transporter ATP binding protein	199
133843		-	-	SAS0117	lipoprotein	23
136572	136572	+	-	SAS0118	hypothetical protein	189
138158		+	-	SAS0119	nucleotidase	80
143980	144317	+	-	SAS0123	bifunctional acetaldehyde-CoA/alcohol dehydrogenase	141
144986	145001	+	-	SAS0124	capsular polysaccharide synthesis enzyme	146
145688	145688	+	-	SAS0125	capsular polysaccharide synthesis enzyme	117
146455		+	-	SAS0126	capsular polysaccharide synthesis enzyme	54
148298		+	-	SAS0127	capsular polysaccharide synthesis enzyme	117
149316		+	-	SAS0128	capsular polysaccharide synthesis enzyme	25
150438	150441	+	-	SAS0129	capsular polysaccharide synthesis enzyme	208
151566		+	-	SAS0130	capsular polysaccharide synthesis enzyme	82
152648		+	-	SAS0131	capsular polysaccharide synthesis enzyme	24
154035		+	-	SAS0132	capsular polysaccharide synthesis enzyme	54
154589		+	-	SAS0133	capsular polysaccharide synthesis enzyme	22
155836		+	-	SAS0134	capsular polysaccharide synthesis enzyme	18
157075		+	-	SAS0135	capsular polysaccharide synthesis enzyme	67

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
157643		+	-	SAS0136	capsular polysaccharide synthesis enzyme	62
158530	158530	+	-	SAS0137	capsular polysaccharide synthesis enzyme	165
159846	159846	+	-	SAS0138	capsular polysaccharide synthesis enzyme	202
161068		+	-	SAS0139	capsular polysaccharide synthesis enzyme	37
161133	161104	-	-	SAS0140	heme-degrading monooxygenase IsdI	104
161466		-	-	SAS0141	hypothetical protein	21
163763	163763	+	-	SAS0142	aldehyde dehydrogenase	197
165368		+	-	SAS0143	cation efflux system protein	60
165702	165702	+	-	SAS0144	hypothetical protein	159
165662	165444	-	-	SAS0145	hypothetical protein	124
166555		+	-	SAS0146	hypothetical protein	0
167637	167637	+	-	SAS0147	ABC transporter ATP-binding protein	179
168625		+	-	SAS0148	lipoprotein	194
169383		+	-	SAS0149	transport system permease	59
170427		+	-	SAS0150	hypothetical protein	52
171003		+	-	SAS0151	hypothetical protein	50
172202		+	-	SAS0152	formate dehydrogenase	55
173838		+	-	SAS0153	transporter protein	24
181460	181460	+	-	SAS0154	non-ribosomal peptide synthetase	196
182117	182395	+	-	SAS0155	4'-phosphopantetheinyl transferase	112
182445		-	-	SAS0156	hypothetical protein	26
183194		-	-	SAS0157	amino acid kinase	59
183974	183974	-	*argJ*	SAS0158	bifunctional ornithine acetyltransferase/N-acetylglutamate synthase	175
185227	185227	-	*argC*	SAS0159	N-acetyl-gamma-glutamyl-phosphate reductase	111
186297	186297	-	-	SAS0160	ornithine aminotransferase	164
187734	187667	-	-	SAS0161	branched-chain amino acid transport system carrier protein	105
189365		-	-	SAS0162	isochorismatase	82
189989	189923	-	-	SAS0163	thiamine pyrophosphate enzyme	137
191902	191630	-	-	SAS0164	glucose-specific PTS transporter protein, IIABC component	249
	193224	+	-	predicted RNA	antisense: SAS0164	753
195587		+	-	SAS0165	hypothetical protein	183
196483	196483	+	*murQ*	SAS0166	N-acetylmuramic acid 6-phosphate etherase	246
197949		+	-	SAS0167	sucrose-specific PTS transporter protein	189
198827	198827	+	-	SAS0168	transcriptional regulator	234
199021	197806	-	-	SAS0169	hypothetical protein	149
202304		+	-	SAS0170	type I restriction enzyme	74
202451		-	-	SAS0171	hypothetical protein	13

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
203931		+	-	SAS0172	hypothetical protein	11
204608		+	-	SAS0173	ABC transporter ATP-binding protein	16
205783		+	-	SAS0174	permease	31
206211		+	-	SAS0175	hypothetical protein	18
207967		+	-	SAS0176	hypothetical protein	68
208469		+	-	SAS0177	hypothetical protein	28
209203		+	-	SAS0178	ATP-binding transport protein	75
209366		+	-	SAS0179	hypothetical protein	46
209413		-	-	SAS0180	hypothetical protein	1
210076	210076	-	-	SAS0181	ABC transporter ATP-binding protein	237
213103	213108	+	-	SAS0182	transport system permease	115
214272		+	-	SAS0183	transport system permease	93
216064		+	-	SAS0184	RGD-containing lipoprotein	67
218108	218288	+	-	SAS0185	gamma-glutamyltranspeptidase	231
218421		-	-	SAS0186	hypothetical protein	86
219384		-	*acpD*	SAS0187	azoreductase	48
220797		+	-	SAS0188	peptidase	65
222277		+	-	SAS0189	ABC transporter ATP-binding protein	58
223561		+	-	SAS0190	extracellular sugar-binding lipoprotein	36
224832	224832	+	-	SAS0191	sugar transport system permease	145
225673	225673	+	-	SAS0192	sugar transport system permease	159
226823	226834	+	-	SAS0193	oxidoreductase	194
227888		+	-	SAS0194	oxidoreductase	97
228911	229081	+	-	SAS0195	hypothetical protein	113
229272		-	-	SAS0196	hypothetical protein	25
231378	232551	+	*uhpT*	SAS0197	sugar phosphate antiporter	114
231737		-	-	SAS0198	response regulator	70
232488		-	-	SAS0199	sensor kinase	39
234041		-	-	SAS0200	lipoprotein	196
237846	237868	+	-	SAS0201	formate acetyltransferase	167
238624		+	-	SAS0202	pyruvate formate-lyase activating enzyme	98
238922	238959	+	-	SAS0203	hypothetical protein	166
240723		+	-	SAS0204	hypothetical protein	61
240887		-	-	SAS0205	hypothetical protein	102
243322		+	-	SAS0206	staphylocoagulase precursor	76
243925	243925	-	-	SAS0207	thiolase	249
245139	245139	-	-	SAS0208	fatty oxidation complex protein	145
247587	247587	-	-	SAS0209	acyl-CoA dehydrogenase	148
248910	248799	-	-	SAS0210	acyl-CoA synthetase	153
250441	250441	-	-	SAS0211	acetyl-CoA transferase	242
253601		+	-	SAS0212	hypothetical protein	81
253915		-	-	SAS0213	extracellular solute-binding lipoprotein	72
255588		-	-	SAS0214	hypothetical protein	35

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
256101		-	-	SAS0215	hypothetical protein	30
256300		-	-	SAS0216	flavohemoprotein	89
258971	258971	+	-	SAS0217	L-lactate dehydrogenase	182
259291	259291	-	-	SAS0218	PTS transport system, IIBC component	184
262117	262117	+	-	SAS0219	inosine-uridine preferring nucleoside hydrolase	311
262323		+	-	SAS0219a	hypothetical protein	90
264550		+	-	SAS0220	PTS regulator	86
265002	265024	+	-	SAS0221	PTS transport system, IIA component	274
265303	265303	+	-	SAS0222	PTS transport system, IIB component	127
266789	266789	+	-	SAS0223	PTS transport system, IIC component	154
267862		+	-	SAS0224	zinc-binding dehydrogenase	69
268010		+	-	SAS0225	hypothetical protein	62
269077	269077	+	-	SAS0226	zinc-binding dehydrogenase	220
270321		+	*ispD*	SAS0227	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	69
271339		+	-	SAS0228	zinc-binding dehydrogenase	34
273055		+	-	SAS0229	hypothetical protein	22
273367	273307	-	-	SAS0230	hypothetical protein	104
273828		+	-	SAS0230a	hypothetical protein	49
275250	275525	+	-	SAS0231	teichoic acid biosynthesis protein	145
276242		+	*ispD*	SAS0232	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	47
277260		+	-	SAS0233	zinc-binding dehydrogenase	65
278970		+	-	SAS0234	hypothetical protein	50
280724		+	-	SAS0235	glycosyl transferase family protein	58
281542		+	-	SAS0236	cell wall biosynthesis protein ScdA	11
283540		+	-	SAS0237	autolysin sensor kinase	80
284283	284335	+	-	SAS0238	two-component response regulator	109
284839		+	-	SAS0239	murein hydrolase regulator LrgA	122
285533	285639	+	-	SAS0240	antiholin-like protein LrgB	136
285641		-	-	SAS0241	GntR family transcriptional regulator	79
287285	287300	+	-	SAS0242	PTS transport system protein	183
288737	288761	+	-	SAS0243	6-phospho-beta-glucosidase	211
289230		-	-	SAS0244	hypothetical protein	93
290242	290092	-	-	SAS0245	ribokinase	124
291184		-	-	SAS0246	D-ribose pyranase	35
291603	291603	-	-	SAS0247	sugar transport protein	114
292716	292586	-	-	SAS0248	LacI family regulatory protein	150
294045		+	-	SAS0248a	hypothetical protein	0
294487		+	-	SAS0249	hypothetical protein	9
294615	294615	-	-	SAS0250	transport protein	148
297217		+	-	SAS0251	choloylglycine hydrolase	83
298493	298894	+	-	SAS0252	peptidoglycan hydrolase	113
298545		-	-	SAS0253	ABC transporter ATP-binding protein	90

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
299218	299218	-	-	SAS0254	hypothetical protein	108
300135		-	-	SAS0255	hypothetical protein	65
301369		-	-	SAS0256	hypothetical protein	23
303223		-	-	SAS0257	hypothetical protein	100
304657		+	-	SAS0258	hypothetical protein	78
307769		+	-	SAS0259	hypothetical protein	50
308227		+	-	SAS0260	hypothetical protein	18
308441		+	-	SAS0261	hypothetical protein	0
309788		+	-	SAS0262	hypothetical protein	21
314249	314249	+	-	SAS0263	hypothetical protein	126
314671		+	-	SAS0264	hypothetical protein	3
315001		+	-	SAS0265	hypothetical protein	43
315675		+	-	SAS0266	hypothetical protein	1
315992		+	-	SAS0267	hypothetical protein	95
317846		+	-	SAS0268	hypothetical protein	101
318351		+	-	SAS0269	hypothetical protein	3
319245		+	-	SAS0270	hypothetical protein	41
320003		+	-	SAS0271	hypothetical protein	10
320702		+	-	SAS0272	hypothetical protein	59
321213		+	-	SAS0273	hypothetical protein	18
321724		+	-	SAS0274	hypothetical protein	27
322235		+	-	SAS0275	hypothetical protein	41
322746		+	-	SAS0276	hypothetical protein	26
323257		+	-	SAS0277	hypothetical protein	3
323768		+	-	SAS0278	hypothetical protein	7
324267		+	-	SAS0279	hypothetical protein	0
325266		+	-	SAS0280	hypothetical protein	9
325814		+	-	SAS0281	hypothetical protein	3
326064		-	-	SAS0282	formate/nitrite transporter	12
327127		-	-	SAS0283	amino acid transport system	49
329908		+	-	SAS0284	hypothetical protein	40
331207		+	-	SAS0285	hypothetical protein	22
331897		+	-	SAS0286	ABC transporter ATP-binding protein	0
333136	333287	+	-	SAS0287	hypothetical protein	136
334597		+	-	SAS0288	PfkB family carbohydrate kinase	80
335495	335495	+	-	SAS0289	hypothetical protein	214
336726	336831	+	-	SAS0290	nucleoside permease	167
336832	336330	-	-	SAS0291	sodium:solute symporter family protein	125
338404		-	-	SAS0292	N-acetylneuraminate lyase	60
340303		+	-	SAS0293	ROK family protein	77
340580	340438	-	-	SAS0294	hypothetical protein	193
342189	342568	+	-	SAS0295	N-acetylmannosamine-6-phosphate 2-epimerase	110
342320	342320	-	-	SAS0296	hypothetical protein	151

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
346123		+	-	SAS0297	lipase precursor	92
346365	345024	-	-	SAS0298	hypothetical protein	110
347265	347265	-	-	SAS0299	NADH:flavin oxidoreductase / NADH oxidase family protein	208
349767	349800	+	-	SAS0300	luciferase family protein	178
350133		+	-	SAS0301	glycine cleavage H-protein	236
350934		+	-	SAS0302	hypothetical protein	143
351868		+	-	SAS0303	hypothetical protein	184
352868		+	-	SAS0304	lipoate-protein ligase A	63
354209	357757	+	-	SAS0305	reductase	116
354302	354302	-	*ulaA*	SAS0306	PTS system ascorbate-specific transporter subunit IIC	313
355663	355653	-	-	SAS0307	PTS transport system protein	184
355949	355949	-	-	SAS0308	PTS transport system IIA component	152
356397	356393	-	-	SAS0309	PTS regulator	179
358983	358983	+	-	SAS0310	MarR family transcriptional regulator	140
360445		+	-	SAS0311	hypothetical protein	69
360992		+	-	SAS0312	hypothetical protein	27
361075	361075	-	-	SAS0313	glycerol-3-phosphate transporter	117
363671		+	-	SAS0314	dioxygenase	58
364746	364759	+	-	SAS0315	luciferase-like monooxygenase	126
365326		+	-	SAS0316	NADH-dependent FMN reductase	85
365386		-	-	SAS0317	hypothetical protein	68
367231		+	-	SAS0318	acetyltransferase	29
368352		+	-	SAS0319	lipoprotein	44
369578		+	-	SAS0320	Sec-independent exported protein	69
371271		+	-	SAS0321	hypothetical protein	60
371363		-	-	SAS0322	Sec-independent protein translocase	30
372081		-	-	SAS0323	Sec-independent protein translocase	51
372404		-	-	SAS0324	hypothetical protein	5
373236		+	-	SAS0325	DNA-binding protein	161
373946	373946	+	-	SAS0326	hypothetical protein	147
374813		+	-	SAS0327	ABC transporter ATP-binding protein	49
375442		+	-	SAS0328	hypothetical protein	73
375832		-	-	SAS0329	hypothetical protein	81
378685	378744	+	-	SAS0330	acetyl-CoA acetyltransferase	291
378768	378709	-	-	SAS0331	hypothetical protein	154
379563	379563	-	-	SAS0332	5-methyltetrahydropteroyltriglutamate/ homocysteine S-methyltransferase	178
381788		-	-	SAS0333	bifunctional homocysteine S-methyltransferase/5,10-methylenetetrahydrofolate reductase	97
383598		-	-	SAS0334	Cys/Met metabolism PLP-dependent enzyme	128
384755		-	-	SAS0335	Cys/Met metabolism PLP-dependent enzyme	143

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
	386022	-	-	SASs003	-	131
387367		+	-	SAS0336	DNA-binding protein	32
388405		+	-	SAS0337	hypothetical protein	15
388638		+	-	SAS0338	hypothetical protein	58
389747		+	-	SAS0339	GTP-dependent nucleic acid-binding protein EngD	95
389833		-	-	SAS0340	hypothetical protein	23
390564	390583	+	*rpsF*	SAS0341	30S ribosomal protein S6	181
391088	391088	+	-	SAS0342	single-strand DNA-binding protein	105
391382	391444	+	*rpsR*	SAS0343	30S ribosomal protein S18	128
391648		-	-	SAS0344	hypothetical protein	51
394156	394176	+	-	SAS0347	hypothetical protein	109
394926		+	-	SAS0348	hypothetical protein	5
395646		+	-	SAS0349	hypothetical protein	44
395784		-	-	SAS0350	hypothetical protein	67
396592		+	-	SAS0351	hypothetical protein	22
396631		-	-	SAS0352	hypothetical protein	0
397599		+	-	SAS0353	phosphoglycerate mutase family protein	30
397665		-	-	SAS0354	hypothetical protein	69
398234		+	-	SAS0354a	hypothetical protein	0
398303		-	-	SAS0355	hypothetical protein	41
399002		-	-	SAS0356	hypothetical protein	7
400727		-	-	SAS0357	alkyl hydroperoxide reductase	100
402266		-	-	SAS0358	alkyl hydroperoxide reductase	93
404082	405395	+	-	SAS0359	nitroreductase family protein	107
404163	404163	-	-	SAS0360	sodium:dicarboxylate symporter protein	302
405734		+	-	SAS0360a	hypothetical protein	0
406725	406725	-	-	SAS0361	hypothetical protein	217
407799		-	-	SAS0362	hypothetical protein	58
408604		-	-	SAS0363	hypothetical protein	44
	409294	+	-	SASs004	-	0
410102		+	-	SAS0364	xanthine phosphoribosyltransferase	70
411370		+	-	SAS0365	xanthine permease	58
412874	412874	+	-	SAS0366	inosine-5'-monophosphate dehydrogenase	148
414440	414498	+	*guaA*	SAS0367	GMP synthase	137
416058		+	-	SAS0368	hypothetical protein	35
416687		+	-	SAS0369	hypothetical protein	17
416684		-	-	SAS0370	hypothetical protein	89
417098		-	-	SAS0371	hypothetical protein	21
417703		-	-	SAS0372	hypothetical protein	54
418835		+	-	SAS0373	hypothetical protein	19
421766		+	-	SAS0375	hypothetical protein	27
422304		-	-	SAS0376	hypothetical protein	24
423051		-	-	SAS0377	hypothetical protein	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
423580		-	-	SAS0378	hypothetical protein	28
424502		+	-	SAS0379	hypothetical protein	6
425031		+	-	SAS0380	hypothetical protein	0
425920		+	-	SAS0381	hypothetical protein	15
426094		-	-	SAS0381a	hypothetical protein	25
426195		-	-	SAS0382	hypothetical protein	28
426573		-	-	SAS0383	hypothetical protein	100
428570		+	-	SAS0384	superantigen-like protein	30
429551		+	-	SAS0385	superantigen-like protein	46
430912		+	-	SAS0386	superantigen-like protein	16
432224		+	-	SAS0387	superantigen-like protein	84
433292		+	-	SAS0388	superantigen-like protein 5	75
434436		+	-	SAS0389	superantigen-like protein	0
435553		+	-	SAS0390	superantigen-like protein 7	11
436590		+	-	SAS0391	superantigen-like protein	15
437667		+	-	SAS0392	superantigen-like protein	8
438709		+	-	SAS0393	superantigen-like protein	28
438918		+	-	SAS0393a	hypothetical protein	83
440527		+	-	SAS0394	restriction enzyme modification protein	134
441779		+	-	SAS0395	restriction and modification system specificity protein	62
442877		+	-	SAS0396	superantigen-like protein	21
444401		+	-	SAS0397	hypothetical protein	36
444508		-	-	SAS0398	hypothetical protein	0
445964		+	-	SAS0399	lipoprotein	7
446782		+	-	SAS0400	hypothetical protein	4
447605		+	-	SAS0401	lipoprotein	37
448454		+	-	SAS0402	lipoprotein	30
449448		+	-	SAS0403	lipoprotein	12
450763		+	-	SAS0404	hypothetical protein	30
451083		+	-	SAS0405	hypothetical protein	0
451416		+	-	SAS0406	hypothetical protein	7
451700		-	-	SAS0407	hypothetical protein	18
453294		+	-	SAS0408	cobalamin synthesis protein	74
453691		+	-	SAS0409	hypothetical protein	62
456364		+	-	SAS0410	NADH dehydrogenase subunit 5	72
459082	459243	+	-	SAS0411	hypothetical protein	293
	458954	-	-	predicted RNA	antisense: SAS0411	644
459606	459694	+	-	SAS0412	hypothetical protein	521
459835		-	-	SAS0413	hypothetical protein	9
460963	460963	+	-	SAS0414	hypothetical protein	187
461734		+	-	SAS0415	hypothetical protein	65
462025		+	-	SAS0415a	hypothetical protein	102

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
463527		+	-	SAS0416	sodium:neurotransmitter symporter family protein	73
464649		+	-	SAS0417	pyridoxal-phosphate dependent protein	113
465784		+	-	SAS0418	Cys/Met metabolism PLP-dependent enzyme	95
467106		+	-	SAS0419	ABC transporter ATP-binding protein	57
467769		+	-	SAS0420	transport system membrane protein	92
468648		+	-	SAS0421	lipoprotein	85
469984		+	-	SAS0422	hypothetical protein	42
470169		-	-	SAS0423	hypothetical protein	0
470982		+	-	SAS0424	MutT domain-containing protein	69
471457	471590	+	-	SAS0425	acetyltransferase	132
471626		-	-	SAS0426	hypothetical protein	90
472405		-	-	SAS0427	hypothetical protein	141
473638	473638	-	-	SAS0428	LysR family regulatory protein	130
479203	479220	+	-	SAS0429	glutamate synthase	147
480684	480684	+	*gltD*	SAS0430	glutamate synthase	131
	481045	+	-	SASt003	Ser tRNA	57
482942	482987	+	-	SAS0431	sugar-specific PTS transport system, IIBC component	224
484646		+	-	SAS0432	glycosyl hydrolase	78
485399		+	-	SAS0433	GntR family transcriptional regulator	64
	485680	+	-	SASs005	-	163
486566		+	-	SAS0434	acetyltransferase (GNAT) family protein	90
488332		+	-	SAS0435	DNA polymerase III, tau subunit	62
488739		+	-	SAS0436	hypothetical protein	19
489342		+	*recR*	SAS0437	recombination protein RecR	50
	491750	+	-	SASr001	16S ribosomal RNA	1791
	490527	-	-	predicted RNA	antisense: SASr001	1869
	494975	+	-	SASr002	23S ribosomal RNA	1040
	493495	-	-	predicted RNA	antisense: SASr002	840
	493972	-	-	predicted RNA	antisense: SASr003 SASr002	1992
	495162	+	-	SASr003	5S ribosomal RNA	148
497403		+	-	SAS0438	Orn/Lys/Arg decarboxylase family protein	78
498022		+	*tmk*	SAS0439	thymidylate kinase	26
498379		+	-	SAS0440	hypothetical protein	72
499519		+	-	SAS0441	DNA polymerase III subunit delta'	89
500323		+	-	SAS0442	hypothetical protein	33
500687	500705	+	-	SAS0443	DNA replication intiation control protein YabA	124
501686		+	-	SAS0444	hypothetical protein	16
501927		+	-	SAS0445	hypothetical protein	52

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
502768	503052	+	-	SAS0446	tetrapyrrole (corrin/porphyrin) methylase family protein	126
505026		+	-	SAS0447	methionyl-tRNA synthetase	89
505830	505891	+	-	SAS0448	TatD related DNase	129
506533		+	-	SAS0449	hypothetical protein	90
507437		+	*ksgA*	SAS0450	dimethyladenosine transferase	53
507800		+	-	SAS0451	hypothetical protein	7
508958		+	*ipk*	SAS0452	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	77
509796		+	-	SAS0453	pur operon repressor	42
510193		+	-	SAS0454	regulatory protein	74
510567		+	-	SAS0455	regulatory protein SpoVG	31
512263		+	*glmU*	SAS0456	bifunctional N-acetylglucosamine-1-phosphate uridyltransferase/glucosamine-1-phosphate acetyltransferase	83
513375		+	-	SAS0457	ribose-phosphate pyrophosphokinase	58
514178		+	-	SAS0458	50S ribosomal protein L25	100
515030		+	-	SAS0459	peptidyl-tRNA hydrolase	137
518536		+	-	SAS0460	transcription-repair coupling factor	95
520052		+	-	SAS0461	polysaccharide biosynthesis protein	173
521245		+	-	SAS0462	tetrapyrrole (corrin/porphyrin) methylase	127
521505	521522	+	-	SAS0463	S4 domain-containing protein	125
521915		+	-	SAS0464	cell division protein	102
522421		+	-	SAS0465	hypothetical protein	32
523896		+	-	SAS0466	hypothetical protein	37
524440		+	-	SAS0467	hypoxanthine phosphoribosyltransferase	2
526790	526805	+	-	SAS0468	cell division protein	119
527900		+	*hslO*	SAS0469	Hsp33-like chaperonin	80
529011		+	-	SAS0470	O-acetylserine (thiol)-lyase	57
530030		+	-	SAS0471	dihydropteroate synthase	81
530373		+	-	SAS0472	dihydroneopterin aldolase	16
530846		+	-	SAS0473	2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	72
532872	532904	+	*lysS*	SAS0474	lysyl-tRNA synthetase	103
	533544	+	-	SASr004	5S ribosomal RNA	130
	533632	+	-	SASt004	Val tRNA	170
	533724	+	-	SASt005	Thr tRNA	461
	533803	+	-	SASt006	Lys tRNA	716
	533911	+	-	SASt007	Gly tRNA	1950
	533882	-	-	predicted RNA	antisense: SASt011 SASt010 SASt009 SASt008 SASt007	1689
	534007	+	-	SASt008	Leu tRNA	2723
	534089	+	-	SASt009	Arg tRNA	2604
	534183	+	-	SASt010	Pro tRNA	1306

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
	534282	+	-	SASt011	Ala tRNA	588
	535958	+	-	SASr005	16S ribosomal RNA	1812
	534703	-	-	predicted RNA	antisense: SASt012 SASr005	1872
	536126	+	-	SASt012	Ile tRNA	173
	539216	+	-	SASr006	23S ribosomal RNA	1022
	537687	-	-	predicted RNA	antisense: SASr006	737
	538199	-	-	predicted RNA	antisense: SASr007 SASr006	2166
	539403	+	-	SASr007	5S ribosomal RNA	290
	541169	+	-	SASr008	16S ribosomal RNA	1788
	539964	-	-	predicted RNA	antisense: SASr008	1961
	544410	+	-	SASr009	23S ribosomal RNA	1026
	542871	-	-	predicted RNA	antisense: SASr009	842
	543436	-	-	predicted RNA	antisense: SASr010 SASr009	2155
	544597	+	-	SASr010	5S ribosomal RNA	104
545308		-	-	SAS0475	GntR family transcriptional regulator	20
547681	547681	+	-	SAS0476	pyridoxal biosynthesis lyase PdxS	192
548245		+	-	SAS0477	glutamine amidotransferase subunit PdxT	64
548451		-	-	SAS0478	nucleoside permease	10
550284		+	-	SAS0479	DNA-binding protein	12
550869		+	-	SAS0480	hypothetical protein	132
551866	551866	+	-	SAS0481	ATP:guanido phosphotransferase	107
554336		+	-	SAS0482	stress response-related Clp ATPase	59
556184	556208	+	-	SAS0483	DNA repair protein RadA	157
557282		+	-	SAS0484	hypothetical protein	33
559293	559338	+	*gltX*	SAS0485	glutamyl-tRNA synthetase	150
560363		+	-	SAS0486	serine acetyltransferase	30
561747		+	*cysS*	SAS0487	cysteinyl-tRNA synthetase	107
562144		+	-	SAS0488	hypothetical protein	33
562898		+	-	SAS0489	SpoU rRNA methylase family protein	20
563422		+	-	SAS0490	hypothetical protein	42
564072		+	-	SAS0491	hypothetical protein	46
564330		+	*rpmG*	SAS0492	50S ribosomal protein L33	60
564568		+	*secE*	SAS0493	preprotein translocase subunit SecE	19
565129		+	-	SAS0494	transcription antitermination protein	102
565732	565785	+	*rplK*	SAS0495	50S ribosomal protein L11	128
566632		+	*rplA*	SAS0496	50S ribosomal protein L1	98
567404		+	*rplJ*	SAS0497	50S ribosomal protein L10	98
567815		+	*rplL*	SAS0498	50S ribosomal protein L7/L12	65
568598		+	-	SAS0499	hypothetical protein	79

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
572364	572364	+	*rpoB*	SAS0500	DNA-directed RNA polymerase subunit beta	218
576124	576124	+	-	SAS0501	DNA-directed RNA polymerase subunit beta'	128
576515		+	-	SAS0502	ribosomal protein L7Ae-like	47
577026		+	*rpsL*	SAS0503	30S ribosomal protein S12	92
577562		+	-	SAS0504	30S ribosomal protein S7	76
579766	579766	+	-	SAS0505	elongation factor G	201
581167	581353	+	*tuf*	SAS0506	elongation factor Tu	283
581449	581449	-	-	SAS0507	peptidase	182
583982		+	-	SAS0508	2-amino-3-ketobutyrate CoA ligase	15
585127		+	-	SAS0509	chaperone protein HchA	80
586923		+	-	SAS0510	ribulokinase	31
588102		+	-	SAS0511	hypothetical protein	45
589513		+	-	SAS0512	branched-chain amino acid aminotransferase	47
590447		+	-	SAS0513	haloacid dehalogenase	67
590563		-	-	SAS0514	deoxyadenosine kinase	41
591218		-	-	SAS0515	deoxyadenosine kinase	66
592372		+	-	SAS0516	deaminase	45
593388		+	-	SAS0517	haloacid dehalogenase	24
593975		+	-	SAS0518	hypothetical protein	29
597279		+	-	SAS0519	surface anchored protein	58
601743		+	-	SAS0520	surface anchored protein	69
605562		+	-	SAS0521	bone sialoprotein-binding protein	101
607153		+	-	SAS0522	glycosyl transferase family protein	58
607277		-	-	SAS0523	glycosyl transferase family protein	40
609049	608974	-	-	SAS0524	GTP cyclohydrolase	143
609940		-	-	SAS0525	hypothetical protein	61
610619	610619	-	-	SAS0526	hypothetical protein	129
612015		+	-	SAS0527	glucosamine-6-phosphate isomerase	59
612724		+	-	SAS0528	hexulose-6-phosphate synthase	86
613274		+	-	SAS0529	6-phospho 3-hexuloisomerase	67
614034		+	-	SAS0530	haloacid dehalogenase	45
615936		+	-	SAS0531	proline/betaine transporter	100
616480		+	-	SAS0532	hypothetical protein	50
617856		+	-	SAS0533	AMP-binding protein	30
618997		+	-	SAS0534	ketoacyl-CoA thiolase	104
619337		+	-	SAS0535	hypothetical protein	52
619609		+	-	SAS0536	hypothetical protein	82
619810		-	-	SAS0537	hypothetical protein	24
620484		-	-	SAS0538	phosphomethylpyrimidine kinase	74
622154		+	-	SAS0539	uracil-DNA glycosylase	39
622535		+	-	SAS0540	hypothetical protein	100
623036		+	-	SAS0541	hypothetical protein	100

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
624641		+	-	SAS0542	amino acid permease	83
624782		-	-	SAS0543	hypothetical protein	43
625247		-	-	SAS0544	hypothetical protein	66
626519		+	-	SAS0545	hypothetical protein	44
626560		-	-	SAS0546	heme peroxidase	52
628466	628466	+	*eutD*	SAS0547	phosphotransacetylase	217
629305	629351	+	-	SAS0548	lipoate-protein ligase A	105
630812		+	-	SAS0549	mevalonate kinase	94
631800	631800	+	-	SAS0550	mevalonate diphosphate decarboxylase	143
632889		+	-	SAS0551	phosphomevalonate kinase	69
633407		+	-	SAS0552	hypothetical protein	59
633459	633459	-	-	SAS0553	pyridine nucleotide-disulfide oxidoreductase	112
634768		-	-	SAS0554	hypothetical protein	264
637242	637242	+	-	SAS0555	hypothetical protein	105
637872		+	-	SAS0556	hypothetical protein	66
638640		+	-	SAS0557	hypothetical protein	29
639345		+	-	SAS0558	hypothetical protein	0
640049		+	-	SAS0559	hypothetical protein	9
641638		+	-	SAS0562	hypothetical protein	2
642314		+	-	SAS0563	hypothetical protein	9
643396	643451	+	-	SAS0564	hypothetical protein	144
644161		+	-	SAS0565	hypothetical protein	25
644833		+	-	SAS0566	hypothetical protein	45
645694		+	-	SAS0567	hypothetical protein	26
646902		+	-	SAS0568	aldo/keto reductase	86
647649	647649	+	-	SAS0569	hypothetical protein	117
648294	648333	+	-	SAS0570	acetyltransferase	115
649629		+	-	SAS0571	phosphohydrolase	56
650178		+	-	SAS0572	hypothetical protein	47
651687	651738	+	-	SAS0573	alcohol dehydrogenase	136
651871		+	-	SAS0573a	hypothetical protein	7
652380		+	-	SAS0574	hypothetical protein	0
654038		+	*argS*	SAS0575	arginyl-tRNA synthetase	85
655064		+	-	SAS0576	DNA repair protein	24
656202		+	-	SAS0577	transport system lipoprotein	9
657301		+	-	SAS0578	FecCD transport family protein	36
658075		+	-	SAS0579	haloacid dehalogenase	89
658859	658859	+	-	SAS0580	hydrolase	126
659501		+	-	SAS0581	hypothetical protein	20
660379		+	-	SAS0582	hypothetical protein	18
661336		+	-	SAS0583	esterase	49
661586		-	-	SAS0584	accessory regulator A	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
662908		-	-	SAS0585	hypothetical protein	30
664032		-	-	SAS0586	hypothetical protein	0
664273		-	-	SAS0587	hypothetical protein	9
665195		+	-	SAS0588	phage integrase family protein	16
667616		+	-	SAS0589	monovalent cation/H+ antiporter subunit A	82
668028		+	-	SAS0590	monovalent cation/H+ antiporter subunit B	81
668369		+	-	SAS0591	monovalent cation/H+ antiporter subunit C	85
669855		+	-	SAS0592	monovalent cation/H+ antiporter subunit D	82
670338		+	-	SAS0593	monovalent cation/H+ antiporter subunit E	44
670637		+	-	SAS0594	monovalent cation/H+ antiporter subunit F	57
671049		+	-	SAS0595	monovalent cation/H+ antiporter subunit G	2
673431		+	-	SAS0596	sodium/hydrogen exchanger family protein	46
674592		-	-	SAS0597	ABC transporter extracellular binding protein	90
675518		-	-	SAS0598	ABC transporter permease	67
676348		-	-	SAS0599	ABC transporter ATP-binding protein	114
677857		+	-	SAS0600	metalloregulator	16
677937		-	-	SAS0601	hypothetical protein	28
679689	679828	+	-	SAS0602	teichoic acid biosynthesis protein	106
679750		-	-	SAS0603	teichoic acids export protein ATP-binding subunit	96
681703		+	-	SAS0604	teichoic acid ABC transporter permease	98
682905		+	-	SAS0605	teichoic acid biosynthesis protein	28
683963		+	-	SAS0606	glycosyl transferase family protein	56
684424		+	-	SAS0607	glycerol-3-phosphate cytidylyltransferase	53
684541		-	-	SAS0608	penicillin-binding protein 4	60
687984		+	-	SAS0609	ABC transporter ATP-binding protein	77
689570		+	-	SAS0610	Na+ dependent nucleoside transporter	101
690928		+	-	SAS0611	hypothetical protein	52
692007		+	-	SAS0612	ferrichrome ABC transporter ATP-binding protein	75
693047		+	-	SAS0613	ferrichrome transport permease	79
694060		+	-	SAS0614	ferrichrome transport permease	93
695262	695262	+	-	SAS0615	dihydroxyacetone kinase subunit DhaK	107
695888		+	-	SAS0616	dihydroxyacetone kinase	38
696243		+	-	SAS0617	PTS system mannnose-specific transporter subunit IIA	66
696856		+	-	SAS0618	hypothetical protein	12
698380		+	-	SAS0619	hypothetical protein	71

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
699574		+	-	SAS0620	esterase	46
700331		+	-	SAS0621	hypothetical protein	52
700540		-	-	SAS0622	hypothetical protein	58
702082		+	-	SAS0623	hypothetical protein	63
702772		+	-	SAS0624	response regulator protein	75
703805		+	-	SAS0625	sensor histidine kinase	20
704710		+	-	SAS0626	ABC transporter	43
706589		+	-	SAS0627	ABC transporter permease	21
707931		+	-	SAS0628	hypothetical protein	22
708954		+	-	SAS0629	phosphate transport protein	84
709542	709542	-	-	SAS0630	hypothetical protein	115
	709939	+	-	predicted RNA	antisense: SAS0630	641
711344		+	-	SAS0631	hypothetical protein	37
714191		+	-	SAS0632	AraC family transcriptional regulator	38
714696		+	-	SAS0633	hypothetical protein	11
715602		+	-	SAS0634	hypothetical protein	145
716075		+	-	SAS0635	hypothetical protein	40
717072		+	-	SAS0636	hypothetical protein	21
718054		+	-	SAS0637	LysR family regulatory protein	6
719613		+	-	SAS0638	sugar efflux transporter	75
720098		+	-	SAS0639	hypothetical protein	29
720197		-	-	SAS0640	hypothetical protein	24
721456		+	-	SAS0641	acetyltransferase	20
721918	722055	+	-	SAS0642	hypothetical protein	124
722355	722367	+	-	SAS0643	hypothetical protein	128
722977		+	-	SAS0644	acetyltransferase	50
723073		-	-	SAS0645	hypothetical protein	87
723641		-	-	SAS0646	hypothetical protein	69
724102		-	-	SAS0647	hypothetical protein	70
724957		-	*uppP*	SAS0648	undecaprenyl pyrophosphate phosphatase	25
727682		+	-	SAS0649	ABC transporter ATP-binding protein	47
729352		+	-	SAS0650	ABC transporter ATP-binding protein	56
729479		-	-	SAS0651	MarR family transcriptional regulator	31
731075		+	-	SAS0652	cobalamin synthesis protein	92
732086		+	-	SAS0653	aldo/keto reductase	75
732408		+	-	SAS0654	hypothetical protein	23
734244	734244	+	-	SAS0655	sodium:sulfate symporter protein	152
735703		+	-	SAS0656	DNA photolyase	95
735858		-	-	SAS0657	hypothetical protein	87
736139		-	-	SAS0658	hypothetical protein	24
736942		-	-	SAS0659	hypothetical protein	27
738739	738739	+	-	SAS0660	fluoroquinolone resistance protein	114

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
739484	739656	+	-	SAS0661	hypothetical protein	104
740139		+	-	SAS0662	hypothetical protein	1
741153		+	-	SAS0663	DeoR ramily regulatory protein	27
742070		+	-	SAS0664	phosphofructokinase	59
744034	744160	+	-	SAS0665	PTS transport system, fructose-specific IIABC component	174
745523		+	-	SAS0666	N-acetylglucosamine-6-phosphate deacetylase	54
747093		+	-	SAS0667	hypothetical protein	55
748154		+	-	SAS0668	aldo/keto reductase	23
749269		+	-	SAS0669	glucosyl transferase	31
749341		-	-	SAS0670	histidine kinase	73
750396		-	-	SAS0671	response regulator protein	41
751057		-	-	SAS0672	hypothetical protein	71
751873		-	-	SAS0673	hypothetical protein	45
752593	752466	-	-	SAS0674	hypothetical protein	132
753276		-	-	SAS0675	radical activating enzyme	95
753993		-	-	SAS0676	6-pyruvoyl tetrahydrobiopterin synthase	36
754414		-	-	SAS0677	hypothetical protein	25
756026		+	-	SAS0678	glutamine amidotransferase class-I protein	58
757161		+	-	SAS0679	para-aminobenzoate synthase component	87
757769		+	-	SAS0680	para-aminobenzoate synthetase component	0
758041		+	-	SAS0681	hypothetical protein	25
758839		+	-	SAS0682	hypothetical protein	125
759827		+	-	SAS0683	hypothetical protein	52
762241		+	-	SAS0684	sulfatase	39
764395		+	-	SAS0685	ABC transporter ATP-binding protein	40
766188		+	-	SAS0686	ATP-dependent DNA helicase	27
767387		+	-	SAS0687	ABC transporter ATP-binding protein	75
768894		+	-	SAS0688	ABC transporter permease	74
770192	770253	+	-	SAS0689	histidinol-phosphate aminotransferase	148
771029	771029	+	-	SAS0690	hypothetical protein	184
771710		-	-	SAS0691	diacylglycerol kinase	39
772896		-	-	SAS0692	proton-dependent oligopeptide transport protein	69
774742		-	-	SAS0693	7-cyano-7-deazaguanine reductase	29
775262		-	-	SAS0694	hypothetical protein	44
777327		+	*nrdI*	SAS0695	ribonucleotide reductase stimulatory protein	22
779395		+	-	SAS0696	ribonucleotide-diphosphate reductase subunit alpha	57
780484	780884	+	*nrdF*	SAS0697	ribonucleotide-diphosphate reductase subunit beta	142
782163		+	-	SAS0698	FecCD transport family protein	92

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
783106		+	-	SAS0699	FecCD transport family protein	8
783864		+	-	SAS0700	ABC transporter ATP-binding protein	30
785011		+	-	SAS0701	lipoprotein	20
785328		-	-	SAS0702	hypothetical protein	24
785660		-	*murB*	SAS0703	UDP-N-acetylenolpyruvoylglucosamine reductase	87
786710	786584	-	-	SAS0704	hypothetical protein	116
788226		+	-	SAS0705	hypothetical protein	15
788700		+	-	SAS0706	hypothetical protein	0
790249		+	-	SAS0707	glycerate kinase	40
790431	790423	-	-	SAS0708	peptidase T	116
791671		-	-	SAS0709	hypothetical protein	51
792183		-	-	SAS0710	hypothetical protein	77
793140		-	-	SAS0711	hypothetical protein	55
795583		+	-	SAS0712	glycosyl transferase	52
795755		-	-	SAS0713	hypothetical protein	50
797406		+	-	SAS0714	hypothetical protein	49
798852		+	-	SAS0715	helicase	44
799519		+	-	SAS0716	hypothetical protein	37
800152		+	-	SAS0717	S30EA family ribosomal protein	21
803097	803114	+	*secA*	SAS0718	preprotein translocase subunit SecA	144
804522	804522	+	*prfB*	SAS0719	peptide chain release factor 2	255
805770		+	-	SAS0720	hypothetical protein	92
806594		+	-	SAS0721	phosphohydrolase	15
806827		+	-	SAS0722	hypothetical protein	10
809081		+	-	SAS0723	excinuclease ABC subunit B	69
811935		+	*uvrA*	SAS0724	excinuclease ABC subunit A	90
813474		+	-	SAS0725	HPr kinase/phosphorylase	21
814319		+	-	SAS0726	prolipoprotein diacylglyceryl transferase	17
814812		+	-	SAS0727	acetyltransferase	28
816259		+	-	SAS0728	hypothetical protein	65
817261		+	-	SAS0729	thioredoxin reductase	57
818937		+	-	SAS0730	hypothetical protein	56
819929		+	-	SAS0731	hypothetical protein	45
820984		+	-	SAS0732	hypothetical protein	50
	821299	-	-	SASt013	Arg tRNA	0
822144	822275	+	*clpP*	SAS0733	ATP-dependent Clp protease proteolytic subunit	140
822387		-	-	SAS0734	hypothetical protein	71
824621		+	-	SAS0736	hypothetical protein	54
826331	826359	+	-	SAS0737	glycolytic operon regulator	164
827394	827394	+	-	SAS0738	glyceraldehyde 3-phosphate dehydrogenase 1	183
828723		+	*pgk*	SAS0739	phosphoglycerate kinase	69

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
829606		+	*tpiA*	SAS0740	triosephosphate isomerase	79
831126	831126	+	-	SAS0741	phosphoglyceromutase	158
832560	832898	+	*eno*	SAS0742	phosphopyruvate hydratase	116
833357		+	-	SAS0743	hypothetical protein	35
833657		+	*secG*	SAS0744	preprotein translocase subunit SecG	56
834513		+	-	SAS0745	carboxylesterase	64
836919	836919	+	-	SAS0746	ribonuclease R	188
837405	837405	+	*smpB*	SAS0747	SsrA-binding protein	250
	837854	+	*tmRNA*	SASs006	-	167
838270	838270	-	-	SAS0749	hypothetical protein	123
840117		+	-	SAS0750	hypothetical protein	0
840786		+	-	SAS0751	acetyltransferase	48
843835	843835	+	-	SAS0752	clumping factor	138
845582		+	-	SAS0753	hypothetical protein	15
846955		+	-	SAS0754	hypothetical protein	48
847811		+	-	SAS0755	hypothetical protein	39
848838		+	-	SAS0756	thermonuclease precursor	54
849395		+	-	SAS0757	cold shock protein	0
849891	849698	-	-	SAS0758	hypothetical protein	110
850171		-	-	SAS0759	hypothetical protein	19
850543		-	-	SAS0760	hypothetical protein	60
851300		-	-	SAS0761	hypothetical protein	45
851517		-	-	SAS0762	hypothetical protein	11
852320		+	-	SAS0763	hypothetical protein	0
852317		-	-	SAS0764	hypothetical protein	7
853358		+	-	SAS0765	phosphoglycerate mutase family protein	59
853407		-	-	SAS0766	LysE type translocator protein	40
854179		-	-	SAS0767	hypothetical protein	37
855072		-	-	SAS0768	OsmC-like protein	34
856358		+	-	SAS0769	3-dehydroquinate dehydratase	32
856980		+	-	SAS0770	nitroreductase family protein	12
857130		-	-	SAS0771	thioredoxin	11
857950		+	-	SAS0772	hypothetical protein	3
858488		+	-	SAS0773	glycine cleavage system protein H	93
859545		+	-	SAS0774	hypothetical protein	50
859569		-	-	SAS0775	hypothetical protein	68
860672		+	-	SAS0776	hypothetical protein	29
860961		+	-	SAS0777	hypothetical protein	26
	861122	+	-	SASs007	-	0
862236		+	-	SAS0779	ABC transporter ATP-binding protein	20
862924		+	-	SAS0780	ABC transporter permease	61
863763		+	-	SAS0781	lipoprotein	14
864311		+	-	SAS0782	hypothetical protein	58

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
864544		-	-	SAS0783	hypothetical protein	46
866484		+	-	SAS0784	ABC transporter ATP-binding protein	42
867889	867912	+	-	SAS0785	hypothetical protein	124
869245		+	-	SAS0786	selenocysteine lyase	249
869699		+	-	SAS0787	NifU-like protein	42
871247	871247	+	-	SAS0788	hypothetical protein	115
871571	871571	+	-	SAS0788a	hypothetical protein	137
871577		-	-	SAS0789	hypothetical protein	50
873296	873302	+	-	SAS0790	hypothetical protein	113
874377		+	-	SAS0791	dioxygenase	65
875401		+	-	SAS0792	hypothetical protein	75
876241	876241	+	-	SAS0793	hypothetical protein	181
877587		+	-	SAS0794	5'-nucleotidase	68
878588		+	-	SAS0795	lipoyl synthase	43
879101	879459	+	-	SAS0796	hypothetical protein	127
879180	879118	-	-	SAS0797	hypothetical protein	110
879978		+	-	SAS0798	hypothetical protein	77
880757		+	-	SAS0799	haloacid dehalogenase	86
881749		+	-	SAS0800	D-isomer specific 2-hydroxyacid dehydrogenase	86
882313		+	-	SAS0801	hypothetical protein	15
883786		+	-	SAS0802	D-alanine--poly(phosphoribitol) ligase subunit 1	66
884997		+	-	SAS0803	activated D-alanine transport protein	47
885251		+	-	SAS0804	D-alanine--poly(phosphoribitol) ligase subunit 2	6
886423		+	-	SAS0805	lipoteichoic acid biosynthesis protein	26
886686		-	-	SAS0806	hypothetical protein	90
887352	887904	+	-	SAS0807	hypothetical protein	137
887411		-	-	SAS0808	pyridine nucleotide-disulfide oxidoreductase	83
889028		+	-	SAS0809	hypothetical protein	82
889400	889631	+	-	SAS0810	hypothetical protein	130
891062		+	-	SAS0811	pyridine nucleotide-disulfide oxidoreductase	88
892668		+	-	SAS0812	cytosol aminopeptidase	95
894395		+	-	SAS0813	transporter protein	58
894788	894808	+	-	SAS0814	hypothetical protein	137
894844		-	-	SAS0815	hypothetical protein	71
896247		-	-	SAS0816	monovalent cation/H+ antiporter subunit G	53
896581		-	-	SAS0817	monovalent cation/H+ antiporter subunit F	18
896874		-	-	SAS0818	monovalent cation/H+ antiporter subunit E	83
897355		-	-	SAS0819	monovalent cation/H+ antiporter subunit D	76

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
898844		-	-	SAS0820	monovalent cation/H+ antiporter subunit C	169
899185		-	-	SAS0821	monovalent cation/H+ antiporter subunit B	145
899606		-	-	SAS0822	monovalent cation/H+ antiporter subunit A	98
902142		-	-	SAS0823	hypothetical protein	9
903182		+	-	SAS0824	cyclophilin type peptidyl-prolyl cis-trans isomerase	24
903974		+	-	SAS0825	S1 RNA-binding domain-containing protein	64
905463	905731	+	-	SAS0826	NADH:flavin oxidoreductase / NADH oxidase family protein	104
906961		+	*rocD*	SAS0827	ornithine--oxo-acid transaminase	37
908314	908343	+	-	SAS0828	NAD-specific glutamate dehydrogenase	169
908704		-	-	SAS0829	glycerophosphoryl diester phosphodiesterase	64
909875		-	-	SAS0830	argininosuccinate lyase	98
911244		-	-	SAS0831	argininosuccinate synthase	79
914131	914131	+	*pgi*	SAS0832	glucose-6-phosphate isomerase	126
915031		+	-	SAS0833	hypothetical protein	47
915560		+	-	SAS0834	signal peptidase Ia	20
916151		+	-	SAS0835	signal peptidase Ib	15
919787		+	-	SAS0836	hypothetical protein	61
923441		+	-	SAS0837	hypothetical protein	41
924509		+	-	SAS0838	fumarylacetoacetate (FAA) hydrolase family protein	46
925227		+	-	SAS0839	hypothetical protein	88
925399		-	-	SAS0840	coenzyme A disulfide reductase	75
926767		-	-	SAS0841	haloacid dehalogenase	48
928013		+	-	SAS0842	hypothetical protein	1
928010		-	-	SAS0843	hypothetical protein	25
930375		+	-	SAS0844	hypothetical protein	59
933187		+	-	SAS0845	ATPase of an ATP-dependent protease	75
933246		-	-	SAS0846	LysR family regulatory protein	20
935370		+	-	SAS0847	hypothetical protein	42
935995		+	-	SAS0848	hypothetical protein	76
937106		+	-	SAS0849	hypothetical protein	36
937605		+	-	SAS0850	hypothetical protein	52
938350		+	-	SAS0851	hypothetical protein	12
938607		-	-	SAS0852	hypothetical protein	44
940028		+	-	SAS0853	3-oxoacyl-ACP synthase	48
941284	941284	+	-	SAS0854	3-oxoacyl-ACP synthase	186
941339		-	-	SAS0855	hypothetical protein	93
942879		+	-	SAS0856	oligopeptide transport system permease	91
943949		+	-	SAS0857	oligopeptide transport system permease	57

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
945047		+	-	SAS0858	oligopeptide transport ATP-binding protein	160
945978	945996	+	-	SAS0859	oligopeptide transport ATP-binding protein	136
947652		+	-	SAS0860	transport system extracellular binding lipoprotein	74
949579		+	-	SAS0861	transport system extracellular binding lipoprotein	40
950616		+	-	SAS0862	oligopeptide transport ATP-binding protein	28
951599		+	-	SAS0863	oligopeptide transport ATP-binding protein	29
952554		+	-	SAS0864	oligopeptide transport system permease	18
953447		+	-	SAS0865	oligopeptide transport system permease	20
953489		-	-	SAS0866	tryptophanyl-tRNA synthetase	66
955168		+	*spxA*	SAS0867	transcriptional regulator Spx	5
956258		+	-	SAS0868	adaptor protein	37
957365		+	-	SAS0869	hypothetical protein	24
959221		+	-	SAS0870	oligopeptidase	55
959681		-	-	SAS0871	hypothetical protein	13
960510		-	-	SAS0872	hypothetical protein	23
960979		-	-	SAS0873	hypothetical protein	17
962105		+	-	SAS0874	hypothetical protein	5
962757		+	-	SAS0875	GTP pyrophosphokinase	20
963583		+	*ppnK*	SAS0876	inorganic polyphosphate/ATP-NAD kinase	85
964434		+	-	SAS0877	RNA pseudouridylate synthase	15
965840		+	-	SAS0878	divalent cation transport protein	66
967694		+	-	SAS0879	cation transport protein	67
968742		+	-	SAS0880	enoyl-(acyl carrier protein) reductase	25
968937		-	-	SAS0881	hypothetical protein	49
971932		+	-	SAS0882	sodium:alanine symporter family protein	61
972835		+	-	SAS0883	hypothetical protein	27
973538		+	-	SAS0884	hypothetical protein	29
973651		-	-	SAS0885	hypothetical protein	73
974819		-	-	SAS0886	diacylglycerol glucosyltransferase	141
977911		+	-	SAS0887	UDP-N-acetylmuramoylalanyl-D-glutamate--L-lysine ligase	87
978152		+	-	SAS0888	hypothetical protein	57
979714		+	*prfC*	SAS0889	peptide chain release factor 3	75
980819		+	-	SAS0890	hypothetical protein	62
981108		-	-	SAS0891	phage integrase	36
983063		+	-	SAS0892	hypothetical protein	59
983050		-	-	SAS0893	hypothetical protein	7
983262		-	-	SAS0894	hypothetical protein	35
983686		-	-	SAS0895	lipoprotein	56

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
984138		-	-	SAS0896	hypothetical protein	75
984612		-	-	SAS0897	phage regulatory protein	33
985293	985337	+	-	SAS0898	phage regulatory protein	135
985553		+	-	SAS0899	hypothetical protein	0
985550		-	-	SAS0900	hypothetical protein	45
986051		+	-	SAS0901	hypothetical protein	71
986339		+	-	SAS0902	DNA-binding protein	13
986512		+	-	SAS0903	hypothetical protein	41
987165		+	-	SAS0905	hypothetical protein	64
987541		+	-	SAS0906	hypothetical protein	17
988704	988729	+	-	SAS0907	hypothetical protein	193
989287		+	-	SAS0908	hypothetical protein	103
991308	991311	+	-	SAS0909	DNA polymerase	163
991506		+	-	SAS0910	hypothetical protein	0
991907		+	-	SAS0911	DNA-binding protein	123
992161	992166	+	-	SAS0912	hypothetical protein	208
992409	992409	+	-	SAS0913	hypothetical protein	149
992629	992629	+	-	SAS0914	hypothetical protein	103
993036		+	-	SAS0915	hypothetical protein	128
993380		+	-	SAS0916	hypothetical protein	30
993685		+	-	SAS0917	hypothetical protein	29
993926		+	-	SAS0918	hypothetical protein	142
994455	994491	+	-	SAS0919	hypothetical protein	119
994665		+	-	SAS0920	hypothetical protein	82
994876		+	-	SAS0921	hypothetical protein	32
995400		+	-	SAS0922	hypothetical protein	59
995600		+	-	SAS0923	hypothetical protein	60
995868		+	-	SAS0924	exported phage protein	60
995895		-	-	SAS0925	hypothetical protein	66
998603	998943	+	-	SAS0926	hypothetical protein	136
999234		+	-	SAS0927	hypothetical protein	68
1000591	1000591	+	-	SAS0928	hypothetical protein	155
1001041	1001138	+	-	SAS0929	phage regulatory protein	142
1001522		+	-	SAS0930	hypothetical protein	72
1001953		+	-	SAS0931	terminase small subunit	81658
1003634	1003634	+	-	SAS0932	terminase large subunit	236552
1004877		+	-	SAS0933	portal protein	131150
1005634	1005634	+	-	SAS0934	phage protease	70278
1006809	1006809	+	-	SAS0935	hypothetical protein	68090
1007156	1007156	+	-	SAS0936	hypothetical protein	37280
1007500	1007526	+	-	SAS0937	hypothetical protein	17258
1007898		+	-	SAS0938	hypothetical protein	23265
1008294	1008294	+	-	SAS0939	hypothetical protein	9346

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1008970	1009031	+	-	SAS0940	major tail protein	19122
1009517	1009517	+	-	SAS0941	major tail protein	12542
1009925	1009966	+	-	SAS0942	hypothetical protein	6985
1010125	1010138	+	-	SAS0943	hypothetical protein	7629
1016339		+	-	SAS0944	hypothetical protein	3438
1017163	1017163	+	-	SAS0945	hypothetical protein	189
1018755		+	-	SAS0946	hypothetical protein	165
1019045	1019045	+	-	SAS0947	hypothetical protein	156
1020971		+	-	SAS0948	hypothetical protein	142
1022437		+	-	SAS0949	hypothetical protein	78
1022826		+	-	SAS0950	hypothetical protein	37
1022983		+	-	SAS0951	hypothetical protein	101
1023328	1023463	+	-	SAS0952	hypothetical protein	120
1023766		+	-	SAS0953	holin	85
1025231		+	-	SAS0954	amidase	15
1028116		+	-	SAS0955	protease	25
1029491		+	-	SAS0956	sodium transport protein	100
1031141		+	-	SAS0957	5'-nucleotidase	36
	1031321	-	-	SASt014	Ser tRNA	46
	1031412	-	-	SASt015	Asn tRNA	369
	1031480	+	-	predicted RNA	antisense: SASt015	787
1031628	1031487	-	-	SAS0958	regulatory protein	147
1032625		+	-	SAS0959	hypothetical protein	61
1032706		-	-	SAS0960	lipoate-protein ligase A	61
1034067		+	-	SAS0961	hypothetical protein	0
1034684		+	-	SAS0962	hypothetical protein	82
1035002		+	-	SAS0963	hypothetical protein	11
1035924		+	-	SAS0964	hypothetical protein	7
1038254		+	-	SAS0967	hypothetical protein	14
1038892		+	-	SAS0968	ABC transporter ATP-binding protein	25
1038980		-	-	SAS0969	hypothetical protein	30
1039898		+	-	SAS0970	hypothetical protein	1
1041385		+	-	SAS0971	glycosyl transferase family protein	19
1041474		-	-	SAS0972	hypothetical protein	38
1043277		+	-	SAS0973	transport system extracellular binding lipoprotein	60
1043323		-	-	SAS0974	hypothetical protein	0
1043518		-	-	SAS0975	hypothetical protein	30
1044449		+	-	SAS0976	hypothetical protein	10
1044500		-	-	SAS0977	1,4-dihydroxy-2-naphthoate octaprenyltransferase	66
1046981		+	-	SAS0978	chorismate binding protein	41
1048641		+	-	SAS0979	menaquinone biosynthesis bifunctional protein	48

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1049431		+	-	SAS0980	hydrolase	47
1050245		+	-	SAS0981	naphthoate synthase	48
1050483		-	-	SAS0982	hypothetical protein	52
1050850		-	-	SAS0983	cysteine protease precursor	46
1052113		-	-	SAS0984	glutamyl endopeptidase precursor	52
1053642	1053642	-	-	SAS0985	aminotransferase	119
1054992		-	-	SAS0986	hypothetical protein	98
1056574		+	-	SAS0987	MarR family transcriptional regulator	48
1056781		-	-	SAS0988	bifunctional autolysin precursor	93
1060761		-	-	SAS0989	acetyltransferase (GNAT) family protein	86
1061350		-	-	SAS0990	hypothetical protein	34
1061868		-	-	SAS0991	hypothetical protein	38
1064729		+	-	SAS0992	autolysis and methicillin resistant-related protein	32
1065266		-	-	SAS0993	quinol oxidase polypeptide IV	72
1065553		-	-	SAS0994	quinol oxidase polypeptide III	91
1066148		-	-	SAS0995	quinol oxidase polypeptide I	218
1068136		-	-	SAS0996	quinol oxidase polypeptide II precursor	227
1069808		-	-	SAS0997	hypothetical protein	74
1070908		-	-	SAS0999	bifunctional 5,10-methylene-tetrahydrofolate dehydrogenase/ 5,10-methylene-tetrahydrofolate cyclohydrolase	4
1072451		+	-	SAS1000	phosphoribosylaminoimidazole carboxylase catalytic subunit	9
1073562		+	-	SAS1001	phosphoribosylaminoimidazole carboxylase ATPase subunit	27
1074270		+	-	SAS1002	phosphoribosylaminoimidazole-succinocarboxamide synthase	83
1074533		+	-	SAS1003	hypothetical protein	48
1075206		+	-	SAS1004	phosphoribosylformylglycinamidine synthase I	52
1077388		+	-	SAS1005	phosphoribosylformylglycinamidine synthase II	50
1078851		+	-	SAS1006	amidophosphoribosyltransferase	115
1079872		+	-	SAS1007	phosphoribosylaminoimidazole synthetase	61
1080441		+	-	SAS1008	phosphoribosylglycinamide formyltransferase	45
1081934		+	*purH*	SAS1009	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	69
1083203	1083302	+	-	SAS1010	phosphoribosylamine--glycine ligase	126
1083468		-	-	SAS1011	cobalt transport protein	77
1084267		-	-	SAS1012	ABC transporter ATP-binding protein	49
1085682		-	-	SAS1013	hypothetical protein	66
	1086334	-	-	SASs008	-	46
1086841		-	-	SAS1014	hypothetical protein	69

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1088422		+	-	SAS1015	hypothetical protein	32
1090019		+	-	SAS1016	hypothetical protein	17
1090615		+	-	SAS1017	hypothetical protein	38
1091035		+	-	SAS1018	phosphocarrier protein HPr	0
1092756	1092820	+	-	SAS1019	phosphoenolpyruvate-protein phosphotransferase	112
1092993		-	-	SAS1020	glutaredoxin	80
1094785		+	-	SAS1021	cytochrome ubiquinol oxidase	67
1095801		+	-	SAS1022	hypothetical protein	23
1096596		+	-	SAS1023	potassium transport protein	34
1096945		-	-	SAS1024	hypothetical protein	59
1098642		-	-	SAS1025	hypothetical protein	9
1099342		-	*def*	SAS1026	peptide deformylase	34
1100884		+	-	SAS1027	hypothetical protein	33
1102167	1102167	+	-	SAS1028	pyruvate dehydrogenase E1 component subunit alpha	118
1103148	1103238	+	-	SAS1029	pyruvate dehydrogenase E1 component subunit beta	249
1104531	1104531	+	-	SAS1030	branched-chain alpha-keto acid dehydrogenase E2	178
1105941	1105941	+	-	SAS1031	dihydrolipoamide dehydrogenase	202
1106384		+	-	SAS1032	hypothetical protein	49
1107067		+	-	SAS1033	DNA-binding protein	3
1108174		+	-	SAS1034	ABC transporter ATP binding protein	50
1108964		+	-	SAS1035	ABC transporter permease	0
1109779		+	-	SAS1036	ABC transporter permease	24
1110852		+	-	SAS1037	ABC transporter extracellular binding protein	14
1111945		+	-	SAS1038	hypothetical protein	41
1112647		+	-	SAS1039	hypothetical protein	26
1112729		-	-	SAS1040	manganese transport protein MntH	101
1114265		-	-	SAS1041	hypothetical protein	44
1115863		+	-	SAS1042	inositol monophosphatase	9
1116017		-	-	SAS1043	hypothetical protein	20
1118157		+	-	SAS1044	BipA family GTPase	85
1118297		-	-	SAS1045	hypothetical protein	54
1118466		-	-	SAS1046	hypothetical protein	47
1119364		+	-	SAS1047	hypothetical protein	0
1120904		+	-	SAS1048	cell division protein	37
1124910		+	-	SAS1049	pyruvate carboxylase	87
1125050		-	-	SAS1050	heme A synthase	71
1127312		+	-	SAS1051	protoheme IX farnesyltransferase	32
1127798		+	-	SAS1052	hypothetical protein	37
1129162		+	-	SAS1053	hypothetical protein	49
1129612		+	-	SAS1054	hypothetical protein	52

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1129675	1129675	-	-	SAS1055	hypothetical protein	198
1131094		+	-	SAS1056	hypothetical protein	6
1131097		-	-	SAS1057	hypothetical protein	3
1132098		+	-	SAS1058	methylase	38
1132582		+	*coaD*	SAS1059	phosphopantetheine adenylyltransferase	42
1132644		-	-	SAS1060	hypothetical protein	30
1134467		+	-	SAS1061	hypothetical protein	29
1134720		+	*rpmF*	SAS1062	50S ribosomal protein L32	90
1134874		-	-	SAS1063	iron-regulated heme-iron binding protein	58
1137014		-	-	SAS1064	iron-regulated heme-iron binding protein	102
1138958		+	-	SAS1065	surface anchored protein	15
1140034		+	-	SAS1066	hypothetical protein	5
1140909		+	-	SAS1067	transport system extracellular binding lipoprotein	43
1141887		+	-	SAS1068	iron/heme permease	78
1142683		+	-	SAS1069	sortase	20
1143025		+	-	SAS1070	heme-degrading monooxygenase IsdG	0
1143169		+	-	SAS1070a	hypothetical protein	0
1144149	1144246	+	-	SAS1071	SpoU rRNA methylase family protein	107
	1144470	+	-	SASs009	-	108
1145588		+	*pheS*	SAS1072	phenylalanyl-tRNA synthetase subunit alpha	75
1147990	1147990	+	*pheT*	SAS1073	phenylalanyl-tRNA synthetase subunit beta	114
1148225	1148047	-	-	SAS1074	ribonuclease HIII	111
1149799		+	-	SAS1075	hypothetical protein	24
1150321		+	-	SAS1076	hypothetical protein	3
1152106		+	-	SAS1077	hypothetical protein	51
1154464		+	-	SAS1078	recombination and DNA strand exchange inhibitor protein	74
1154951		+	-	SAS1079	thioredoxin	0
1157056		+	*uvrC*	SAS1080	excinuclease ABC subunit C	18
1157994		+	-	SAS1081	succinate dehydrogenase cytochrome b558	22
1159812		+	*sdhA*	SAS1082	succinate dehydrogenase flavoprotein subunit	98
1160627		+	*sdhB*	SAS1083	succinate dehydrogenase iron-sulfur subunit	69
1161663	1161674	+	-	SAS1084	glutamate racemase	111
1162262		+	-	SAS1085	nucleoside-triphosphatase	56
1162758		+	-	SAS1086	hypothetical protein	39
1163053		+	-	SAS1087	hypothetical protein	4
1163564		+	-	SAS1088	hypothetical protein	21
1163934		-	-	SAS1089	formyl peptide receptor-like 1 inhibitory protein	5
1165509		+	-	SAS1090	hypothetical protein	55
1166265		+	-	SAS1091	fibrinogen-binding protein precursor	46

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1166769		+	-	SAS1092	hypothetical protein	41
1167409		+	-	SAS1093	hypothetical protein	40
1167534		-	-	SAS1094	hypothetical protein	11
1167926		-	-	SAS1095	hypothetical protein	48
1168338		-	-	SAS1096	hypothetical protein	60
1169048		-	-	SAS1097	alpha-hemolysin precursor	85
1170826		+	-	SAS1097a	hypothetical protein	30
1171007		+	-	SAS1098	hypothetical protein	5
1171451		-	-	SAS1099	superantigen-like protein	44
1172275		-	-	SAS1100	superantigen-like protein	32
1173095		-	-	SAS1101	superantigen-like protein	74
1175259		+	-	SAS1102	ornithine carbamoyltransferase	62
1176214		+	-	SAS1103	carbamate kinase	67
1177942	1178062	+	-	SAS1104	hypothetical protein	132
1178476		+	-	SAS1105	hypothetical protein	6
1178782		-	-	SAS1106	hypothetical protein	9
1180166		+	-	SAS1107	DNA-binding protein	0
	1180641	-	-	SASt016	Arg tRNA	52
1181212		+	-	SAS1107a	anti protein	4
1181403		+	-	SAS1108	anti protein	23
1182222		+	-	SAS1109	haloacid dehalogenase	29
1182331		-	-	SAS1110	hypothetical protein	0
1184579		+	-	SAS1111	hypothetical protein	48
1185154	1185154	+	-	SAS1112	cell division protein MraZ	152
1186105		+	*mraW*	SAS1113	S-adenosyl-methyltransferase MraW	73
1186520		+	-	SAS1114	cell division protein	73
1188735		+	-	SAS1115	penicillin-binding protein 1	42
1189992		+	*mraY*	SAS1116	phospho-N-acetylmuramoyl-pentapeptide-transferase	20
1191343		+	*murD*	SAS1117	UDP-N-acetylmuramoyl-L-alanyl-D-glutamate synthetase	30
1192678		+	-	SAS1118	cell division protein	39
1194196		+	-	SAS1119	cell division protein	94
1195401		+	-	SAS1120	cell division protein FtsZ	59
1196452		+	-	SAS1121	hypothetical protein	31
1197144		+	-	SAS1122	hypothetical protein	53
1197704	1197704	+	-	SAS1123	hypothetical protein	105
1198006		+	-	SAS1124	hypothetical protein	30
1198895		+	-	SAS1125	hypothetical protein	16
1199536		+	-	SAS1126	hypothetical protein	16
	1199743	+	-	SASs010	-	80
1202510	1202556	+	*ileS*	SAS1127	isoleucyl-tRNA synthetase	109
1203597		+	-	SAS1128	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	23
1204941		+	*lspA*	SAS1130	lipoprotein signal peptidase	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1205858		+	-	SAS1131	RNA pseudouridylate synthase	23
1206785		+	-	SAS1132	bifunctional pyrimidine regulatory protein PyrR/uracil phosphoribosyltransferase	39
1208310		+	-	SAS1133	uracil permease	96
1209219		+	*pyrB*	SAS1134	aspartate carbamoyltransferase	100
1210511		+	*pyrC*	SAS1135	dihydroorotase	86
1211613		+	-	SAS1136	carbamoyl phosphate synthase small subunit	201
1214779		+	*carB*	SAS1137	carbamoyl phosphate synthase large subunit	100
1215581		+	-	SAS1138	orotidine 5'-phosphate decarboxylase	63
1216192		+	*pyrE*	SAS1139	orotate phosphoribosyltransferase	72
1216434	1216496	+	-	SAS1140	hypothetical protein	179
1217272		+	-	SAS1141	hypothetical protein	9
1217535		-	-	SAS1142	hypothetical protein	88
1220130		+	*gmk*	SAS1143	guanylate kinase	14
1220348		+	*rpoZ*	SAS1144	DNA-directed RNA polymerase subunit omega	0
1221763		+	-	SAS1145	flavoprotein	61
1224171	1224229	+	-	SAS1146	primosomal protein n'	156
1225627		+	-	SAS1147	lipoprotein	0
1225790		-	-	SAS1148	hypothetical protein	16
1226780		+	-	SAS1149	peptide deformylase	37
1227708		+	-	SAS1150	methionyl-tRNA formyltransferase	70
1229012		+	-	SAS1151	hypothetical protein	70
1230109	1230109	+	-	SAS1152	ribosomal RNA large subunit methyltransferase N	112
1230859		+	-	SAS1153	protein phosphatase	30
1232850		+	-	SAS1154	serine/threonine-protein kinase	67
1233953		+	-	SAS1155	hypothetical protein	41
1234598		+	-	SAS1156	ribulose-phosphate 3-epimerase	64
1235246		+	-	SAS1157	hypothetical protein	17
1235627		-	*rpmB*	SAS1158	50S ribosomal protein L28	9
1236632		+	-	SAS1159	hypothetical protein	8
1238293		+	-	SAS1160	hypothetical protein	46
1240543		+	-	SAS1161	ATP-dependent DNA helicase RecG	69
1241318		+	-	SAS1162	fatty acid biosynthesis transcriptional regulator	49
1242309		+	-	SAS1163	glycerol-3-phosphate acyltransferase PlsX	110
1243228		+	-	SAS1164	malonyl CoA-acyl carrier protein transacylase	44
1243955		+	-	SAS1165	3-oxoacyl-ACP reductase	77
1244558		+	*acpP*	SAS1166	acyl carrier protein	24
1245405		+	*rnc*	SAS1167	ribonuclease III	30

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1249118		+	-	SAS1168	chromosome partition protein	53
1250368		+	-	SAS1169	cell division protein	73
1250687	1250687	+	-	SAS1170	DNA-binding protein	157
1252080	1252133	+	-	SAS1171	signal recognition particle protein	110
1252790	1252797	+	*rpsP*	SAS1172	30S ribosomal protein S16	152
1253481		+	*rimM*	SAS1173	16S rRNA-processing protein RimM	6
1254218		+	*trmD*	SAS1174	tRNA (guanine-N(1)-)-methyltransferase	23
1254671	1254671	+	*rplS*	SAS1175	50S ribosomal protein L19	150
1255140		-	-	SAS1176	hypothetical protein	45
1259031		+	*rbgA*	SAS1177	ribosomal biogenesis GTPase	0
1259782	1259782	+	*rnhB*	SAS1178	ribonuclease HII	108
1261057		+	*sucC*	SAS1179	succinyl-CoA synthetase subunit beta	63
1261987	1262007	+	-	SAS1180	succinyl-CoA synthetase subunit alpha	174
1263332		+	-	SAS1181	cell wall hydrolase	62
1264604		+	-	SAS1182	FemAB family protein	40
1265649		+	-	SAS1183	SMF family protein	24
1267898		+	-	SAS1184	DNA topoisomerase I	70
1269361		+	-	SAS1185	tRNA (uracil-5-)-methyltransferase Gid	17
1270674		+	-	SAS1186	integrase/recombinase	52
1271216	1271237	+	-	SAS1187	ATP-dependent protease peptidase subunit	210
1272685		+	*hslU*	SAS1188	ATP-dependent protease ATP-binding subunit HslU	56
1273483		+	-	SAS1189	transcriptional repressor CodY	36
1274592		+	*rpsB*	SAS1190	30S ribosomal protein S2	71
1275655	1275680	+	*tsf*	SAS1191	elongation factor Ts	166
1276514	1276532	+	*pyrH*	SAS1192	uridylate kinase	185
1277087	1277395	+	*frr*	SAS1193	ribosome recycling factor	112
1278230		+	-	SAS1194	undecaprenyl pyrophosphate synthase	28
1279019		+	-	SAS1195	phosphatidate cytidylyltransferase	62
1280517		+	-	SAS1196	hypothetical protein	78
1282240		+	-	SAS1197	prolyl-tRNA synthetase	38
1286814		+	*polC*	SAS1198	DNA polymerase III PolC	91
1287571		+	-	SAS1199	hypothetical protein	14
1288767		+	*nusA*	SAS1200	transcription elongation factor NusA	80
1289072		+	-	SAS1201	hypothetical protein	102
1289386		+	-	SAS1202	ribosomal protein	13
1291508	1291567	+	*infB*	SAS1203	translation initiation factor IF-2	127
1292244	1292244	+	*rbfA*	SAS1204	ribosome-binding factor A	154
1293331		+	-	SAS1205	tRNA pseudouridine synthase B	3
1294317		+	-	SAS1206	riboflavin biosynthesis protein	18
1294701		+	*rpsO*	SAS1207	30S ribosomal protein S15	54
1297165		+	-	SAS1208	polynucleotide phosphorylase	96
1299074		+	-	SAS1209	hypothetical protein	56

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1301700		+	-	SAS1210	DNA translocase (FtsK/SpoIIIE family protein)	38
1302418		+	-	SAS1211	hypothetical protein	56
1303714		+	-	SAS1212	hypothetical protein	43
1305000		+	-	SAS1213	protease	52
1305704	1305704	+	-	SAS1214	short chain dehydrogenase	121
1306636		+	-	SAS1215	hypothetical protein	4
1307047		+	-	SAS1216	DNA-binding protein	0
1307659		+	-	SAS1217	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	60
1309037		+	-	SAS1218	hypothetical protein	25
1310245		+	*recA*	SAS1219	recombinase A	81
1312158	1312309	+	-	SAS1220	phosphodiesterase	108
1312455		-	-	SAS1221	hypothetical protein	30
1313641	1313723	+	-	SAS1222	hypothetical protein	104
1315541		+	-	SAS1223	pyruvate flavodoxin/ferredoxin oxidoreductase	86
1316408		+	-	SAS1224	2-oxoglutarate ferredoxin oxidoreductase subunit beta	75
1316795		+	-	SAS1225	hypothetical protein	12
1318473		+	-	SAS1226	(dimethylallyl)adenosine tRNA methylthiotransferase	67
1318839		+	-	SAS1227	hypothetical protein	51
1319357		+	-	SAS1228	hypothetical protein	49
1322278		+	-	SAS1229	DNA mismatch repair protein MutS	50
1324300		+	*mutL*	SAS1230	DNA mismatch repair protein	21
1324848		+	-	SAS1231	glycerol uptake operon antiterminator regulatory protein	25
1324885		-	-	SAS1231a	hypothetical protein	0
1326141	1326141	+	-	SAS1232	glycerol uptake facilitator protein	118
1327766		+	*glpK*	SAS1233	glycerol kinase	65
1329597		+	-	SAS1234	aerobic glycerol-3-phosphate dehydrogenase	68
1330661		+	-	SAS1235	hydrolase	50
1331614		+	*miaA*	SAS1236	tRNA delta(2)-isopentenylpyrophosphate transferase	52
1331862		+	-	SAS1237	hypothetical protein	4
1332084		-	-	SAS1238	glutathione peroxidase	37
1333910		+	-	SAS1239	hypothetical protein	41
1335167		+	-	SAS1240	hypothetical protein	68
1335779	1335797	+	-	SAS1241	glutamine synthetase	114
1337138		+	-	SAS1242	glutamine synthetase	102
1337838		+	-	SAS1243	hypothetical protein	25
1338745		+	-	SAS1244	hypothetical protein	13
1339248		+	-	SAS1245	hypothetical protein	54
1341286		+	-	SAS1246a	hypothetical protein	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1341666		+	-	SAS1247	hypothetical protein	0
1342575		+	-	SAS1249	hypothetical protein	32
1343075		+	-	SAS1250	hypothetical protein	24
1343464	1343476	+	-	SAS1251	hypothetical protein	170
1343599		+	-	SAS1252	hypothetical protein	59
1344522		+	-	SAS1254	hypothetical protein	0
1344918		+	-	SAS1255	hypothetical protein	90
1345083		-	-	SAS1256	hypothetical protein	21
1346575		+	-	SAS1257	hypothetical protein	2
1348113		+	-	SAS1258	cardiolipin synthase	40
1349196		+	-	SAS1259	ABC transporter ATP-binding protein	13
1349896		+	-	SAS1260	hypothetical protein	35
1350991		+	-	SAS1261	sensor kinase	43
1351590		+	-	SAS1262	response regulator	12
1351703		-	-	SAS1263	hypothetical protein	3
1352563		+	-	SAS1264	thermonuclease	0
1352706		-	-	SAS1265	hypothetical protein	30
1354466		+	-	SAS1266	hypothetical protein	48
1354520		-	-	SAS1267	aspartate kinase	33
1357373		+	-	SAS1268	homoserine dehydrogenase	53
1358440		+	-	SAS1269	threonine synthase	63
1359356	1359413	+	-	SAS1270	homoserine kinase	104
1360217	1360217	+	-	SAS1271	haloacid dehalogenase	109
1360510		-	-	SAS1272	hypothetical protein	36
1361042		-	-	SAS1273	amino acid permease	24
1364217		+	-	SAS1274	catalase	91
1364457		+	*rpmG*	SAS1275	50S ribosomal protein L33	18
1365180		+	*rpsN*	SAS1276	30S ribosomal protein S14	4
1366314		+	-	SAS1277	guanosine 5'-monophosphate oxidoreductase	42
1367352		+	-	SAS1278	hypothetical protein	15
1367732		-	-	SAS1279	LexA repressor	22
1368731		+	-	SAS1280	hypothetical protein	0
1369106		+	-	SAS1281	hypothetical protein	7
1371215		+	-	SAS1282	transketolase	80
1371735		+	-	SAS1283	hypothetical protein	22
1372381	1372431	+	-	SAS1284	hypothetical protein	125
1373626		+	-	SAS1285	exonuclease	46
1376659		+	-	SAS1286	exonuclease	12
1376730		-	*mscL*	SAS1287	large-conductance mechanosensitive channel	0
1378942		+	-	SAS1288	glycine betaine transporter 1	101
1382539		+	-	SAS1289	aconitate hydratase	83
1383186		+	-	SAS1290	hypothetical protein	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1383393		-	-	SAS1292	hypothetical protein	23
1384064		-	-	SAS1293	hypothetical protein	35
1386869		+	-	SAS1294	DNA topoisomerase IV subunit B	85
1389271		+	-	SAS1295	DNA topoisomerase IV subunit A	63
1390981		+	-	SAS1296	sodium:alanine symporter family protein	83
1392332		+	-	SAS1297	transcription antiterminator	30
1392474		+	-	SAS1298	hypothetical protein	42
1393675		+	-	SAS1299	hypothetical protein	100
1396678		+	-	SAS1300	hypothetical protein	28
1396881		-	-	SAS1301	methionine sulfoxide reductase A	17
1398510		+	-	SAS1302	hypothetical protein	17
1398598		-	-	SAS1303	4-oxalocrotonate tautomerase	95
1400193		+	-	SAS1304	ImpB/MucB/SamB family protein	41
1400329		-	-	SAS1305	prephenate dehydrogenase	61
1402616		+	-	SAS1306	peptidase	65
	1402880	+	-	SASs011	-	7
	1403094	+	-	SASs012	-	0
1404514		+	-	SAS1307	anthranilate synthase component I	31
1405077	1405077	+	-	SAS1308	anthranilate synthase component II	133
1406081		+	*trpD*	SAS1309	anthranilate phosphoribosyltransferase	80
1406865		+	*trpC*	SAS1310	indole-3-glycerol phosphate synthase	13
1407497		+	-	SAS1311	N-(5'-phosphoribosyl)anthranilate isomerase	81
1408704		+	-	SAS1312	tryptophan synthase subunit beta	139
1409425		+	*trpA*	SAS1313	tryptophan synthase subunit alpha	55
1411009		+	-	SAS1314	factor essential for expression of methicillin resistance	20
1412287		+	-	SAS1315	methicillin resistance expression factor	81
1413232		+	-	SAS1316	hypothetical protein	62
1414319		-	-	SAS1319	hypothetical protein	50
1414916		-	-	SAS1320	oligopeptide transporter ATPase	31
1415610		-	-	SAS1321	oligopeptide transporter ATPase	4
1416370		-	-	SAS1322	oligopeptide transport system permease	45
1417193		-	-	SAS1323	oligopeptide transport system permease	38
1418483		-	-	SAS1324	hypothetical protein	30
1420846		+	-	SAS1325	peptidase	68
1420985		-	-	SAS1326	phosphate transport system protein	51
1421633		-	-	SAS1327	phosphate transporter ATP-binding protein	29
1422531		-	-	SAS1328	ABC transporter permease	32
1423450	1423450	-	-	SAS1329	ABC transporter permease	112
1424567		-	-	SAS1330	phosphate-binding lipoprotein	21
1426526		-	-	SAS1332	hypothetical protein	15
1429176		+	-	SAS1333	ABC transporter ATP-binding protein	11
	1429961	+	-	SASs013	-	0
1431248		+	-	SAS1334	aspartate kinase	59

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1432301	1432301	+	-	SAS1335	aspartate semialdehyde dehydrogenase	208
1433190		+	*dapA*	SAS1336	dihydrodipicolinate synthase	84
1433909		+	-	SAS1337	dihydrodipicolinate reductase	80
1434655	1434674	+	-	SAS1338	tetrahydrodipicolinate acetyltransferase	103
1435949	1435949	+	-	SAS1339	peptidase	122
1437039		+	-	SAS1340	alanine racemase	90
1438294		+	-	SAS1341	diaminopimelate decarboxylase	51
1438534		-	-	SAS1342	hypothetical protein	58
1439132		-	-	SAS1343	cold shock protein	10
1439503		-	-	SAS1344	hypothetical protein	65
1440243		+	-	SAS1345	acylphosphatase	79
1440891		+	-	SAS1346	hypothetical protein	39
1442059		+	-	SAS1347	hypothetical protein	69
1443072		-	-	SAS1350	branched-chain amino acid transporter protein	8
1444640		-	-	SAS1351	hypothetical protein	37
1446540		-	-	SAS1352	hypothetical protein	24
1447512		-	-	SAS1353	hypothetical protein	40
1447744		-	-	SAS1354	hypothetical protein	43
1449133		-	-	SAS1355	dihydrolipoamide succinyltransferase	25
1450415		-	*sucA*	SAS1356	2-oxoglutarate dehydrogenase E1	89
1453497		-	-	SAS1357	sensor kinase	10
1454849		-	-	SAS1358	response regulator protein	35
1455979		-	-	SAS1358a	hypothetical protein	11
1456295		-	-	SAS1359	hypothetical protein	83
1456926		-	*murG*	SAS1360	undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	41
1458008		-	-	SAS1361	acetyltransferase	13
1459705		-	-	SAS1363	protease	17
1461387		-	-	SAS1364	hypothetical protein	0
1461608		-	-	SAS1365	PTS system glucose-specific transporter subunit IIA	50
1462120		-	-	SAS1366	methionine sulfoxide reductase B	96
1462541		-	-	SAS1367	methionine sulfoxide reductase A	64
1463160		-	-	SAS1368	hypothetical protein	90
1464014		-	-	SAS1369	dihydrofolate reductase	87
1464693	1464494	-	*thyA*	SAS1370	thymidylate synthase	122
1466073		-	-	SAS1371	hypothetical protein	70
1466526		-	-	SAS1372	hypothetical protein	89
1467689		-	-	SAS1373	hypothetical protein	78
1467952		-	-	SAS1374	hypothetical protein	88
1469095		+	-	SAS1375	hypothetical protein	13
1469261		+	-	SAS1375a	hypothetical protein	34
1470036		+	-	SAS1376	hypothetical protein	18

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
	1480047	-	-	predicted RNA	-	634
	1479687	+	-	predicted RNA	-	634
	1479775	+	-	predicted RNA	-	650
1497358		-	-	SAS1379	transporter protein	99
1498906		-	-	SAS1380	amino acid permease	83
1500259		-	-	SAS1381	threonine dehydratase	77
1501394		-	-	SAS1382	alanine dehydrogenase	23
1502988		-	-	SAS1383	5'-3' exonuclease	36
1503886		-	-	SAS1384	hypothetical protein	81
1508969		+	-	SAS1385	hypothetical protein	43
1509540		-	-	SAS1386	hypothetical protein	83
1509956		-	-	SAS1387	hypothetical protein	82
	1511315	-	*rnpB*	SASs014	-	20
1511752		-	-	SAS1388	hypothetical protein	49
1512110		-	-	SAS1389	hypothetical protein	44
1512666		-	-	SAS1390	hypothetical protein	8
1513070		-	-	SAS1391	hypothetical protein	0
1514181		+	*recU*	SAS1392	Holliday junction-specific endonuclease	29
1516361	1516361	+	-	SAS1393	penicillin-binding protein 2	109
1516925		-	-	SAS1394	hypothetical protein	23
1517271		-	-	SAS1395	endonuclease	26
1517920		-	-	SAS1396	hypothetical protein	34
1518934		-	*asnC*	SAS1397	asparaginyl-tRNA synthetase	69
1520548		-	-	SAS1398	hypothetical protein	48
1523265		-	-	SAS1399	BirA bifunctional protein [includes: biotin operon repressor; biotin--[acetyl-CoA-carboxylase] synthetase	29
1524223		-	-	SAS1400	tRNA CCA-pyrophosphorylase	25
1525430		-	-	SAS1401	glycosyl transferase family protein	36
1526814		-	-	SAS1402	hypothetical protein	16
1527467		-	-	SAS1403	hypothetical protein	83
1528219		-	-	SAS1404	hypothetical protein	73
1528796		-	-	SAS1405	hypothetical protein	0
1529385		-	-	SAS1406	hypothetical protein	37
1530636	1530636	-	-	SAS1407	3-phosphoshikimate 1-carboxyvinyltransferase	162
1531944	1531944	-	*aroB*	SAS1408	3-dehydroquinate synthase	110
1533034		-	-	SAS1409	chorismate synthase	88
1534588		-	-	SAS1409a	hypothetical protein	75
1534998		-	*ndk*	SAS1410	nucleoside diphosphate kinase	60
1535539		-	-	SAS1411	heptaprenyl diphosphate synthase component II	43
1536500		-	*ubiE*	SAS1412	ubiquinone/menaquinone biosynthesis methyltransferase	11

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1537228		-	-	SAS1413	hypothetical protein	92
1538231		-	-	SAS1414	DNA-binding protein HU	22
1538674		-	*gpsA*	SAS1415	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase	4
1539689		-	*engA*	SAS1416	GTP-binding protein EngA	43
1541221		-	*rpsA*	SAS1417	30S ribosomal protein S1	37
1543094		-	*cmk*	SAS1418	cytidylate kinase	49
1544798		+	-	SAS1419	L-asparaginase	100
1544913	1544745	-	-	SAS1420	pyridine nucleotide-disulfide oxidoreductase	151
1546301	1546301	-	-	SAS1421	cell surface elastin binding protein	117
1547914		-	-	SAS1422	DEAD/DEAH box helicase	33
1549283		-	-	SAS1423	hypothetical protein	33
1550592		+	-	SAS1424	ferredoxin	82
1550698		-	-	SAS1425	hypothetical protein	32
	1551305	-	-	SASs015	-	35
1551653		-	-	SAS1426	lipoprotein	40
1552624		-	-	SAS1427	lipoprotein	27
1553599		-	-	SAS1428	lipoprotein	37
1554595		-	-	SAS1429	hypothetical protein	24
1556769		-	-	SAS1430	lipoprotein	18
1557922		-	-	SAS1431	sensor kinase	51
1559654		-	-	SAS1432	response regulator protein	33
1560512		-	-	SAS1433	ribosomal large subunit pseudouridine synthase B	25
1561242		-	-	SAS1434	hypothetical protein	22
1561777		-	-	SAS1435	hypothetical protein	97
1563106		+	-	SAS1436	hypothetical protein	42
1563184		-	-	SAS1437	integrase/recombinase	102
1564120		-	-	SAS1438	iron uptake regulatory protein	90
1564674		-	-	SAS1439	ADP-ribose pyrophosphatase	52
1566206		+	-	SAS1440	aldo/keto reductase	12
1566422		-	-	SAS1441	hypothetical protein	6
1566686		-	-	SAS1442	short chain dehydrogenase	55
1568398		+	-	SAS1443	pyrroline-5-carboxylate reductase	59
1568501	1568481	-	-	SAS1444	hypothetical protein	105
1571218		+	-	SAS1445	glucose-6-phosphate 1-dehydrogenase	89
1572314		+	-	SAS1446	AraC family transcriptional regulator	48
1572396		-	-	SAS1447	alpha-D-1,4-glucosidase	92
1574061		-	-	SAS1448	maltose operon transcriptional repressor	31
1575274		-	-	SAS1449	glyoxalase/bleomycin resistance protein/dioxygenase superfamily protein	0
1575864		-	-	SAS1450	6-phosphogluconate dehydrogenase	43
1577338	1577271	-	-	SAS1451	peptidase	110
1579040		-	-	SAS1452	hypothetical protein	0
1580034		-	-	SAS1453	hypothetical protein	42

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1580822		-	-	SAS1454	lipoamide acyltransferase component of branched-chain alpha-keto acid dehydrogenase complex	56
1582109	1582109	-	-	SAS1455	2-oxoisovalerate dehydrogenase subunit beta	191
1583092		-	-	SAS1456	2-oxoisovalerate dehydrogenase subunit alpha	174
1584100	1584100	-	-	SAS1457	dihydrolipoamide dehydrogenase	143
1585672		-	-	SAS1458	DNA repair protein	34
1587367		-	-	SAS1459	arginine repressor ArgR	3
1588251		-	-	SAS1460	geranyltranstransferase	29
1589110		-	-	SAS1461	exodeoxyribonuclease VII small subunit	15
1589333		-	*xseA*	SAS1462	exodeoxyribonuclease VII large subunit	39
1590687		-	*nusB*	SAS1463	transcription antitermination protein NusB	40
1591136		-	-	SAS1464	hypothetical protein	0
1591513		-	-	SAS1465	acetyl-CoA carboxylase biotin carboxylase subunit	82
1592868		-	-	SAS1466	biotin carboxyl carrier protein of acetyl-CoA carboxylase	204
1593806		-	-	SAS1467	elongation factor P	6
1594389		-	-	SAS1468	peptidase	61
1596136		+	-	SAS1469	hypothetical protein	11
1596368		+	-	SAS1470	hypothetical protein	0
1596432		-	-	SAS1471	lipoate-protein ligase A	73
1597806		+	-	SAS1472	hypothetical protein	0
1598171		-	-	SAS1473	glycine dehydrogenase subunit 2	48
1599636		-	-	SAS1474	glycine dehydrogenase subunit 1	104
1601002		-	*gcvT*	SAS1475	glycine cleavage system aminomethyltransferase T	59
1602252		-	-	SAS1476	shikimate kinase	20
1603009		-	-	SAS1477	hypothetical protein	7
1603424		-	-	SAS1478	hypothetical protein	7
1603710		-	-	SAS1479	hypothetical protein	22
1604134		-	-	SAS1480	hypothetical protein	33
1604459		-	-	SAS1481	hypothetical protein	9
1605501		-	-	SAS1482	hypothetical protein	28
1606527		-	-	SAS1483	metallo-beta-lactamase superfamily protein	1
1607147		-	-	SAS1484	hypothetical protein	6
1607476		-	-	SAS1485	glucokinase	6
1608459		-	-	SAS1486	hypothetical protein	9
1608643		-	-	SAS1487	hypothetical protein	51
1610118		-	-	SAS1488	5-formyltetrahydrofolate cyclo-ligase	7
1610866		-	*rpmG*	SAS1489	50S ribosomal protein L33	9

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1611128		-	-	SAS1490	penicillin-binding protein 3	72
1613324		-	-	SAS1491	superoxide dismutase	38
1614199		-	-	SAS1492	zinc-specific metalloregulatory protein	17
1614596		-	-	SAS1493	ABC transporter permease	28
1615501	1615460	-	-	SAS1494	ABC transporter ATP-binding protein	137
1616412		-	-	SAS1495	endonuclease IV	26
1617312		-	-	SAS1496	helicase	40
1618772		-	-	SAS1497	hypothetical protein	18
1619875		-	-	SAS1498	hypothetical protein	31
1620683	1620647	-	-	SAS1499	RNA polymerase sigma factor RpoD	116
1622013		-	-	SAS1500	DNA primase	20
1623891		-	-	SAS1501	hypothetical protein	58
1627069		+	-	SAS1503	glycyl-tRNA synthetase	88
1627220		-	-	SAS1504	recombination protein O	88
1627994		-	*era*	SAS1505	GTP-binding protein Era	15
1628894		-	-	SAS1506	cytidine deaminase	35
1629309	1629307	-	-	SAS1507	diacylglycerol kinase	137
1629656		-	-	SAS1508	hypothetical protein	63
1630124		-	-	SAS1509	PhoH-like protein	54
1631375		-	-	SAS1510	hypothetical protein	30
1632090		-	-	SAS1511	hypothetical protein	99
1633097	1633097	-	-	SAS1512	hypothetical protein	178
1634024	1633844	-	*rpsU*	SAS1513	30S ribosomal protein S21	180
1634493		-	-	SAS1514	hypothetical protein	79
1635846	1635840	-	-	SAS1515	16S ribosomal RNA methyltransferase RsmE	146
1636600		-	*prmA*	SAS1516	50S ribosomal protein L11 methyltransferase	65
1637542		-	-	SAS1517	molecular chaperone DnaJ	84
1638817		-	*dnaK*	SAS1518	molecular chaperone DnaK	52
1640718		-	-	SAS1519	heat shock protein GrpE	34
1641376		-	-	SAS1520	heat-inducible transcription repressor	59
1642454		-	-	SAS1521	coproporphyrinogen III oxidase	22
1644144		-	-	SAS1522	GTP-binding protein LepA	70
1646564		+	*rpsT*	SAS1523	30S ribosomal protein S20	38
1646609		-	*holA*	SAS1524	DNA polymerase III subunit delta	48
1647640		-	-	SAS1525	hypothetical protein	19
1649846		-	-	SAS1526	deaminase	75
1650399		-	-	SAS1527	hypothetical protein	36
1651125		-	-	SAS1528	hypothetical protein	67
1651844		-	-	SAS1529	hypothetical protein	24

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1652198		-	-	SAS1530	hypothetical protein	38
1652772		-	-	SAS1531	hypothetical protein	68
1653344		-	-	SAS1532	hypothetical protein	4
1653638		-	-	SAS1533	shikimate 5-dehydrogenase	36
1654458		-	-	SAS1534	GTP-binding protein YqeH	51
1655559		-	-	SAS1535	hypothetical protein	29
1656106		-	-	SAS1536	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase	42
1657375		+	-	SAS1537	hypothetical protein	73
1657577		-	-	SAS1538	enterotoxin	14
1659036		-	-	SAS1539	hypothetical protein	17
1660543		-	-	SAS1540	hypothetical protein	46
1661784	1661784	-	-	SAS1541	LamB/YcsF family protein	151
1662536		-	-	SAS1542	biotin carboxylase	99
1663911		-	-	SAS1543	biotin carboxyl carrier protein of acetyl-CoA carboxylase	42
1664362	1664362	-	-	SAS1544	hypothetical protein	131
1665362		-	-	SAS1545	hypothetical protein	93
1666422		-	*greA*	SAS1546	transcription elongation factor GreA	64
1666926		-	-	SAS1547	uridine kinase	20
1667549		-	-	SAS1548	peptidase	84
1668829		-	-	SAS1549	peptidase	46
1669755		-	-	SAS1550	O-methyltransferase	47
1670678		-	-	SAS1551	hypothetical protein	2
1671001		-	-	SAS1552	Holliday junction resolvase-like protein	29
1671433		-	-	SAS1553	hypothetical protein	25
1671756	1671756	-	*alaS*	SAS1554	alanyl-tRNA synthetase	169
	1674489	-	-	SASs016	-	165
1674729		-	-	SAS1555	hypothetical protein	81
1677208		-	-	SAS1556	hypothetical protein	41
1678457		-	*mnmA*	SAS1557	tRNA-specific 2-thiouridylase MnmA	75
1679576		-	-	SAS1558	cysteine desulfurase	59
1682043	1682191	+	-	SAS1559	oxygenase	130
1682280	1681982	-	-	SAS1560	hypothetical protein	104
1682466		-	-	SAS1561	hypothetical protein	38
1682748		-	-	SAS1562	hypothetical protein	20
1684529		+	-	SAS1563	recombination factor protein RarA	101
1684690		-	-	SAS1564	hypothetical protein	29
1685677		-	-	SAS1565	hypothetical protein	26
1685924	1685779	-	*aspS*	SAS1566	aspartyl-tRNA synthetase	135
1687706		-	*hisS*	SAS1567	histidyl-tRNA synthetase	75
1689429		-	-	SAS1568	N-acetylmuramoyl-L-alanine amidase	31
1690301		-	-	SAS1569	D-tyrosyl-tRNA(Tyr) deacylase	22
1690765		-	-	SAS1570	GTP pyrophosphokinase	68

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1693382		-	-	SAS1571	adenine phosphoribosyltransferase	9
1693922		-	-	SAS1572	single-stranded-DNA-specific exonuclease	102
1696398		-	-	SAS1573	bifunctional preprotein translocase subunit SecD/SecF	65
1698952		-	-	SAS1574	hypothetical protein	53
1699231		-	*tgt*	SAS1575	queuine tRNA-ribosyltransferase	62
1700393		-	*queA*	SAS1576	S-adenosylmethionine--tRNA ribosyltransferase-isomerase	88
1701420	1701419	-	*ruvB*	SAS1577	Holliday junction DNA helicase RuvB	112
1702456		-	*ruvA*	SAS1578	Holliday junction DNA helicase RuvA	27
1703072		-	-	SAS1579	hypothetical protein	52
1703540		-	*obgE*	SAS1580	GTPase ObgE	64
1705317		-	*rpmA*	SAS1581	50S ribosomal protein L27	20
1705613		-	-	SAS1582	hypothetical protein	49
1705939		-	*rplU*	SAS1583	50S ribosomal protein L21	48
1706609		-	-	SAS1584	hypothetical protein	15
1707139		-	-	SAS1585	rod shape-determining protein MreC	35
1708180		-	-	SAS1585a	hypothetical protein	76
1708373		-	-	SAS1586	hypothetical protein	29
1709203		+	-	SAS1587	hypothetical protein	16
1709320		-	-	SAS1588	hypothetical protein	0
1709672		-	*radC*	SAS1589	DNA repair protein RadC	52
1710355		-	-	SAS1590	type III leader peptidase family protein	2
1711332		-	-	SAS1591	folylpolyglutamate synthase	36
1712616		-	*valS*	SAS1592	valyl-tRNA synthetase	39
1716209		+	-	SAS1593	DNA-3-methyladenine glycosylase I	63
1716432		-	-	SAS1594	hypothetical protein	70
1717539		-	-	SAS1595	hypothetical protein	3
1717701	1717701	-	-	SAS1596	glutamate-1-semialdehyde aminotransferase	144
1719035	1718988	-	-	SAS1597	delta-aminolevulinic acid dehydratase	105
1720012		-	-	SAS1598	uroporphyrinogen III synthase	75
1720702		-	*hemC*	SAS1599	porphobilinogen deaminase	94
1721670		-	-	SAS1600	hypothetical protein	28
1722507		-	*hemA*	SAS1601	glutamyl-tRNA reductase	49
1724070		-	*engB*	SAS1602	ribosome biogenesis GTP-binding protein YsxC	28
1724814		-	*clpX*	SAS1603	ATP-dependent protease ATP-binding subunit ClpX	38
1726227	1726227	-	*tig*	SAS1604	trigger factor	120
1727691		-	-	SAS1605	hypothetical protein	54
1728639		-	-	SAS1606	hypothetical protein	34
1729389		-	*rplT*	SAS1607	50S ribosomal protein L20	66
1729792	1729746	-	*rpmI*	SAS1608	50S ribosomal protein L35	121
1730021		-	*infC*	SAS1609	translation initiation factor IF-3	50

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1730777		-	-	SAS1610	lysine-specific permease	55
	1732415	-	-	SASs017	-	118
1732696	1732696	-	*thrS*	SAS1611	threonyl-tRNA synthetase	169
	1734764	-	-	SASs018	-	7
1735045		-	-	SAS1612	primosomal protein DnaI	83
1735965		-	-	SAS1613	chromosome replication initiation/membrane attachment protein	95
1737366		-	*nrdR*	SAS1614	transcriptional regulator NrdR	34
1738047		-	-	SAS1615	glyceraldehyde 3-phosphate dehydrogenase 2	47
1739242		-	*coaE*	SAS1616	dephospho-CoA kinase	28
1739881		-	-	SAS1617	formamidopyrimidine-DNA glycosylase	58
1740769		-	-	SAS1618	DNA polymerase I	68
1743693		-	-	SAS1619	hypothetical protein	5
1745679		-	-	SAS1620	alkaline phosphatase synthesis sensor protein	55
1747340		-	-	SAS1621	alkaline phosphatase synthesis transcriptional regulatory protein	67
1748434		-	-	SAS1622	isocitrate dehydrogenase	53
1749751		-	-	SAS1623	citrate synthase	80
1752581		+	-	SAS1624	D-serine/D-alanine/glycine transporter	55
1752929	1752108	-	-	SAS1625	pyruvate kinase	126
1754708		-	*pfkA*	SAS1626	6-phosphofructokinase	76
1755945		-	-	SAS1627	acetyl-CoA carboxylase carboxyltransferase subunit alpha	66
1756889		-	-	SAS1628	acetyl-CoA carboxylase subunit beta	59
1757941		-	-	SAS1629	NAD-dependent malic enzyme	72
1759620		-	-	SAS1630	DNA polymerase III subunit alpha	47
1762838		-	-	SAS1631	hypothetical protein	96
1764233		-	-	SAS1632	DNA-binding protein	51
1766149		+	-	SAS1633	universal stress protein	89
1766454		-	-	SAS1634	metal-dependent hydrolase	81
1768373		+	-	SAS1635	metallopeptidase	60
1768689	1768547	-	-	SAS1636	alanine dehydrogenase	108
1770448		+	-	SAS1637	universal stress protein	0
1770694	1770694	-	-	SAS1638	acetate kinase	125
1771984		-	-	SAS1639	hypothetical protein	21
1773054		-	*tpx*	SAS1640	thiol peroxidase	16
1773646		-	-	SAS1641	hypothetical protein	48
1774460	1774417	-	-	SAS1642	thiamine biosynthesis protein ThiI	142
1775683		-	-	SAS1643	aminotransferase class-V protein	40
1777194	1777194	-	-	SAS1644	septation ring formation regulator EzrA	116
1779489		+	-	SAS1645	hypothetical protein	51
1780335		+	*rpsD*	SAS1646	30S ribosomal protein S4	42
1780551		-	-	SAS1647	hypothetical protein	60
1781820	1781820	+	-	SAS1648	OsmC-like protein	153

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1783094		+	-	SAS1649	soluble hydrogenase subunit	50
1784685	1784886	+	-	SAS1650	D-3-phosphoglycerate dehydrogenase	116
1784835		-	-	SAS1651	hypothetical protein	33
1786072		-	-	SAS1652	PTS system IIBC component	84
1787666		-	-	SAS1653	acyltransferase	67
1789729		+	-	SAS1654	protease	75
1789823		-	-	SAS1655	tyrosyl-tRNA synthetase	75
	1791148	-	-	SASs019	-	66
1792372		+	-	SAS1656	transglycosylase	52
1792575		-	-	SAS1657	haptoglobin-binding surface anchored protein	49
1795350		+	-	SAS1657a	hypothetical protein	30
1795596		-	-	SAS1658	formate--tetrahydrofolate ligase	77
1797703	1797703	-	-	SAS1659	acetyl-CoA synthetase	123
1800210		+	-	SAS1660	acetoin utilization protein	73
1801404		+	-	SAS1661	histone deacetylase family protein	44
1801502	1801502	-	-	SAS1662	catabolite control protein A	131
1803031		-	-	SAS1663	bifunctional 3-deoxy-7-phosphoheptulonate synthase/chorismate mutase	77
1804822		-	-	SAS1664	hypothetical protein	44
1805942		-	-	SAS1665	hypothetical protein	40
1806507		-	*murC*	SAS1666	UDP-N-acetylmuramate--L-alanine ligase	38
1807844	1807824	-	-	SAS1667	FtsK/SpoIIIE family protein	112
1811689		-	-	SAS1668	hypothetical protein	38
1812314		-	-	SAS1669	hypothetical protein	0
1813271		-	-	SAS1670	thioredoxin	17
1813647		-	-	SAS1671	aminopeptidase	50
1815121		+	-	SAS1672	hypothetical protein	31
1815246		-	-	SAS1673	metallo-beta-lactamase superfamily protein	89
1816558		-	*trmB*	SAS1674	tRNA (guanine-N(7)-)-methyltransferase	26
1817217		-	-	SAS1675	hypothetical protein	12
1818559		-	-	SAS1676	D-alanine aminotransferase	24
1819411		-	-	SAS1677	dipeptidase PepV	43
1821513		-	-	SAS1678	hypothetical protein	19
1821952		-	-	SAS1679	RNA pseudouridine synthase	0
1822644		-	-	SAS1680	polysaccharide biosynthesis protein	31
1825980		+	-	SAS1681	hypothetical protein	95
1826097	1826051	-	-	SAS1682	surface anchored protein	202
	1832245	+	-	predicted RNA	antisense: SAS1682	974
1832983		-	-	SAS1683	hypothetical protein	87
1833316	1833316	-	*leuS*	SAS1684	leucyl-tRNA synthetase	104

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1836027		-	-	SAS1685	hypothetical protein	40
1838271		+	-	SAS1686	hypothetical protein	115
1838831		+	-	SAS1687	hypothetical protein	14
1838951		-	-	SAS1688	repressor of toxins	27
1839926		-	-	SAS1689	hypothetical protein	16
1841988		+	-	SAS1690	proline dehydrogenase	55
1842110		-	*ribH*	SAS1691	6,7-dimethyl-8-ribityllumazine synthase	24
1842587		-	-	SAS1692	riboflavin biosynthesis protein	38
1843779		-	-	SAS1693	riboflavin synthase subunit alpha	40
1844418		-	-	SAS1694	bifunctional riboflavin biosynthesis protein	84
	1845588	-	-	SASs020	-	239
1845942		-	-	SAS1695	hypothetical protein	48
1848281		+	-	SAS1696	arsenical resistance operon repressor 2	39
1849573		+	-	SAS1697	arsenical pump membrane protein 2	63
1849660		-	-	SAS1698	hypothetical protein	16
1850790		-	-	SAS1699	hypothetical protein	17
1851683		+	-	SAS1700	DNA-binding protein	0
1852239		+	-	SAS1701	hypothetical protein	55
1852226		-	-	SAS1702	hypothetical protein	78
1854039		-	-	SAS1704	translaldolase	56
1855314		+	-	SAS1705	hypothetical protein	0
1855935		+	-	SAS1706	camphor resistance protein CrcB	56
1856285		+	-	SAS1707	CrcB-like protein	51
1856656		+	-	SAS1708	hypothetical protein	38
1856668		-	-	SAS1709	aldo/keto reductase	61
1857713		-	-	SAS1710	hypothetical protein	35
1858746		-	-	SAS1711	S-adenosylmethionine synthetase	59
	1860045	-	-	SASs021	-	72
1861903		+	-	SAS1712	phosphoenolpyruvate carboxykinase	98
1862203	1862326	+	-	SAS1712a	hypothetical protein	278
1862281	1862044	-	-	SAS1713	hypothetical protein	156
1863032		-	-	SAS1714	hypothetical protein	121
1863828	1864979	+	-	SAS1715	hypothetical protein	112
1863825	1863795	-	-	SAS1716	hypothetical protein	229
1864831	1864831	-	-	SAS1717	AMP-binding protein	138
1866468		-	-	SAS1718	lipoprotein	28
1867876		+	-	SAS1719	hypothetical protein	38
1868952		+	-	SAS1720	hypothetical protein	69
1869652		+	-	SAS1721	lipoprotein	86
1870034		+	-	SAS1722	hypothetical protein	43
1870707		+	-	SAS1723	hypothetical protein	22
1870995		-	-	SAS1724	hypothetical protein	0
1871836		-	-	SAS1725	hypothetical protein	0

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1872023		-	-	SAS1726	hypothetical protein	7
1879882		-	-	SAS1731	restriction modification DNA specificity protein	40
1881074		-	-	SAS1732	type I restriction enzyme modification protein	155
1882993	1882926	-	-	SAS1733	serine protease	115
1883833		-	-	SAS1734	serine protease	90
1884610		-	-	SAS1735	serine protease	67
1885457		-	-	SAS1736	serine protease	68
1886600		-	-	SAS1737	hypothetical protein	56
1887694		+	-	SAS1738	hypothetical protein	41
1888168		-	-	SAS1739	lantibiotic ABC transporter protein	0
1888863		-	-	SAS1740	lantibiotic ABC transporter protein	15
1889621		-	-	SAS1741	lantibiotic transport ATP-binding protein	65
1890336		-	-	SAS1742	lantibiotic leader peptide processing serine protease	80
1891719		-	-	SAS1743	lantibiotic modifying enzyme	41
1892253		-	-	SAS1744	lantibiotic biosynthesis protein	27
1893490		-	-	SAS1745	lantibiotic biosynthesis protein	42
1896548		-	-	SAS1746	lantibiotic protein	16
	1896904	-	-	predicted RNA	-	905
	1896958	+	-	predicted RNA	-	695
1897428		-	-	SAS1747a	lantibiotic protein	0
1897813		-	*hlgB*	SAS1748	gamma-hemolysin component B precursor	31
1898798		-	*hlgA*	SAS1749	gamma-hemolysin component A precursor	64
1901085		+	-	SAS1750	hypothetical protein	25
1901886		+	-	SAS1751	hypothetical protein	16
	1903131	-	-	SASt017	Ser tRNA	219
	1903761	+	-	predicted RNA	antisense: SASt023 SASt022 SASt021 SASt020 SASt019 SASt018	1135
	1903274	-	-	SASt018	Glu tRNA	352
	1903347	-	-	SASt019	Asn tRNA	576
	1903436	-	-	SASt020	Gly tRNA	915
	1903525	-	-	SASt021	His tRNA	935
	1903614	-	-	SASt022	Phe tRNA	964
	1903703	-	-	SASt023	Asp tRNA	1151
	1903800	-	-	SASt024	Met tRNA	1042
1904343	1903874	-	-	SAS1752	hypothetical protein	136
1905219		-	-	SAS1753	protoporphyrinogen oxidase	64
1906643		-	*hemH*	SAS1754	ferrochelatase	82
1907624		-	*hemE*	SAS1755	uroporphyrinogen decarboxylase	63
1909427		+	-	SAS1756	signal transduction protein	65

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1909551		-	-	SAS1757	transporter protein	56
1910767		-	-	SAS1758	ABC transporter ATP-binding protein	181
1912063		+	-	SAS1759	HIT-family protein	41
1912570		+	-	SAS1760	hypothetical protein	24
1912754		+	-	SAS1760a	hypothetical protein	0
1913860		+	-	SAS1761	hypothetical protein	5
1915027		+	-	SAS1762	peptidyl-prolyl cis-isomerase	7
1915148	1915094	-	-	SAS1763	3'-5' exoribonuclease	121
1916086		-	-	SAS1764	hypothetical protein	61
1919012		-	-	SAS1765	DNA repair exonuclease	77
1921090		-	-	SAS1766	hypothetical protein	23
1921503		-	-	SAS1767	hypothetical protein	12
1922806		-	-	SAS1768	DNA-binding protein	35
1923626		-	-	SAS1769	response regulator	35
1924271		-	-	SAS1770	histidine kinase	53
1926367		+	-	SAS1771	RNA pseudouridylate synthase	54
1926828		-	*fumC*	SAS1772	fumarate hydratase	39
1928409		-	-	SAS1773	hypothetical protein	87
1929075		+	-	SAS1774	hypothetical protein	44
1929345		+	-	SAS1775	hypothetical protein	67
1929410	1929225	-	-	SAS1776	hypothetical protein	125
1929587		-	-	SAS1777	hypothetical protein	13
1930345		-	-	SAS1778	SpoU rRNA methylase family protein	90
1930820		-	-	SAS1779	(Fe-S)-binding protein	97
1932098		-	-	SAS1780	glutamine transport ATP-binding protein	90
1932813		-	-	SAS1781	extracellular glutamine-binding protein	28
1934528		-	-	SAS1782	hypothetical protein	7
	1936137	-	-	SASt025	Leu tRNA	41
	1938446	+	-	predicted RNA	antisense: SASt031 SASt030 SASt050 SASt028 SASt027 SASt049 SASt026 SASt048 SASt047 SASt046 SASt045 SASt044 SASt043 SASt029 SASt042 SASt041 SASt040 SASt039 SASt038 SASt037 SASt036 SASt035 SASt034 SASt033 SASt032	3011
	1936262	-	-	SASt026	Xaa tRNA	241
	1936349	-	-	SASt027	Gly tRNA	490
	1936431	-	-	SASt028	Cys tRNA	647
	1936510	-	-	SASt029	Gln tRNA	645
	1936593	-	-	SASt030	His tRNA	967
	1936668	-	-	SASt031	Trp tRNA	1894
	1936757	-	-	SASt032	Tyr tRNA	2911
	1936843	-	-	SASt033	Thr tRNA	3180
	1936926	-	-	SASt034	Phe tRNA	3398
	1937017	-	-	SASt035	Asp tRNA	3248

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
	1937102	-	-	SASt036	Met tRNA	3531
	1937190	-	-	SASt037	Ser tRNA	4674
	1937321	-	-	SASt038	Asp tRNA	4801
	1937407	-	-	SASt039	Ser tRNA	4584
	1937506	-	-	SASt040	Met tRNA	4577
	1937605	-	-	SASt041	Met tRNA	3947
	1937703	-	-	SASt042	Ala tRNA	3200
	1937796	-	-	SASt043	Pro tRNA	2361
	1937879	-	-	SASt044	Arg tRNA	2189
	1937971	-	-	SASt045	Leu tRNA	2005
	1938070	-	-	SASt046	Gly tRNA	2216
	1938148	-	-	SASt047	Leu tRNA	2981
	1938233	-	-	SASt048	Lys tRNA	3107
	1938314	-	-	SASt049	Thr tRNA	2899
	1938406	-	-	SASt050	Val tRNA	2507
	1938493	-	-	SASr011	5S ribosomal RNA	1962
	1938680	-	-	SASr012	23S ribosomal RNA	1093
	1939936	+	-	predicted RNA	antisense: SASr012	2314
	1940469	+	-	predicted RNA	antisense: SASr012	1067
	1941894	-	-	SASt051	Ala tRNA	0
	1941988	-	-	SASt052	Ile tRNA	70
	1942154	-	-	SASr013	16S ribosomal RNA	1602
	1943577	+	-	predicted RNA	antisense: SASr013	2100
1944450		-	-	SAS1783	peroxide operon regulator	28
1944993		-	-	SAS1784	hypothetical protein	11
1945949		-	-	SAS1785	AhpC/TSA family protein	39
1947774	1947774	+	-	SAS1786	glutamate-1-semialdehyde aminotransferase	109
1949161		+	-	SAS1787	hypothetical protein	43
1949353		-	-	SAS1788	ABC transporter ATP-binding protein	67
1951380		-	-	SAS1789	hypothetical protein	38
1952224		-	-	SAS1790	HhH-GPD superfamily base excision DNA repair protein	19
1954390		+	-	SAS1791	hypothetical protein	70
1954651		-	-	SAS1792	hypothetical protein	10
1955496		-	-	SAS1793	hypothetical protein	22
1957028		-	-	SAS1794	hypothetical protein	8
1957320		-	*recX*	SAS1795	recombination regulator RecX	15
1958399		-	-	SAS1796	glycosyltransferase	29
1959547		-	-	SAS1797	ThiJ/PfpI family protein	36
1960193		-	-	SAS1798	hypothetical protein	31
1961729		+	-	SAS1799	hypothetical protein	64

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
1961989		-	-	SAS1800	hypothetical protein	52
1962608	1962608	-	-	SAS1801	aminopeptidase	140
1963867		-	-	SAS1802	hypothetical protein	32
1964678		+	-	SAS1803	low molecular weight phosphotyrosine protein phosphatase	70
1964960		+	-	SAS1804	hypothetical protein	28
1966449		+	-	SAS1805	hypothetical protein	52
1966561		-	-	SAS1806	response regulator	59
1967180		-	-	SAS1807	histidine kinase sensor	68
1968220		-	-	SAS1808	hypothetical protein	37
1968936		-	-	SAS1809	hypothetical protein	28
1969539		-	-	SAS1810	methionine aminopeptidase	26
1971814		+	-	SAS1811	hypothetical protein	91
1972112		-	-	SAS1811a	hypothetical protein	50
1972304		-	-	SAS1812	hypothetical protein	50
1972943		-	-	SAS1813	hypothetical protein	29
1973676		-	-	SAS1814	Mur ligase family protein	65
1975781		+	-	SAS1815	ferritin	44
1976725		+	-	SAS1816	exonuclease	52
1976792		-	-	SAS1817	DNA polymerase IV	41
1978109		-	-	SAS1818	hypothetical protein	73
1978809		-	-	SAS1819	RNA methyltransferase	22
1980251		-	-	SAS1820	lipid kinase	69
1981852	1981852	-	*gatB*	SAS1822	aspartyl/glutamyl-tRNA amidotransferase subunit B	113
1983292		-	*gatA*	SAS1823	aspartyl/glutamyl-tRNA amidotransferase subunit A	91
1984751		-	*gatC*	SAS1824	aspartyl/glutamyl-tRNA amidotransferase subunit C	2
1986958		+	-	SAS1825	high affinity proline permease	77
1987047		-	-	SAS1826	lipoprotein	76
1988259		-	-	SAS1827	DNA ligase	79
1990266		-	-	SAS1828	ATP-dependent DNA helicase	77
1992455		-	-	SAS1829	geranylgeranylglyceryl phosphate synthase	18
1993319		-	-	SAS1830	hypothetical protein	41
1993730		-	-	SAS1831	adenylosuccinate lyase	74
1997052		+	-	SAS1832	staphopain protease	24
1997409		+	-	SAS1833	hypothetical protein	10
1997701		-	-	SAS1834	hypothetical protein	44
1997855		-	-	SAS1835	hypothetical protein	82
1998728		-	*nadE*	SAS1836	NAD synthetase	50
1999542		-	-	SAS1837	nicotinate phosphoribosyltransferase	54
2002271		+	-	SAS1838	oxygenase	29
2003085		+	-	SAS1839	prephenate dehydratase	96

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2003275		-	-	SAS1840	sodium:sulfate symporter	101
2005042		-	-	SAS1841	hypothetical protein	60
2007071		+	-	SAS1842	isochorismatase family protein	45
2008053		+	-	SAS1843	manganese-dependent inorganic pyrophosphatase	50
2009852	2009852	+	-	SAS1844	aldehyde dehydrogenase	240
2009972		-	-	SAS1845	hypothetical protein	68
2011671		+	-	SAS1846	hypothetical protein	18
2012244		-	-	SAS1848	hypothetical protein	21
2013908		+	-	SAS1849	hypothetical protein	72
2013965		-	-	SAS1850	hypothetical protein	26
2014804		-	-	SAS1851	hypothetical protein	12
2015421		-	-	SAS1852	hypothetical protein	0
2016629	2016633	+	-	SAS1853	hypothetical protein	168
2016695		-	-	SAS1854	hypothetical protein	16
2017435		-	-	SAS1855	ABC transporter ATP-binding protein	31
2018304		-	-	SAS1856	hypothetical protein	36
2018985		-	-	SAS1857	ABC transporter ATP-binding protein	6
2019878		-	-	SAS1858	GntR family transcriptional regulator	14
2020517		-	-	SAS1859	hypothetical protein	45
2020936	2020905	-	-	SAS1859.1	hypothetical protein	413
2022520		+	-	SAS1860	hypothetical protein	51
2026030		+	-	SAS1863a	hypothetical protein	13
2026362		+	-	SAS1864	hypothetical protein	5
2026601		+	-	SAS1865	hypothetical protein	0
2026654		-	-	SAS1866	hypothetical protein	42
2028502	2028502	-	-	SAS1868	staphylokinase precursor	117
2029184		-	-	SAS1869	autolysin	61
2029951	2029940	-	-	SAS1870	holin	141
2030417	2030206	-	-	SAS1871	hypothetical protein	106
2030702		-	-	SAS1872	enterotoxin type A precursor	68
2031848	2031476	-	-	SAS1873	hypothetical protein	109
2032278	2032232	-	-	SAS1874	hypothetical protein	177
2032611		-	-	SAS1875	hypothetical protein	103
2032756		-	-	SAS1876	hypothetical protein	128
	2033666	+	-	predicted RNA	antisense: SAS1876	635
	2033881	+	-	predicted RNA	antisense: SAS1876	734
2036554	2036539	-	-	SAS1877	hypothetical protein	127
2038044		-	-	SAS1878	hypothetical protein	250
	2038523	+	-	predicted RNA	antisense: SAS1878	634
	2041190	+	-	predicted RNA	antisense: SAS1878	680

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2042749	2042749	-	-	SAS1879	hypothetical protein	374
2042931	2042931	-	-	SAS1880	hypothetical protein	207
2043442	2043378	-	-	SAS1881	hypothetical protein	288
2044396		-	-	SAS1882	hypothetical protein	166
2044773		-	-	SAS1883	hypothetical protein	166
2045150		-	-	SAS1884	hypothetical protein	243
2045472		-	-	SAS1885	hypothetical protein	263
2045813	2045805	-	-	SAS1886	hypothetical protein	334
2046007	2046007	-	-	SAS1887	capsid protein	200
2047342	2047342	-	-	SAS1888	prohead protease	241
	2048033	+	-	predicted RNA	antisense: SAS1888 SAS1889	870
2047919		-	-	SAS1889	portal protein	375
2049175	2049175	-	-	SAS1890	hypothetical protein	413
	2049495	+	-	predicted RNA	antisense: SAS1891	724
2049389	2049389	-	-	SAS1891	terminase-large subunit	1026
	2051480	+	-	predicted RNA	antisense: SAS1891 SAS1892	1513
2051086	2051084	-	-	SAS1892	hypothetical protein	2837
2051682	2051682	-	-	SAS1893	hypothetical protein	909
2052042	2052036	-	-	SAS1894	hypothetical protein	177
2052609		-	-	SAS1895	hypothetical protein	67
2053102		-	-	SAS1896	hypothetical protein	31
2053302		-	-	SAS1897	hypothetical protein	48
2053959		-	-	SAS1898	hypothetical protein	61
2054111		-	-	SAS1899	hypothetical protein	30
2054601	2054599	-	-	SAS1901	hypothetical protein	263
2055014	2055006	-	-	SAS1902	hypothetical protein	218
2055239	2055239	-	-	SAS1903	phage regulatory protein	211
2056162	2056162	-	-	SAS1904	single-strand DNA-binding protein	153
2056633		-	-	SAS1905	hypothetical protein	170
2057331	2057331	-	-	SAS1906	hypothetical protein	182
2058253		-	-	SAS1907	hypothetical protein	81
2060205		-	-	SAS1908	hypothetical protein	52
2060477		-	-	SAS1909	hypothetical protein	41
2060830		-	-	SAS1910	hypothetical protein	16
2060988		-	-	SAS1911	hypothetical protein	56
2061999		+	-	SAS1912	hypothetical protein	80
2062014		-	-	SAS1912a	hypothetical protein	56
2062185		-	-	SAS1913	hypothetical protein	49
2063739		+	-	SAS1916	hypothetical protein	33
2063763		-	-	SAS1917	hypothetical protein	95
2064041		-	-	SAS1918	regulatory protein	110

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2065194	2065389	+	-	SAS1919	repressor	134
2066118		+	-	SAS1920	enterotoxin	73
2066870		+	-	SAS1921	enterotoxin	15
2067991		+	-	SAS1922	integrase	93
2069110		-	-	SAS1924	leukocidin F subunit	26
2070148		-	-	SAS1925	leukocidin S subunit	58
2072859		+	-	SAS1926	succinyl-diaminopimelate desuccinylase	60
2073231		-	-	SAS1927	hypothetical protein	58
2074138		-	-	SAS1928	ferrichrome-binding protein precursor	30
2076517		+	-	SAS1929	sodium transport protein	41
2077371		-	-	SAS1930	GNAT family acetyltransferase	12
2077920		-	-	SAS1931	hypothetical protein	11
2078674		-	-	SAS1932	terminase small subunit	25
2079261		-	-	SAS1933	hypothetical protein	5
2080175	2080073	-	*groEL*	SAS1935	molecular chaperone GroEL	187
2081867		-	*groES*	SAS1936	co-chaperonin GroES	41
2083069		+	-	SAS1937	hypothetical protein	62
2083094	2083094	-	-	SAS1938	membrane anchored protein	201
2085059		+	-	SAS1939	hypothetical protein	65
2086205		+	-	SAS1940	carbon-nitrogen hydrolase	55
	2086458	-	-	SASs022	-	37
2086804		-	-	SAS1940a	delta-hemolysin	60
2087737		+	-	SAS1941	autoinducer processing protein	28
2087881		+	-	SAS1942	autoinducer peptide	30
2089198		+	-	SAS1943	autoinducer sensor protein	73
2089933		+	-	SAS1944	autoinducer sensor protein response regulator protein	25
2090302		-	-	SAS1945	fructokinase	34
2091258		-	-	SAS1946	sucrose-6-phosphate hydrolase	38
2092891		-	-	SAS1947	sucrose operon repressor	70
2094024		-	-	SAS1948	ammonium transporter family protein	68
2095482		-	-	SAS1949	hypothetical protein	102
2095766		-	-	SAS1950	hypothetical protein	94
2097149		-	-	SAS1951	redox-sensing transcriptional repressor Rex	40
2099965		+	-	SAS1952	ABC transporter ATP-binding protein	37
2101937		+	-	SAS1953	hypothetical protein	68
2102039		-	-	SAS1954	DNA-binding/iron metalloprotein/AP endonuclease	54
2103057		-	-	SAS1955	acetyltransferase	51
2103494		-	-	SAS1956	hypothetical protein	97
2104137		-	-	SAS1957	hypothetical protein	40
2106797	2106797	+	-	SAS1958	dihydroxy-acid dehydratase	217
2108594	2108746	+	-	SAS1959	acetolactate synthase large subunit	117

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2109989	2109989	+	-	SAS1961	ketol-acid reductoisomerase	152
2111548	2111550	+	-	SAS1962	2-isopropylmalate synthase	164
2112597	2112610	+	-	SAS1963	3-isopropylmalate dehydrogenase	168
2113981		+	-	SAS1964	isopropylmalate isomerase large subunit	86
2114554		+	*leuD*	SAS1965	isopropylmalate isomerase small subunit	46
2115837		+	-	SAS1966	threonine dehydratase	73
	2116304	-	-	SASr014	5S ribosomal RNA	177
	2116491	-	-	SASr015	23S ribosomal RNA	1019
	2117678	+	-	predicted RNA	antisense: SASr015	2574
	2117898	+	-	predicted RNA	antisense: SASr015	668
	2118260	+	-	predicted RNA	antisense: SASr015	921
	2119960	-	-	SASr016	16S ribosomal RNA	1710
	2121480	+	-	predicted RNA	antisense: SASr016	2267
	2121632	-	-	SASt053	Gly tRNA	805
	2121724	-	-	SASt054	Leu tRNA	689
2122456	2122456	-	-	SAS1967	hypothetical protein	120
2122904		-	-	SAS1968	RNA binding protein	91
2125487		-	-	SAS1969	RNA polymerase sigma factor SigB	54
2126232		-	-	SAS1970	serine-protein kinase RsbW	5
2126713	2126712	-	-	SAS1971	anti-sigma B factor antagonist	169
2127158		-	-	SAS1972	sigma factor sigB regulation protein	53
2128508		-	-	SAS1973	hypothetical protein	49
2128867		-	-	SAS1974	hypothetical protein	90
2129122	2129050	-	-	SAS1975	alanine racemase	153
2130336		-	*acpS*	SAS1976	4'-phosphopantetheinyl transferase	11
2130699		-	-	SAS1977	hypothetical protein	38
2131177		-	-	SAS1978	hypothetical protein	91
2132753		-	-	SAS1979	hypothetical protein	2
2133441		-	-	SAS1980	potassium-transporting ATPase subunit C	29
2134021		-	-	SAS1981	potassium-transporting ATPase subunit B	87
2136067		-	-	SAS1982	potassium-transporting ATPase subunit A	80
2140672		+	-	SAS1983	sensor kinase	89
2141367		+	-	SAS1984	response regulator protein	70
2141736		-	-	SAS1985	helicase	54
2143773		-	-	SAS1986	UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-- D-alanyl-D-alanyl ligase	37
2145146		-	*ddl*	SAS1987	D-alanyl-alanine synthetase A	49
2147715	2147768	+	-	SAS1988	hypothetical protein	115
2147978		-	-	SAS1989	hypothetical protein	8

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2148243		-	-	SAS1990	hypothetical protein	0
2148464		-	-	SAS1991	hypothetical protein	41
2150407		+	-	SAS1992	cardiolipin synthetase	55
2151082	2151955	+	-	SAS1993	hypothetical protein	113
2151696	2151058	-	-	SAS1994	hypothetical protein	125
2152654		-	*thiE*	SAS1995	thiamine-phosphate pyrophosphorylase	101
2153288		-	-	SAS1996	hydroxyethylthiazole kinase	91
2154072		-	-	SAS1997	phosphomethylpyrimidine kinase	170
2154895		-	-	SAS1998	transcriptional activator	147
	2155664	-	-	SASs023	-	68
2156092		+	-	SAS1998b	hypothetical protein	5
2156108	2156108	-	-	SAS1999	hypothetical protein	217
2157192		-	-	SAS2000	ssDNA-binding protein	10
2158221		+	-	SAS2001	hypothetical protein	24
2158279	2158279	-	*fabZ*	SAS2002	(3R)-hydroxymyristoyl-ACP dehydratase	118
2158753		-	-	SAS2003	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	71
2160129		-	-	SAS2004	hypothetical protein	24
2160924		-	*atpC*	SAS2005	ATP synthase F0F1 subunit epsilon	62
2161348		-	-	SAS2006	ATP synthase F0F1 subunit beta	93
2162782	2162779	-	-	SAS2007	ATP synthase F0F1 subunit gamma	117
2163679		-	-	SAS2008	ATP synthase F0F1 subunit alpha	93
2165209		-	-	SAS2009	ATP synthase F0F1 subunit delta	46
2165748		-	-	SAS2010	ATP synthase F0F1 subunit B	28
2166466		-	-	SAS2011	F0F1 ATP synthase subunit C	59
2166721		-	-	SAS2012	ATP synthase F0F1 subunit A	97
2167470	2167450	-	-	SAS2013	ATP synthase I	123
2167990		-	-	SAS2014	UDP-GlcNAc 2-epimerase	78
2169141		-	*upp*	SAS2015	uracil phosphoribosyltransferase	74
2169798		-	*glyA*	SAS2016	serine hydroxymethyltransferase	62
2171063		-	-	SAS2017	hypothetical protein	37
2171694		-	-	SAS2018	low molecular weight protein-tyrosine-phosphatase	92
2172110		-	-	SAS2019	hypothetical protein	88
2173240		-	-	SAS2020	hypothetical protein	49
2174063		-	*prfA*	SAS2021	peptide chain release factor 1	90
2175140		-	-	SAS2022	thymidine kinase	231
2176086		-	*rpmE2*	SAS2023	50S ribosomal protein L31	40
2176458		-	*rho*	SAS2024	transcription termination factor Rho	46
2178021		-	-	SAS2025	aldehyde dehydrogenase	36
2179686		-	-	SAS2026	hypothetical protein	71
2180110	2180107	-	-	SAS2027	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	116
2181835		-	-	SAS2028	fructose-bisphosphate aldolase	85

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2183434		+	-	SAS2029	hypothetical protein	46
2183543		-	*pyrG*	SAS2030	CTP synthetase	41
2185489		-	-	SAS2031	DNA-directed RNA polymerase subunit delta	78
2186131		-	-	SAS2032	acetyltransferase	69
2188218	2188218	+	-	SAS2033	pantothenate kinase	117
2188348	2188049	-	-	SAS2034	hypothetical protein	142
2189418		-	-	SAS2035	hypothetical protein	28
2190611		-	-	SAS2036	peptidase	53
2192576		+	-	SAS2037	S-ribosylhomocysteinase	52
2192728	2192686	-	-	SAS2038	hypothetical protein	176
2193092	2193079	-	-	SAS2039	pyrimidine-nucleoside phosphorylase	118
2194674		-	-	SAS2040	deoxyribose-phosphate aldolase	67
2196361	2196445	+	*deoD*	SAS2041	purine nucleoside phosphorylase	113
2196482		-	-	SAS2042	non-heme iron-containing ferritin	4
2197125		-	-	SAS2043	hypothetical protein	6
2197996		-	-	SAS2044	hypothetical protein	31
2200041		-	-	SAS2045	hypothetical protein	38
2200302		-	-	SAS2046	mannose-6-phosphate isomerase	38
2201676		-	-	SAS2047	hypothetical protein	9
2202813		+	-	SAS2048	zinc and cobalt transport repressor protein	69
2203795	2203888	+	-	SAS2049	zinc resistance protein	137
2205152		+	-	SAS2050	hypothetical protein	23
2207056		+	-	SAS2052a	hypothetical protein	0
2207186		-	-	SAS2053	haloacid dehalogenase	74
2208111		-	-	SAS2054	ABC transporter ATP-binding protein	35
2209125		-	-	SAS2055	glucosamine--fructose-6-phosphate aminotransferase	75
	2211132	-	-	SASs024	-	0
2211553		+	-	SAS2056	hypothetical protein	0
2213149	2213149	+	-	SAS2057	PTS system mannitol-specific transporter subunit IIBC	182
2215316		+	-	SAS2058	transcriptional antiterminator	99
2215762		+	-	SAS2059	PTS system mannitol-specific transporter subunit IIA	86
2216868		+	-	SAS2060	mannitol-1-phosphate 5-dehydrogenase	63
	2217774	+	-	predicted RNA	-	685
2224979	2223844	-	*glmM*	SAS2063	phosphoglucosamine mutase	348
	2224800	+	-	predicted RNA	-	6549
2226361		-	-	SAS2064	hypothetical protein	25
2227295		-	-	SAS2065	hypothetical protein	39
2228293		-	-	SAS2066	arginase	50
	2229336	-	-	SASt055	Lys tRNA	27

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
	2229415	-	-	SASt056	Gln tRNA	47
	2229499	-	-	SASt057	Tyr tRNA	191
	2229891	+	-	predicted RNA	antisense: SASt059 SASt058 SASt057 SASt060 SASr017	994
	2229612	-	-	SASt058	Val tRNA	420
	2229695	-	-	SASt059	Glu tRNA	651
	2229768	-	-	SASt060	Asn tRNA	939
	2229853	-	-	SASr017	5S ribosomal RNA	1219
	2230040	-	-	SASr018	23S ribosomal RNA	1084
	2231284	+	-	predicted RNA	antisense: SASr018	2411
	2231404	+	-	predicted RNA	antisense: SASr018	684
	2231819	+	-	predicted RNA	antisense: SASr018	1169
	2233386	-	-	SASr019	16S ribosomal RNA	1635
	2234839	+	-	predicted RNA	antisense: SASr019	1848
2235396	2234940	-	-	SAS2067	hypothetical protein	110
2236608		-	-	SAS2068	transport protein	80
2238373		-	-	SAS2069	hypothetical protein	46
2238945		-	-	SAS2070	transport protein	32
2240465		-	-	SAS2071	hypothetical protein	2
2241167		-	-	SAS2072	UTP-glucose-1-phosphate uridylyltransferase	44
2242398		-	-	SAS2073	hypothetical protein	26
2243350		+	-	SAS2074	hypothetical protein	60
2243482		-	-	SAS2075	hypothetical protein	40
2245088		-	-	SAS2076	FecCD transport family protein	62
2246053		-	-	SAS2077	FecCD transport family protein	59
2247096		-	-	SAS2078	transport system binding lipoprotein	44
2248471		-	-	SAS2079	hypothetical protein	44
2249548		-	-	SAS2080	hypothetical protein	67
2251292		-	-	SAS2081	hypothetical protein	29
2254563		+	-	SAS2082	hypothetical protein	40
2254723		-	-	SAS2083	alkaline shock protein 23	51
2255295		-	-	SAS2084	hypothetical protein	32
2255547		-	-	SAS2085	hypothetical protein	35
2256258	2256258	-	-	SAS2086	glycine betaine transporter 2	104
2258128		-	-	SAS2087	zinc-binding dehydrogenase	47
2259369	2259136	-	-	SAS2088	zinc-binding dehydrogenase	145
2260653		-	-	SAS2089	hypothetical protein	25
2261788	2261535	-	-	SAS2090	6-phospho-beta-galactosidase	120
2263218	2263218	-	-	SAS2091	PTS system lactose-specific transporter subunit IIBC	152
2264936		-	-	SAS2092	PTS system lactose-specific transporter subunit IIA	70

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2265269		-	-	SAS2093	tagatose 1,6-diphosphate aldolase	92
2266253	2266250	-	-	SAS2094	tagatose-6-phosphate kinase	142
2267198		-	-	SAS2095	galactose-6-phosphate isomerase subunit LacB	93
2267729		-	-	SAS2096	galactose-6-phosphate isomerase subunit LacA	82
2268525		-	-	SAS2097	lactose phosphotransferase system repressor	16
2270262	2270262	+	-	SAS2098	NAD-dependent deacetylase	178
2270460		+	-	SAS2099	hypothetical protein	83
2270553		-	-	SAS2100	hypothetical protein	57
2271423	2271327	-	-	SAS2100a	hypothetical protein	150
2271767		-	-	SAS2101	aldo/keto reductase	51
2272685		-	-	SAS2102	MerR family transcriptional regulator	38
2275957		+	-	SAS2103	hyaluronate lyase precursor 2	51
2276571		+	-	SAS2104	hypothetical protein	41
2276958		-	-	SAS2105	hypothetical protein	35
2277699		-	-	SAS2106	acetolactate synthase	43
2279736		+	-	SAS2107	hypothetical protein	0
2279810		-	-	SAS2107a	hypothetical protein	0
2280339	2280339	-	*rpsI*	SAS2108	30S ribosomal protein S9	112
2280751		-	*rplM*	SAS2109	50S ribosomal protein L13	100
2281428		-	*truA*	SAS2110	tRNA pseudouridine synthase A	74
2282236		-	-	SAS2111	cobalt transport protein	84
2283032		-	*cbiO*	SAS2112	cobalt transporter ATP-binding subunit	93
2283889		-	*cbiO*	SAS2113	cobalt transporter ATP-binding subunit	159
2285236		-	*rplQ*	SAS2114	50S ribosomal protein L17	42
2285621	2285605	-	-	SAS2115	DNA-directed RNA polymerase subunit alpha	163
2286640	2286640	-	-	SAS2116	30S ribosomal protein S11	202
2287053	2287049	-	*rpsM*	SAS2117	30S ribosomal protein S13	209
2287441		-	*rpmJ*	SAS2118	50S ribosomal protein L36	90
2287586	2287555	-	*infA*	SAS2119	translation initiation factor IF-1	161
2287997	2287805	-	*adk*	SAS2120	adenylate kinase	115
2288661	2288661	-	*secY*	SAS2121	preprotein translocase subunit SecY	151
2289953		-	*rplO*	SAS2122	50S ribosomal protein L15	44
2290410	2290410	-	*rpmD*	SAS2123	50S ribosomal protein L30	261
2290606	2290590	-	*rpsE*	SAS2124	30S ribosomal protein S5	346
2291127		-	*rplR*	SAS2125	50S ribosomal protein L18	96
2291517	2291517	-	*rplF*	SAS2126	50S ribosomal protein L6	117
2292078		-	*rpsH*	SAS2127	30S ribosomal protein S8	21
2292508	2292495	-	*rpsN*	SAS2128	30S ribosomal protein S14	130
2292716		-	*rplE*	SAS2129	50S ribosomal protein L5	56
2293282	2293278	-	*rplX*	SAS2130	50S ribosomal protein L24	162

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2293635		-	*rplN*	SAS2131	50S ribosomal protein L14	91
2294035		-	*rpsQ*	SAS2132	30S ribosomal protein S17	20
2294322		-	-	SAS2133	50S ribosomal protein L29	22
2294521		-	*rplP*	SAS2134	50S ribosomal protein L16	101
2294958	2294956	-	*rpsC*	SAS2135	30S ribosomal protein S3	170
2295635	2295635	-	*rplV*	SAS2136	50S ribosomal protein L22	114
2296017		-	*rpsS*	SAS2137	30S ribosomal protein S19	36
2296362	2296362	-	*rplB*	SAS2138	50S ribosomal protein L2	151
	2296703	+	-	predicted RNA	antisense: rplB	634
2297228	2297196	-	*rplW*	SAS2139	50S ribosomal protein L23	311
2297503		-	*rplD*	SAS2140	50S ribosomal protein L4	179
2298153		-	*rplC*	SAS2141	50S ribosomal protein L3	70
2298843		-	*rpsJ*	SAS2142	30S ribosomal protein S10	102
2299897		+	-	SAS2143	hypothetical protein	22
2300088		-	-	SAS2144	xanthine/uracil permeases family protein	67
2301535		-	-	SAS2145	DNA topoisomerase III	47
2304744		+	-	SAS2146	acetyltransferase (GNAT) family protein	29
2305788		+	-	SAS2147	glucose uptake protein	95
2306207		-	-	SAS2148	hypothetical protein	46
2307412		+	-	SAS2149	hypothetical protein	3
2307974		+	-	SAS2150	hypothetical protein	67
2308158		-	-	SAS2151	AcrB/AcrD/AcrF family protein	69
2311442		-	-	SAS2152	peptidoglycan pentaglycine interpeptide biosynthesis protein	30
2313347		+	-	SAS2153	hypothetical protein	6
2313339		-	-	SAS2154	hypothetical protein	35
2314705		+	-	SAS2155	MarR family transcriptional regulator	25
2315909		+	-	SAS2156	transporter protein	62
2316011		-	-	SAS2157	hypothetical protein	98
2316939	2316939	-	-	SAS2158	molybdenum cofactor biosynthesis protein A	197
2317981		-	*mobA*	SAS2159	molybdopterin-guanine dinucleotide biosynthesis protein MobA	41
2318587		-	-	SAS2160	molybdopterin-synthase small subunit	47
2318826		-	-	SAS2161	molybdopterin-synthase large subunit	58
2319286		-	-	SAS2162	molybdopterin-guanine dinucleotide biosynthesis protein B	101
2319768		-	-	SAS2163	molybdenum cofactor biosynthesis protein	160
2321584	2321621	+	*moaC*	SAS2164	molybdenum cofactor biosynthesis protein MoaC	195
2321586	2321586	-	-	SAS2165	molybdenum cofactor biosynthesis protein B	128
2322123	2322093	-	-	SAS2166	molybdopterin synthase sulfurylase	138
2323297		-	-	SAS2167	molybdenum transport ATP-binding protein	93
2323903		-	-	SAS2168	molybdenum transport system permease	164

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2324588		-	-	SAS2169	molybdate-binding lipoprotein precursor	88
2326457		+	-	SAS2170	formate dehydrogenase accessory protein	51
2326629		-	-	SAS2171	acetyltransferase (GNAT) family protein	42
2327476		-	-	SAS2172	BioY family protein	41
2328151		-	-	SAS2173	inosine-uridine preferring nucleoside hydrolase	102
2329490		-	-	SAS2174	ferrichrome-binding lipoprotein precursor	35
2330612		-	-	SAS2175	hypothetical protein	73
2331813	2331767	-	-	SAS2176	hypothetical protein	109
2331983	2331983	-	-	SAS2177	hypothetical protein	141
2333450		+	*ureA*	SAS2178	urease subunit gamma	47
2333874		+	*ureB*	SAS2179	urease subunit beta	67
2335586	2335586	+	*ureC*	SAS2180	urease subunit alpha	185
2336051		+	*ureE*	SAS2181	urease accessory protein UreE	95
2336733		+	-	SAS2182	urease accessory protein UreF	24
2337360		+	-	SAS2183	urease accessory protein UreG	96
2338196		+	-	SAS2184	urease accessory protein UreD	58
2338385		-	-	SAS2185	accessory regulator A	38
2339160		-	-	SAS2186	hypothetical protein	66
2339550		-	-	SAS2187	hypothetical protein	22
2340320		-	-	SAS2188	transcriptional regulator	23
2343694	2343783	+	-	SAS2189	hypothetical protein	239
2343711	2343468	-	-	SAS2190	hypothetical protein	353
2344127	2344127	-	-	SAS2191	Na+/H+ antiporter	136
2345622		-	-	SAS2192	dehydrogenase	40
2347374		+	-	SAS2193	hypothetical protein	38
2348111	2348206	+	-	SAS2194	hypothetical protein	211
2348416	2348416	+	-	SAS2195	hypothetical protein	161
2349426		+	-	SAS2196	glycerate dehydrogenase	81
2349507		-	-	SAS2197	hypothetical protein	23
2351356	2351305	-	-	SAS2198	N-acetylmuramoyl-L-alanine amidase	252
2352154		-	-	SAS2199	hypothetical protein	90
2352615		-	-	SAS2200	hypothetical protein	23
2353088		-	-	SAS2201	bifunctional protein GlmU	167
	2355696	+	-	predicted RNA	antisense: SAS2201	661
2356644		-	-	SAS2202	hypothetical protein	87
2357724		-	-	SAS2203	inositol monophosphatase	20
2359598		+	-	SAS2204	DeoR family regulatory protein	40
2360245		-	-	SAS2206	hypothetical protein	38
2361360		+	-	SAS2207	hypothetical protein	26
2362677		+	-	SAS2208	transcriptional regulator	17
2363064		-	-	SAS2209	amino acid permease	98

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2365079		+	-	SAS2210	hypothetical protein	24
2365261		+	-	SAS2211	hypothetical protein	65
2365357	2364444	-	-	SAS2212	hypothetical protein	115
2365559		-	-	SAS2213	haloacid dehalogenase	40
2366269		-	-	SAS2214	sodium/bile acid symporter family protein	93
2367984		+	-	SAS2215	hypothetical protein	39
2368050		-	-	SAS2216	PTS system, arbutin-like IIBC component	57
2370748		+	-	SAS2217	transcriptional regulator	36
2370806	2370794	-	-	SAS2218	hypothetical protein	154
2371479	2371479	-	-	SAS2219	transporter	130
	2372869	-	-	SASs025	-	78
2373017	2372965	-	-	SAS2220	hypothetical protein	128
2374073		-	-	SAS2221	short chain dehydrogenase	54
2375065		-	-	SAS2222	peptidase	83
2376462	2376187	-	-	SAS2223	imidazolonepropionase	197
2377700		-	-	SAS2224	urocanate hydratase	216
2380405	2380524	+	-	SAS2225	LysR family regulatory protein	108
2380898		-	-	SAS2226	formimidoylglutamase	95
2383382	2383382	+	-	SAS2227	hypothetical protein	112
2383448		-	-	SAS2228	ribose-5-phosphate isomerase A	36
2384961		+	-	SAS2229	hypothetical protein	55
2385197	2385197	-	-	SAS2230	aldose 1-epimerase	117
2386246		-	-	SAS2231	hypothetical protein	96
2386790		-	-	SAS2232	hypothetical protein	83
2388015		-	-	SAS2233	ABC transporter ATP-binding protein	78
2389145		-	-	SAS2234	hypothetical protein	44
2389879		+	-	SAS2234a	hypothetical protein	0
2390076		+	-	SAS2234b	hypothetical protein	92
2390772	2390913	+	-	SAS2235	3-methylpurine glycosylase	117
2392317		+	-	SAS2236	permease	48
2392365	2392365	-	-	SAS2237	isopentenyl pyrophosphate isomerase	219
2393445		-	-	SAS2238	CorA-like Mg2+ transporter protein	88
2394980		+	-	SAS2239	hypothetical protein	16
2395396		+	-	SAS2240	hypothetical protein	78
2395504		-	-	SAS2241	hypothetical protein	50
2397270		+	-	SAS2242	hypothetical protein	41
2397516		-	-	SAS2243	transporter	52
2399460		-	-	SAS2244	hypothetical protein	27
2400779		+	-	SAS2245	TetR family regulatory protein	37
2401009		-	-	SAS2246	teicoplanin resistance associated membrane protein	96
2402486		-	-	SAS2247	teicoplanin resistance associated membrane protein	53

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2404108		-	-	SAS2248	MarR family transcriptional regulator	39
2405726		+	-	SAS2249	hypothetical protein	66
2405957		-	-	SAS2250	ABC transporter ATP-binding protein	63
2406622		-	-	SAS2251	permease	69
2408487		+	-	SAS2252	response regulator protein	62
2409853		+	-	SAS2253	sensor kinase	81
2410459		+	-	SAS2254	hypothetical protein	42
2410911		+	-	SAS2255	hypothetical protein	14
2410977	2410977	-	-	SAS2256	malate:quinone oxidoreductase	111
2412836		-	-	SAS2257	L-lactate permease 2	34
2416424		+	-	SAS2258	hypothetical protein	42
2416578		-	-	SAS2259	hypothetical protein	29
2417506		-	-	SAS2260	acetyltransferase (GNAT) family protein	13
2419216		+	-	SAS2261	zinc-binding dehydrogenase	77
2420706		+	-	SAS2262	hypothetical protein	16
2420982		-	-	SAS2263	acetyltransferase (GNAT) family protein	75
2421419		-	-	SAS2264	pyridine nucleotide-disulfide oxidoreductase family protein	68
2422685		-	-	SAS2265	hypothetical protein	19
2423323		-	-	SAS2266	hypothetical protein	88
2425384		+	-	SAS2267	TetR family regulatory protein	18
2426374		+	-	SAS2268	CorA-like Mg2+ transporter protein	10
2426459		-	-	SAS2269	PTS system, sucrose-specific IIBC component	34
2428467		+	-	SAS2270	hypothetical protein	56
2432060		+	-	SAS2271	AraC family transcriptional regulator	23
2432553		+	-	SAS2272	hypothetical protein	83
2433174		+	-	SAS2273	hypothetical protein	48
2433445		-	-	SAS2274	proton/sodium-glutamate symport protein	61
2434904		-	-	SAS2275	hypothetical protein	22
2436113		+	-	SAS2276	MarR family transcriptional regulator	20
2436757		+	-	SAS2277	small heat shock protein	19
2436864	2436864	-	-	SAS2278	nitrite transport protein	181
2438223		+	-	SAS2278a	hypothetical protein	11
2440036		+	-	SAS2281	MerR family transcriptional regulator	92
2440294		-	-	SAS2282	response regulator	32
2440970	2440970	-	-	SAS2283	histidine kinase	109
2442028		-	-	SAS2284	hypothetical protein	80
2442500		-	-	SAS2285	nitrate reductase gamma chain	77
2443170		-	-	SAS2286	respiratory nitrate reductase delta chain	148
2443738		-	-	SAS2287	nitrate reductase subunit beta	52
2445287		-	-	SAS2288	nitrate reductase subunit alpha	214
2449284		-	-	SAS2289	tetrapyrrole (corrin/porphyrin) methylase family protein	91

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2450252		-	-	SAS2290	assimilatory nitrite reductase small subunit	137
2450570	2450567	-	-	SAS2291	nitrite reductase large subunit	140
2453041		-	-	SAS2292	hypothetical protein	54
2454001		-	-	SAS2293	acetyltransferase (GNAT) family protein	80
2454772		-	-	SAS2294	nitrite transporter	62
2455813	2455729	-	-	SAS2295	hypothetical protein	109
2456079		-	-	SAS2296	hypothetical protein	34
2456732		-	-	SAS2297	solute binding lipoprotein	20
2458576		-	-	SAS2298	hypothetical protein	0
2458842		-	-	SAS2299	hypothetical protein	27
2459366		-	-	SAS2300	lipoprotein	68
2459984		-	-	SAS2301	lipoprotein	98
2461851		+	-	SAS2302	FemAB family protein	53
2461948	2461866	-	-	SAS2303	ABC transporter ATP-binding protein	170
2462676		-	-	SAS2304	transport system membrane protein	84
2463376		-	-	SAS2305	extracellular solute-binding lipoprotein	45
2464276		-	-	SAS2306	transport system protein	99
2466147		-	*gpmA*	SAS2307	phosphoglyceromutase	31
2467160	2467160	-	-	SAS2308	cation efflux family protein	118
2470037		+	-	SAS2309	IgG-binding protein	37
2471502		+	-	SAS2310	gamma-hemolysin component A precursor	82
2473017		+	-	SAS2311	gamma-hemolysin component C precursor	30
2473996		+	-	SAS2312	gamma-hemolysin component C precursor	34
2474048		-	-	SAS2313	hypothetical protein	69
2474526		-	-	SAS2314	6-carboxyhexanoate--CoA ligase	4
2475229		-	-	SAS2315	8-amino-7-oxononanoate synthase	20
2476322		-	-	SAS2316	biotin synthase	52
2477331		-	-	SAS2317	adenosylmethionine-8-amino-7-oxononanoate aminotransferase	34
2478667		-	-	SAS2318	dethiobiotin synthetase	101
2479806		-	-	SAS2319	ABC transporter ATP-binding protein	51
2481564		-	-	SAS2320	ABC transporter ATP-binding protein	40
2483877		-	-	SAS2320a	hypothetical protein	60
2484269		+	-	SAS2322	hypothetical protein	80
2484252		-	-	SAS2323	hypothetical protein	26
2486038	2486097	+	-	SAS2324	glycerate kinase	183
2486757	2486830	+	-	SAS2325	hypothetical protein	123
2488150		+	-	SAS2326	transporter protein	66
2488273		-	-	SAS2327	hypothetical protein	82
2489515	2489752	+	-	SAS2328	hypothetical protein	107
2489578		-	-	SAS2329	hypothetical protein	45

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2492079		+	-	SAS2330	amino acid permease	84
2494295		+	-	SAS2331	sodium/hydrogen exchanger family protein	49
2494435	2494435	-	-	SAS2332	hypothetical protein	111
2497992	2497992	+	-	SAS2333	hypothetical protein	105
2498084		-	-	SAS2334	hypothetical protein	58
2499257		-	-	SAS2335	2-dehydropantoate 2-reductase	21
2500418	2500418	-	-	SAS2336	transport protein	129
2502112		-	-	SAS2337	glycine betaine/carnitine/choline transport system permease	98
2502807		-	-	SAS2338	glycine betaine/carnitine/choline-binding lipoprotein precursor	25
2503765		-	-	SAS2339	glycine betaine/carnitine/choline transport system permease	97
2504397		-	-	SAS2340	glycine betaine/carnitine/choline transport ATP-binding protein	180
2506293		-	-	SAS2341	hypothetical protein	99
2508709		+	-	SAS2342	amino acid permease	71
2510368		+	-	SAS2343	carboxylesterase	95
2510430		-	-	SAS2344	transport protein	76
2512013		-	-	SAS2345	hypothetical protein	77
2512782		-	-	SAS2346	ABC transporter ATP-binding protein	39
2513692		-	-	SAS2347	hypothetical protein	103
2515124		-	-	SAS2348	hypothetical protein	49
2515390		-	-	SAS2349	hypothetical protein	82
2516461		+	-	SAS2350	lipoprotein	51
2516629	2516629	-	-	SAS2351	hypothetical protein	158
2519151		+	-	SAS2352	hypothetical protein	29
2519300		-	-	SAS2352a	hypothetical protein	66
2519513	2519459	-	-	SAS2353	transporter	117
2520718	2520718	-	-	SAS2354	oligopeptide transporter ATPase	116
2521460		-	-	SAS2355	oligopeptide transporter ATPase	120
2522272		-	-	SAS2356	oligopeptide transporter membrane permease	229
2523138		-	-	SAS2357	oligopeptide transporter membrane permease	99
2524086		-	-	SAS2358	oligopeptide transporter substrate binding protein	95
2525824	2525824	-	-	SAS2359	hypothetical protein	200
2527121		-	-	SAS2360	hypothetical protein	56
2527950	2527947	-	-	SAS2361	hypothetical protein	131
2529646		-	-	SAS2362	hypothetical protein	85
2530271		-	-	SAS2363	short chain dehydrogenase	61
2532862		+	-	SAS2364	transporter	102
2532974		-	-	SAS2365	hypothetical protein	58
2533848		-	-	SAS2366	hypothetical protein	22

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2534285		-	-	SAS2367	hypothetical protein	0
2534731		-	-	SAS2368	hypothetical protein	3
2535409		-	-	SAS2369	hypothetical protein	11
2536082	2536082	-	-	SAS2370	short chain dehydrogenase	104
2537347		+	-	SAS2371	hypothetical protein	2
2537607	2537607	-	-	SAS2372	hypothetical protein	150
2538338		-	-	SAS2373	hypothetical protein	9
2539267		-	-	SAS2374	hypothetical protein	22
2540200		-	-	SAS2375	lipoprotein	3
2541264		-	-	SAS2376	helicase	26
2544127		-	-	SAS2377	NUDIX hydrolase	6
2546459		+	-	SAS2378	phosphomannomutase	85
2547009		+	-	SAS2379	hypothetical protein	94
2547461		+	-	SAS2380	hypothetical protein	28
2547569		-	-	SAS2381	hypothetical protein	16
2547783		-	-	SAS2382	hypothetical protein	51
2549359	2549359	-	-	SAS2383	cell wall-anchored protein	286
	2550831	+	-	predicted RNA	antisense: SAS2383	758
	2551788	+	-	predicted RNA	antisense: SAS2383	622
2553779		-	-	SAS2384	accessory regulator	85
2555202		+	-	SAS2385	accessory regulator	73
2555414		-	-	SAS2386	UTP--glucose-1-phosphate uridylyltransferase	66
2556460	2556346	-	*fnbB*	SAS2387	fibronectin-binding protein precursor	494
	2557283	+	-	predicted RNA	antisense: fnbB	1551
	2560952	+	-	predicted RNA	antisense: SAS2388	2723
2560014	2560014	-	-	SAS2388	fibronectin-binding protein precursor	878
2563196		-	-	SAS2389	hypothetical protein	74
2563530	2563530	-	-	SAS2390	gluconate permease	171
2565005	2564949	-	-	SAS2391	gluconokinase	106
2566582	2566559	-	-	SAS2392	gluconate operon transcriptional repressor	143
2567416	2567263	-	-	SAS2393	MerR family transcriptional regulator	174
2568345		-	-	SAS2394	hypothetical protein	44
2569268		-	-	SAS2395	hypothetical protein	24
2569823		-	-	SAS2396	hypothetical protein	45
2572158		-	-	SAS2396a	hypothetical protein	20
2572555	2572555	-	-	SAS2397	transporter protein	109
2574990	2575342	+	-	SAS2398	DedA family protein	107
2576203		+	-	SAS2399	ABC transporter ATP-binding protein	26
2576975		+	-	SAS2400	hypothetical protein	38
2579313		+	-	SAS2401	hypothetical protein	51

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2580734	2581161	+	-	SAS2402	hypothetical protein	108
2580839		-	-	SAS2403	phospholipase/carboxylesterase	65
2581463		-	-	SAS2404	dioxygenase	16
2582499		-	-	SAS2405	MarR family transcriptional regulator	51
2583443		+	-	SAS2406	acetyltransferase (GNAT) family protein	94
2584543		-	-	SAS2409	nitroreductase family protein	48
2586476		+	-	SAS2410	D-lactate dehydrogenase	94
2586723		-	-	SAS2411	haloacid dehalogenase	24
2588479		+	-	SAS2412	ABC transporter ATP-binding protein	0
2589216		+	-	SAS2413	hypothetical protein	52
2589378		-	-	SAS2414	sortase	71
2590683		+	-	SAS2415	acetyltransferase (GNAT) family protein	28
2591249		-	-	SAS2415a	hypothetical protein	85
2591598	2591549	-	-	SAS2416	L-serine dehydratase subunit alpha	564
	2592264	+	-	predicted RNA	antisense: SAS2416	935
2592511		-	-	SAS2417	L-serine dehydratase subunit beta	92
2593194	2593192	-	-	SAS2418	hypothetical protein	132
2594990		+	-	SAS2419	hypothetical protein	45
2596108	2596108	+	-	SAS2420	hypothetical protein	105
2596189	2596079	-	-	SAS2421	hypothetical protein	167
2597290		-	-	SAS2422	hypothetical protein	101
2597676		-	-	SAS2423	hypothetical protein	70
2598451	2598350	-	-	SAS2424	PTS system, glucose-specific IIABC component	152
2600818	2600518	-	-	SAS2425	pyruvate oxidase	109
2602604		-	-	SAS2426	hypothetical protein	40
2603286		-	-	SAS2427	hypothetical protein	45
2603894		-	-	SAS2428	LysR family regulatory protein	67
2605157		+	-	SAS2429	hypothetical protein	51
2605978		+	-	SAS2430	hypothetical protein	42
2606116	2606094	-	-	SAS2431	3-hydroxy-3-methylglutaryl-CoA reductase	139
2608816	2608816	+	-	SAS2432	3-hydroxy-3-methylglutaryl-CoA synthase	160
2608986	2608986	-	-	SAS2433	6-O-methylguanine DNA methyltransferase	262
2612021	2612268	+	-	SAS2434	ATP-dependent protease ATP-binding subunit ClpL	103
2612090		-	-	SAS2435	hypothetical protein	25
2612273		-	-	SAS2436	ferrous iron transport protein B	77
2614279		-	-	SAS2437	hypothetical protein	30
2614722		-	-	SAS2438	transport protein	68
2617904		+	-	SAS2439	TetR family regulatory protein	26
2618259	2618259	-	-	SAS2440	1-pyrroline-5-carboxylate dehydrogenase	134

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2619993	2619993	-	-	SAS2441	acetyltransferase	160
2621022	2621065	+	-	SAS2442	hypothetical protein	107
2623670	2623682	+	-	SAS2443	copper importing ATPase A	297
2624225	2624557	+	-	SAS2444	heavy-metal-associated protein	477
2624315	2624248	-	-	SAS2445	D-lactate dehydrogenase	233
2625336	2625325	-	-	SAS2446	aminotransferase	209
2626917		-	-	SAS2447	squalene synthase	43
2628437		-	-	SAS2448	squalene desaturase	75
2629337		-	-	SAS2449	glycosyl transferase	100
2630470		-	-	SAS2450	phytoene dehydrogenase related protein	50
2631956		-	-	SAS2451	hypothetical protein	66
2632619		-	-	SAS2452	secretory antigen precursor	90
2633747		-	-	SAS2453	hypothetical protein	47
2636070		-	-	SAS2454	acetyltransferase (GNAT) family protein	9
2636834	2636834	-	-	SAS2455	immunodominant antigen A	146
2638143	2638143	-	-	SAS2456	hypothetical protein	224
2639547		+	-	SAS2457	hypothetical protein	21
2640177	2640311	+	-	SAS2458	TetR family regulatory protein	110
2640875	2641064	+	-	SAS2459	hypothetical protein	117
2641325		+	-	SAS2460	hypothetical protein	43
2641488		-	-	SAS2461	glyoxalase	61
2641882		-	-	SAS2462	hypothetical protein	59
2642723		-	-	SAS2463	hypothetical protein	71
2643711		+	-	SAS2464	TetR family regulatory protein	47
2644408		+	-	SAS2465	short chain dehydrogenase	48
2645516		+	-	SAS2466	hypothetical protein	59
2646354		+	-	SAS2467	hydrolase	93
2646501		-	-	SAS2468	cobalamin synthesis protein/P47K family protein	96
2647488		-	-	SAS2469	hypothetical protein	91
2648822		-	-	SAS2470	hypothetical protein	112
2650223		-	-	SAS2471	hypothetical protein	80
2650507		-	-	SAS2472	hypothetical protein	119
2650890		-	-	SAS2473	hypothetical protein	18
2651507	2651507	-	-	SAS2474	hypothetical protein	151
2653655		+	-	SAS2475	dihydroorotate dehydrogenase 2	55
2654085	2654085	+	-	SAS2476	hypothetical protein	167
2654271		-	-	SAS2477	hypothetical protein	68
2655796		+	-	SAS2478	hypothetical protein	34
2655877		-	-	SAS2479	DNA-binding protein	48
2658289	2658304	+	-	SAS2480	hypothetical protein	172
2659104		-	-	SAS2482	aspartate alpha-decarboxylase	43
2659489		-	*panC*	SAS2483	pantoate--beta-alanine ligase	100

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2660333		-	*panB*	SAS2484	3-methyl-2-oxobutanoate hydroxymethyltransferase	156
2662084	2662175	+	-	SAS2485	2-dehydropantoate 2-reductase	170
2662170	2661800	-	-	SAS2486	alpha-acetolactate decarboxylase	133
2663152		-	-	SAS2487	L-lactate dehydrogenase 2	62
2666246	2666246	+	-	SAS2488	amino acid permease family protein	250
2666326	2666292	-	-	SAS2489	4-aminobutyrate aminotransferase	115
2668337		+	-	SAS2490	hypothetical protein	55
2669350		+	-	SAS2491	fructose-1,6-bisphosphate aldolase	61
2669544	2669544	-	-	SAS2492	malate:quinone oxidoreductase	162
2671665	2672385	+	-	SAS2493	hypothetical protein	137
2671725	2671725	-	-	SAS2494	AMP-binding protein	232
2673517		-	-	SAS2495	hypothetical protein	68
2674240		-	-	SAS2496	hypothetical protein	0
2674730	2674730	-	-	SAS2497	choline dehydrogenase	141
2676700	2676440	-	-	SAS2498	betaine aldehyde dehydrogenase	107
2679006		+	-	SAS2499	hypothetical protein	78
2679194	2678864	-	-	SAS2500	metallo-beta-lactamase superfamily protein	122
2680139		-	-	SAS2501	choline transporter	70
2682280		-	-	SAS2502	anaerobic ribonucleotide reductase activating protein	11
2682813		-	-	SAS2503	anaerobic ribonucleoside triphosphate reductase	117
2684909		-	-	SAS2504	transporter protein	73
2686755		-	-	SAS2505	precorrin-2 dehydrogenase	15
2687423	2687361	-	-	SAS2506	sulfite reductase [NADPH] flavoprotein alpha-component	145
2689698		-	-	SAS2507	glutathione peroxidase	0
2691619		-	-	SAS2508	hypothetical protein	51
2693616		-	-	SAS2509	ABC transporter ATP-binding protein	40
2694479		-	-	SAS2510	sensor kinase	65
2695377		-	-	SAS2511	response regulator protein	40
2696068		-	-	SAS2512	hypothetical protein	58
2697890		+	-	SAS2513	alkaline phosphatase III precursor	43
2697950		-	-	SAS2513a	hypothetical protein	76
2698173		-	-	SAS2514	regulatory protein	77
2698873	2698859	-	-	SAS2515	esterase	135
2699750	2699750	-	-	SAS2516	fibrinogen and keratin-10 binding surface anchored protein	2397
	2701150	+	-	predicted RNA	antisense: SAS2516	7270
2702817		-	-	SAS2517	regulatory protein	44
2703620	2703620	-	-	SAS2518	carbamate kinase	144
2704578	2704578	-	-	SAS2519	arginine/ornithine antiporter	139
2706099		-	-	SAS2520	ornithine carbamoyltransferase	88
2707142		-	-	SAS2521	arginine deiminase	58

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2708724		-	-	SAS2522	arginine repressor ArgR	27
2709540		-	-	SAS2523	zinc metalloproteinase aureolysin	48
2711493		-	-	SAS2524	immunodominant antigen B	0
2712736		+	-	SAS2525	hypothetical protein	72
2714850		+	-	SAS2526	transcriptional regulator	50
2716879	2716892	+	-	SAS2527	PTS transport system, IIABC component	233
	2716234	-	-	predicted RNA	antisense: SAS2527	699
2717831	2717832	+	-	SAS2528	mannose-6-phosphate isomerase	117
2717943	2717699	-	-	SAS2529	hypothetical protein	109
2722994		+	-	SAS2530	N-acetylmuramoyl-L-alanine amidase	91
2723259		-	-	SAS2531	isochorismatase family protein	47
2723988		-	-	SAS2532	surface anchored protein	101
2726137	2726137	-	-	SAS2533	hypothetical protein	172
2727488		-	-	SAS2534	hypothetical protein	128
2729014		-	-	SAS2535	preprotein translocase subunit SecA	44
2731394		-	-	SAS2536	hypothetical protein	253
2732331		-	-	SAS2537	hypothetical protein	108
2733889		-	-	SAS2538	hypothetical protein	195
2735453	2735443	-	*secY*	SAS2539	preprotein translocase subunit SecY	116
2736794	2736794	-	-	SAS2540	cell wall-anchored protein	358
	2738345	+	-	predicted RNA	antisense: SAS2540	799
	2738744	+	-	predicted RNA	antisense: SAS2540	659
	2739008	+	-	predicted RNA	antisense: SAS2540	783
	2739600	+	-	predicted RNA	antisense: SAS2540	800
	2739983	+	-	predicted RNA	antisense: SAS2540	953
	2740487	+	-	predicted RNA	antisense: SAS2540	788
	2740558	+	-	predicted RNA	antisense: SAS2540	655
2744166		-	-	SAS2541	hypothetical protein	44
2746115		+	-	SAS2542	lipoprotein	80
2746404		+	-	SAS2543	hypothetical protein	79
2746917		+	-	SAS2544	hypothetical protein	22
2747002	2746988	-	-	SAS2545	hypothetical protein	149
2747692		-	-	SAS2546	methionine sulfoxide reductase A	33
2748171		-	-	SAS2547	acetyltransferase (GNAT) family protein	65
2748906		-	-	SAS2548	capsule synthesis protein	29
2749670		-	-	SAS2549	capsule synthesis protein	54
2750379		-	-	SAS2550	capsule synthesis protein	44
2751890		-	-	SAS2551	ica operon transcriptional regulator	28
2753852		+	-	SAS2552	N-glycosyltransferase	143

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2754121		+	-	SAS2553	intercellular adhesion protein D	69
2754990		+	-	SAS2554	intercellular adhesion protein B	40
2756029		+	-	SAS2555	intercellular adhesion protein C	9
2756475		-	-	SAS2556	lipase precursor	97
2759610	2759362	-	-	SAS2557	bifunctional phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP	117
2760239		-	-	SAS2558	imidazole glycerol phosphate synthase subunit HisF	143
2760994		-	-	SAS2559	1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)methylideneamino] imidazole-4-carboxamide isomerase	139
2761691		-	*hisH*	SAS2560	imidazole glycerol phosphate synthase subunit HisH	174
2762266		-	*hisB*	SAS2561	imidazoleglycerol-phosphate dehydratase	299
2762813		-	-	SAS2562	aminotransferase	97
2763842		-	-	SAS2563	histidinol dehydrogenase	83
2765085		-	*hisG*	SAS2564	ATP phosphoribosyltransferase	47
2765717	2765717	-	*hisZ*	SAS2565	ATP phosphoribosyltransferase	112
2766839		-	-	SAS2566	hypothetical protein	64
2768081		-	-	SAS2567	hypothetical protein	42
2768625		-	-	SAS2568	ABC transporter permease	34
2769455		-	-	SAS2569	ABC transporter ATP-binding protein	41
2771220		-	-	SAS2570	hypothetical protein	23
2771803		-	-	SAS2571	hypothetical protein	45
2773546		+	-	SAS2572	hypothetical protein	88
2773657		-	-	SAS2573	hypothetical protein	22
2774026		-	-	SAS2574	hypothetical protein	52
2776197		+	-	SAS2575	hypothetical protein	90
2776987	2777009	+	-	SAS2576	pyrrolidone-carboxylate peptidase	131
2777555		+	-	SAS2577	hypothetical protein	37
2777716		-	-	SAS2578	collagen adhesin precursor	78
2783063		+	-	SAS2579	sodium:sulfate symporter family protein	40
2783476		-	-	SAS2580	hypothetical protein	19
2785181		+	-	SAS2581	hypothetical protein	70
2786363	2786416	+	-	SAS2582	nickel transport protein	225
2787400		+	-	SAS2583	N-acetyltransferase	72
2787534		-	-	SAS2584	hypothetical protein	59
2789087		+	-	SAS2585	ABC transporter ATP-binding protein	158
2790957		+	-	SAS2586	ABC transporter permease	45
2791241		+	-	SAS2586a	hypothetical protein	26
2791834		+	-	SAS2587	cold shock protein	19
2791952		-	-	SAS2588	hypothetical protein	44
2792781		-	-	SAS2589	hypothetical protein	78
2793244		-	-	SAS2590	hypothetical protein	70
2793914		-	-	SAS2591	DNA-binding protein	30
2794796		-	*gidB*	SAS2592	16S rRNA methyltransferase GidB	75

Translation Stop	Transcription Stop	Strand	Name	Synonym	Product	Expression
2795515		-	-	SAS2593	tRNA uridine 5-carboxymethylaminomethyl modification protein GidA	107
2797459		-	*trmE*	SAS2594	tRNA modification GTPase TrmE	45
2798977		-	*rnpA*	SAS2595	ribonuclease P	44
2799451		-	*rpmH*	SAS2596	50S ribosomal protein L34	11

TABLE S2, Sheet 5: "tRNA in EVs isolated from isolated from MSSA476

Element	Start	Stop	Full name	Cove score	Read count	GC content	Bases of selection
tRNA	1941988	1942064	tRNA Ile (GAT)	101.60	42	59.74	gggcctatagctcagctggttagagcgcacgcctgataagcgtgaggtcggtggttcgagtccacttaggcccacca
tRNA	1938233	1938308	tRNA Lys (TTT)	98.23	379	47.37	gagccattagctcagttggtagagcatctgacttttaatcagagggtcagaggttcgaatcctctatggctcacca
tRNA	2229612	2229687	tRNA Val (TAC)	95.74	213	56.58	ggagaattagctcagctgggagagcatctgccttacaagcagagggtcggcggttcgaacccgtcattctccacca
tRNA	1936843	1936918	tRNA Thr (TGT)	92.93	560	55.26	gccggcctagctcaattggtagagcaactgacttgtaatcagtaggttgggggttcaagtcctctggccggcacca
tRNA	2229336	2229411	tRNA Lys (TTT)	90.55	45	46.05	gagccattagctcagttggtagagcatctgacttttaatcagagggtcagaggttcgaatcctctatggctcacta
tRNA	533731	533803	tRNA Lys (TTT)	89.08	150	47.95	gagccattagctcagctggtagagcatctgacttttaatcagagggtcagaggttcgaatcctctatggctca
tRNA	1937703	1937778	tRNA Ala (TGC)	89.05	244	64.47	gggggcttagctcagctgggagagcgcctgctttgcacgcaggaggtcagcggttcgatcccgctagtctccacca
tRNA	1937506	1937579	tRNA Met (CAT)	88.12	463	54.05	ggacctttagctcagttggttagagctaacggctcataaccgttcggtcgcaggttcgagtcctgcaaggtcca
tRNA	1937796	1937869	tRNA Pro (TGG)	86.85	346	56.76	cgggaagtagctcagcttggtagagcacttggtttgggaccaaggggtcgcaggttcgaatcctgtcttcccga
tRNA	1938070	1938144	tRNA Gly (GCC)	86.82	586	54.67	gcagaagtagttcagcggtagaatacaaccttgccaaggttggggtcgcgggttcgaatcccgtcttctgctcca
tRNA	1903347	1903421	tRNA Asn (GTT)	85.44	216	54.67	tccacagtagctcagtggtagagctatcggctgttaaccgatcggtcgtaggttcgagtcctacctgtggagcca
tRNA	1937017	1937092	tRNA Asp (GTC)	83.32	859	61.84	ggtctcgtagtgtagcggttaacacgcctgcctgtcacgcaggagatcgcgggttcgattcccgtcgagaccgcca
tRNA	1937605	1937681	tRNA Met (CAT)	82.22	284	63.64	ggcggtgtagctcagctggctagagcgtacggttcatacccgtgaggtcgggggttcgatcccctccaccgccacta
tRNA	1180641	1180714	tRNA Arg (TCT)	81.85	8	59.46	gtcctggtagctcagctggatagagcaatggccttctaagccatcggtcgggggttcgaatccctcccaggacg
tRNA	1936431	1936504	tRNA Cys (GCA)	79.57	400	55.41	ggcggcatagccaagtggtaaggcagaggtctgcaaaacctttatcaccggttcaaatccggttgccgcctcca
tRNA	1937971	1938059	tRNA Leu (TAA)	76.44	590	61.80	gccggggtggcggaactggcagacgcacaggacttaaaatcctgcggtgagtgatcaccgtaccggttcgattccggtcctcggcacca
tRNA	1936926	1936998	tRNA Phe (GAA)	76.42	635	50.68	ggttcagtagctcagttggtagagcaatggattgaagctccatgtgtcggcagttcgactctgtcctgaacca
tRNA	1937102	1937175	tRNA Met (CAT)	75.92	984	62.16	cgcgggatggagcagttcggtagctcgtcgggctcataacccgaaggtcggtggttcaaatccgcctcccgcaa
tRNA	1903436	1903509	tRNA Gly (TCC)	75.68	220	52.70	gcgggtgtagtttaatggcaaaacctcagccttccaagctgatgttgtgggttcgattcccatcacccgctcca
tRNA	533919	534007	tRNA Leu (TAA)	75.33	607	61.80	gccggggtggcggaactggcagacgcacaggacttaaaatcctgcggtgagagatcaccgtaccggttcgattccggtcctcggcacca
tRNA	24238	24310	tRNA Asp(GTC)	75.02	217	61.64	ggtctcgtagtgtagcggttaacacgcctgcctgtcacgcaggagatcgcgggttcgattcccgtcgagaccg
tRNA	1937190	1937282	tRNA Ser (TGA)	74.09	829	61.29	ggaggaatacccaagtccggctgaagggatcggtcttgaaaaccgacagggccttaacgggccgcgggggttcgaatccctcttcctccgcca
tRNA	1903525	1903600	tRNA His (GTG)	72.71	229	56.58	gcggttgtggtgaagtggttaacacatcggattgtggttccgacattcgagggttcgatccccttcagccgcccca
tRNA	1936510	1936581	tRNA Gln (TTG)	70.48	449	55.56	tgggctatagccaagcggtaaggcaacggactttgactccgtcactcgttggttcgaatccagctagcccag
tRNA	1938148	1938229	tRNA Leu (TAG)	70.09	473	62.20	gcgggtgtggcggaattggcagacgcactagacttaggatctagcgccttacggcgtgggggttcgactcccttcacccgca

Element	Start	Stop	Full name	Cove score	Read count	GC content	Bases of selection
tRNA	1936668	1936741	tRNA Trp (CCA)	69.76	422	51.35	aggggcatagttcaacggtagaatagaggtctccaaaaccttggtgtgggttcgattcctactgcccctgcca
tRNA	1936757	1936837	tRNA Tyr (GTA)	69.29	525	61.73	ggaggggtagcgaagtggctaaacgcggcggactgtaaatccgctccttcgggttcggcagttcgaatctgccccctcca
tRNA	1937879	1937952	tRNA Arg (ACG)	69.23	445	55.41	gcgcccgtagctcaattggatagagcgtttgactacggatcaagaggttatgggttcgactcctatcgggcgcg
tRNA	2229695	2229766	tRNA Glu (TTC)	68.58	259	59.72	ggctccttggtcaagcggttaagacaccgccctttcacggcggtaacacgggttcgagtcccgtaggagtca
tRNA	1936593	1936665	tRNA His (GTG)	68.08	446	57.53	gcggctgtggtgaagtggttaacacatcggattgtggttccgacattcgagggttcgatccccttcagccgcc
tRNA	1031321	1031409	tRNA Ser (GCT)	63.03	131	60.67	ggaaacgtactcaagttggctgaagaggcgcccctgctaagggtgtaggtcgcgaaagcggcgcgagggttcgaatccctccgtttccg
tRNA	1937407	1937496	tRNA Ser (TGA)	61.04	657	60.00	ggaggaatacccaagtccggctgaagggatcggtcttgaaaaccgacaggggcttaacggctcgcgggggttcgaatccctcttcctccg
tRNA	1936137	1936220	tRNA Leu (CAA)	59.46	89	63.10	gccggtgtggcggaattggcagacgcgcgggactcaaaatcccgttccacttgtggagtgtcggttcgaccccgaccaccggta
tRNA	2121724	2121809	tRNA Leu (GAG)	57.05	29	62.79	gcggtcgtggcggaatggcagacgcgctaggttgagggcctagtgggagaaatcccgtggaggttcaagtcctctcggccgcatca
tRNA	1903131	1903219	tRNA Ser (GGA)	51.22	97	58.43	ggaaggttgtccgagctggccgaaggagcacgcctggaaagtgtgtaggcgtcacaagcgtctcaagggttcgaatcccttaccttccg
tRNA	821299	821370	tRNA Arg (CCG)	45.36	4	44.44	ctccttgtggtgtaatggataacacgtaagattccggttcttaagataggggttcaattcccttcaaggagg
tRNA	1936349	1936423	tRNA Gly (TCC)	41.66	280	49.33	gcgggagtagttcaacttttagaacacgttccttcccggaacgaggtataggtgcaaatcctatcttccgctcca
tRNA	1936262	1936333	tRNA pseudo anticodon (TCC)	36.67	175	41.67	gcgggagtatttcaactcttagaatacattccttcctggaatgaggtataggtgtaaatcctatcttccgct

Table S2, Sheet 6: Small RNA in MVs isolated from MSSA476

sRNA Id	RFAM Accession	Type	Description	Genomic coordinates according to BSRD	Gene length (nt)	Strand	Read count	Bit score	GC content in %
ssas1686.1	RF00013	protein-binding small RNA	6s RNA	1685656..1685846	197	R	80	97.5	42.41
ssas838.1	RF00023	protein-binding small RNA	SsrA (tmRNA)	837496..837857	362	F	65	162.6	43.92
ssas474.1	RF0188	trans-encoded antisense RNA	RsaC	673626..674066	441	R	33	555.6	35.37
ssas1675.1	RF00230	regulatory element	T-box	1674489..1674687	178	R	32	90.55	34.27
ssas386.6	RF00230	Regulatory element	T-box	386022..386281	200	F	24	96.6	31.52
ssas486.1	RF00169	Trans-encoded antisense RNA	small cytoplasmic RNA(4.5sRNA, ffs)	485461..485730	270	F	24	69.1	48.52
ssas1552.1	RF00050	regulatory element	FMN riboswitch	1551305..1551439	135	R	23	121.7	46.67
ssas387.1	RF00230	Regulatory element	T-box	386093..386293	201	R	22	114.2	39.80
ssas2373.1	RF00162	regulatory element	SAM riboswitch	2372869..2372964	96	R	21	78.2	47.92
ssas1874.1	RF01797	trans-encoded antisense RNA	fstAT	1873399..1873493	95	R	19	90.2	42.11
ssas2206.1	RF01492	trans-encoded antisense RNA	rli28	2205592..2205772	181	R	19	123.8	34.25
ssas1255.1	RF00556	regulatory element	L19_leader	1254259..1254301	43	F	18	50.0	41.86
ssas424.1	RF01764	Regulatory element	yjdF	423980..424080	101	F	17	92.9	38.61
ssas2031.1	RF01492	trans-encoded antisense RNA	rli28	2030445..2030623	179	R	17	85.6	37.43
ssas1200.1	RF00230	regulatory element	T-box	1199542..1199714	173	F	16	80.94	34.10
ssas2156.1	RF00059	regulatory element	TPP riboswitch	2155664..2155766	103	F	16	70.9	40.78
ssas1792.1	RF00230	regulatory element	T-box leader	1791148..1791366	203	R	15	80.52	31.53
ssas2087.1	RF00503	trans-encoded antisense RNA	RNAIII	2086458..2086973	516	R	14	472.2	28.68
ssas13.1	RF00230	Regulatory element	T-box	12486..12696	211	F	13	93.3	33.65
ssas2480.1	RF01822	trans-encoded antisense RNA	RsaJ	2479454..2479740	287	F	13	332.1	30.66
ssas1733.1	RF00168	regulatory element	Lysine riboswitch	1732415..1732590	176	F	12	104.6	31.82
ssas2484.1	RF01797	trans-encoded antisense RNA	fstAT	2483979..2484075	97	F	12	78.12	40.21
ssas567.1	RF00557	Regulatory element	L10_Leader	566720..566866	127	F	10	86.8	31.21
ssas756.1	RF00552	Regulatory element	PreQ1-I Riboswitch	755252..755298	47	R	9	38.61	36.17
ssas1716.2	RF00230	regulatory element	T-box	1715403..1715591	189	R	9	70.41	33.86
ssas2028.1	RF01828	trans-encoded antisense RNA	SprD	2027163..2027304	142	R	9	161.8	37.32
ssas560.1	RF00230	Regulatory element	T-box	559398..559623	226	F	8	68.35	35.40
ssas16.1	RF00162	Regulatory element	SAM riboswitch	159449..16047	99	F	7	74.3	44.42
ssas1861.1	RF00162	regulatory element	SAM riboswitch	1860045..1860156	112	F	7	67.3	43.75
ssas2580.1	RF01797	trans-encoded antisense RNA	fstAT	2579344..2579434	91	R	7	71.47	46.15

sRNA Id	RFAM Accession	Type	Description	Genomic coordinates according to BSRD	Gene length (nt)	Strand	Read count	Bit score	GC content in %
sssas980.1	RF00080	regulatory element	yybP-ykoY leader	979834..979962	129	F	6	56.9	35.66
ssas1145.1	RF00230	regulatory element	T-box	1144228..1144441	214	F	6	75.8	31.31
ssas1035.1	RF01492	trans-encoded antisense RNA	rli28	1034785..1034968	184	F	5	66.9	34.24
ssas1512.1	RF00010	regulatory element	RNaseP_bact_b	1511315..1511662	380	R	5	273.3	39.47
ssas1716.1	RF00230	regulatory element	T-box	1715293..1715324	32	R	5	24.87	43.75
ssas1731.1	RF00558	regulatory element	L20_leader	1730577..1730702	126	R	5	82.9	44.44
ssas1087.1	RF00059	regulatory element	TPP riboswitch	1086334..1086434	101	R	4	71.0	43.56
ssas1846.1	RF00050	regulatory element	FMN riboswitch	1845588..1845723	136	R	4	116.8	43.38
ssas410.1	RF00167	Regulatory element	Purine riboswitch	409191..409293	103	F	3	77.7	29.13
ssas627.1	RF0186	trans-encoded antisense RNA	RsaA	626252..626390	139	F	2	112.3	33.81
ssas824.1	RF01821	Trans-encoded antisense RNA	RsaH	823438..823561	124	F	2	128.0	38.71
ssas862.1	RF00162	Regulatory element	SAM riboswitch	861019.. 861122	104	F	2	82.8	44.23
ssas960.2	RF01858	trans-encoded antisense RNA	RsaF	959384..959502	119	F	2	140.3	26.05
ssas1403.1	RF00230	Regulatory element	T-box	1402686..1402880	177	F	2	70.61	36.16
ssas1430.1	RF00168	regulatory element	Lysine riboswitch	1429786..1429961	176	F	2	95.8	34.09
ssas1603.1	RF00504	regulatory element	Glycine riboswitch	1602168..1602265	98	R	2	68.14	38.78
ssas2282.1	RF00555	regulatory element	L13_leader	2281316..2281385	70	R	2	53.7	37.14
ssas2359.1	RF01775	trans-encoded antisense RNA	RsaOG	2358615..2358758	144	R	2	174.6	36.11
ssas1735.1	RF00230	regulatory element	T-box leader	1734764..1734991	228	R	1	92.1	31.31

PAPER III

Bacterial Membrane-Derived Vesicles Attenuate Vancomycin Activity Against Methicillin-Resistant *Staphylococcus aureus*

Monika Kumaraswamy[1,2,3], Kamilla Wiull[4†], Bishnu Joshi[5†], George Sakoulas[6], Armin Kousha[6,7], Gustav Vaaje-Kolstad[4], Mona Johannessen[5], Kristin Hegstad[5,8], Victor Nizet[3,6,9], Fatemeh Askarian[4,5,6]

[1]Infectious Diseases Section, VA San Diego Healthcare System, San Diego, CA 92161, USA
[2]Department of Medicine, University of California San Diego, La Jolla, CA 92093, USA
[3]Collaborative to Halt Antibiotic Resistant Microbes (CHARM), University of California San Diego, La Jolla, CA 92093, USA
[4]Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Oslo, Norway
[5]Research Group for Host-Microbe Interactions, UiT - The Arctic University of Norway, Tromsø, Norway
[6]Department of Pediatrics, University of California San Diego, La Jolla, CA 92093, USA
[7]Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway
[8]Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, University Hospital of North-Norway, Tromsø, Norway
[9]Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California San Diego, La Jolla, CA 92093, USA

†These authors contributed equally.

Running Title: Membrane Vesicles Protect MRSA Against Vancomycin

Corresponding Author:
Fatemeh Askarian, PhD, Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Oslo, Norway; Phone: TBD ; E-mail: Fatemeh.askarian@nmbu.no

Alternate Corresponding Author:
Monika Kumaraswamy, MD, Division of Infectious Diseases, Department of Medicine, University of California San Diego, 9500 Gilman Drive, Mail Code 0711, La Jolla, CA 92093-0711; Phone: (858)534-2325; Fax: (858)246-1868; E-mail: mkumaraswamy@ucsd.edu