5-25-2022 9:30 AM

Screening for purine transporters in S. aureus

Tothong Sonpaveerawong, *The University of Western Ontario*

Supervisor: Dr. David Heinrichs, *The University of Western Ontario*

A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

Abstract

Staphylococcus aureus is an important human pathogen and crucial part of its pathogenesis depends on its ability to acquire purines to cause disease. In this study, I screened a library of individual mutants under purine import-dependent conditions by inhibiting the purine biosynthesis using the pharmacological agents methotrexate and 6-mercaptopurine and supplementing inosine monophosphate and/or guanine as an exogenous purine source. I identified an ATP-Binding Cassette transporter mutant that failed to grow under the selective purine transport conditions. Further growth characterization of the mutant revealed that the growth defect was not due to an inability to transport purines but rather to downstream effects related to the toxicity of the purine biosynthesis inhibitors. A thorough understanding of *S. aureus* purine acquisition will allow for development of antimicrobials with prolonged effectiveness.

Key words: *Staphylococcus aureus*, ABC transporter, methotrexate, 6-mercaptopurine

Lay Summary

Staphylococcus aureus is a bacterium that usually colonizes the skin of humans but is capable of causing various invasive and life-threatening diseases. To cause disease, *S. aureus* requires nutrients that allow the bacterium to grow. One such type of nutrients are called purines that are building blocks of DNA and compounds used as energy by the cells. While *S. aureus* can make purines itself, it can also import them from the environment through transporters; however, purine transport in *S. aureus* has not been well characterized. This study aimed to identify a *S. aureus* purine transporter. This was done by using specific drugs to block the ability of the bacteria make their own purines rendering them dependent on taking up purines from the environment. I screened over 2,000 mutants and looked for specific mutants that could not grow in the presence of these drugs, indicating that they cannot take up purines, and thus, could be the mutants of our interest. Thorough understanding of how *S. aureus* acquire purines is essential in developing antimicrobials with prolonged effectiveness.

Acknowledgement

I would like to thank everyone who has supported me in various ways throughout my academic journey. First of all, I would like to thank Dr. David Heinrichs for giving me the opportunity to conduct my fourth year thesis in his lab and to pursue my MSc there. His guidance, support and encouragement in the past three years were crucial for my growth as a scientist. Secondly, I would like to thank Dr. Mariya Goncheva who kindly mentored me in my first year and continued to support me thereafter. Her guidance in conducting various scientific techniques, forming sound rationale and communicating the results was a critical foundation in my graduate research. Furthermore, I greatly appreciate her encouragement in other aspects of the graduate student life – from planning for post-graduate career to preparing for interviews. Thirdly, I would like to thank Dr. Ronald Flannagan who provided tremendous help in directing the many turns of this project and provided helpful suggestions and encouragement throughout the writing process. His expertise in scientific principles, experimental design and troubleshooting has considerably helped me navigate through the ambiguity I encountered with this project.

Next, I would like to thank my committee members; Dr. John McCormick and Dr. Martin McGavin. I appreciate their insights in suggesting experiments to conduct and providing thoughtful feedback on all my reports and assignments throughout this degree.

I am grateful to work alongside my amazing coworkers, the members of the Heinrichs lab, who were always willing to help with experiments and to discuss the many aspects of my projects. Special thanks to the graduate students – Jaclyn, Camille, Denny, Iza, Thiago and Grace, for their wonderful friendship, fun memories and emotional support. I am grateful to have come to know such intelligent and kind people and hope that our paths will cross in the future.

Finally, I would like to thank my family, ป๊า ม๊า เหมียว, for believing in me as a scientist and supporting me emotionally throughout the degree. โดยเฉพาะม๊าที่คอยถามถึงความคืบหน้าในการเรียนปริญญาโท และให้การสนับสนุนมาตลอดสามปี ขอบคุณค่ะ

Table of Contents

List of figures

List of Tables

List of Abbreviations

6MP	6-mercaptopurine
ADAM10	A distegrin and metalloprotease 10
AMP	antimicrobial peptide
BCAAs	branched-chain amino acids
CDM	chemically defined media
CF	cystic fibrosis
CFU	colony forming unit
EDTA	ethylenediaminetetraacetic acid
HNP	human neutrophil peptides
HOCl	hypochlorous acid
IMP	inosine monophosphate
IPTG	isopropyl ß-D-1-thiogalactopyranoside
MIC	minimal inhibitory concentration
Mn^{2+}	manganese ions
MntC	substrate-binding unit of manganese ATP-Binding Cassette transporter
MntH	NRAMP manganese transporter
MPO	myeloperoxidase
MRSA	methicillin-resistant *S. aureus*
MSCRAMMs	microbial surface components recognizing adhesive matrix molecules
MTX	methotrexate
NET	neutrophil extracellular trap
NO	nitric oxide
Nox2	NADPH oxidase
NRAMP	natural resistance-associated macrophage protein
O_2^-	superoxide ions
OD_{600}	optical density at measured at a wavelength of 600 nm
PPP	pentose phosphate pathway
PRPP	phosphoribosyl-alpha-1-pyrophosphate
PSM	phenol soluble modulins

PVL	Panton-Valentine leucocydin
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute 1640
SCV	small colony variants
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
SPIN	staphylococcal peroxidase inhibitor
TSA	tryptic soy agar
TSB	tryptic soy broth

Chapter 1: Introduction

1.1 *Staphylococcus aureus* is an important human pathogen.

Staphylococcus aureus is a Gram-positive, coagulase-positive coccoid bacterium that chronically colonizes approximately 30% of the human population (Wertheim et al., 2005). Although it is often a commensal organism that asymptomatically colonizes the skin sebaceous glands, nose and guts of healthy individuals, *S. aureus* can also act as an opportunistic pathogen and cause a wide range of infections that can vary from minor skin and soft tissue infections to life-threatening diseases (Liu, 2009; Le Loir, Baron, and Gautier, 2003). The infections *S. aureus* causes can be classified as: 1. superficial infections consisting of mild pimples and boils to styes, abscesses and carbuncles; 2. toxin-mediated infections that cause food poisoning; 3. toxic shock and scalded skin syndromes due to various toxins produced by the bacterium; and 4. invasive infections that penetrate the skin causing serious internal conditions such as pneumonia, bacteraemia, endocarditis and septicaemia (Wise, 1964; Otto, 2014). Moreover the ability of *S. aureus* to cause such an array of diseases can in part be attributed to the extensive collection of virulence factors that *S. aureus* possesses.

The emergence of "superbugs", such as methicillin-resistant *S. aureus* (MRSA), that can display resistance to multiple antibiotics has complicated treatment of infection and their emergence poses a serious threat to public health (Lowy, 1998; Ruffing et al., 2012; Mongodin et al., 2003). A recent study published this year estimated that in 2019, MRSA was one of the pathogens in which resistance was attributable to 100,000 deaths and 3.5 million disability-adjusted life-years (Murray et al., 2022). Furthermore, patients suffering from MRSA infections are associated with higher morbidity, higher treatment cost, hospital resources utilization and longer hospital stays have been associated with MRSA infections (Engemann et al., 2003). Attempts to eradicate MRSA are complicated because it can persist within a home environment and nearly any item in contact with skin can serve as a fomite in transmission. Additionally, colonization can persist over long periods of time in which strains can evolve and even be replaced with another strain within the same host (Azarian et al., 2016). This leads to development of persistent strains that are able to resist host-imposed nutrient starvation conditions and develop resistance to antibiotics. Therefore, thorough understanding of how *S. aureus*, and especially MRSA, controls its virulence factors and causes disease is crucial in

developing effective or novel drug targets and reduce the impact of this versatile pathogen.

1.2 Virulence factors of *S. aureus*

S. aureus is a prolific pathogen due to an extraordinarily high number of virulence factors that it can express compared to other staphylococci. The ability of *S. aureus* to sense its environment and produce a vast arsenal of virulence factors such as protein A, hemolysins, leukocidins, proteases and immune-modulatory factors allows it to infect virtually any host tissues (Peacock et al., 2002; Zecconi and Scali 2013; Turner et al., 2019). These virulence factors allow it to successfully establish infection, avoid killing by host immune cells, invade tissues, and maintain infections (DuMont et al., 2011; Foster et al., 2014). Furthermore, distinct mechanisms such as inhibition of uptake and killing by phagocytic cells, dissemination in the bloodstream and abscesses or biofilms formation allow *S. aureu*s to survive in different host microenvironments (Thammavongsa et al., 2009).

1.2.1 Origin of infection

S. aureus infections usually originate from asymptomatic colonization, infected fomites (particularly in hospital settings), or transfer from other individuals (von Eiff et al., 2001). To establish a systemic infection, a breach of the protective dermis typically needs to occur (Stryjewski and Chambers, 2008). While infections can develop from minor skin scratches, *S. aureus* can also actively promote epithelial disruption by secreting α-toxin, a major cytolytic toxin in *S. aureus*. This activity of α-toxin is dependent on the host metalloproteinase and distegrin domain-containing protein 10 (ADAM10). While ADAM10 functions in the host to cleave membrane proteins at cellular surface, it also acts as a specific receptor for the bacterial protein (Wilke and Wardenburg, 2010).

Binding of α-toxin to ADAM10 can have two distinct outcomes in affected cells. Firstly, α-toxin causes disruption of epithelial barriers by triggering the breakdown of adherens junctions between neighboring epithelial cells (Popov et al., 2015). By activation of ADAM10, disruption of adherence junctions occurs through cleavage of E(epithelial)-cadherin, a host cell adhesion molecules (CAMs) protein that mediates cell-cell adhesion and is critical for tissue formation, adhesion and maintenance (Takeichi, 1977; Van Roy and Berx, 2008). As a result of α-toxin function in this capacity, the actin cytoskeleton de-polymerizes and epithelial barrier integrity is lost (N. Inoshima, Wang, and Wardenburg, 2012). Secondly, α-toxin also functions

as a pore forming toxin by forming heptameric pores that penetrates the lipid-bilayer in a series of target cells (Inoshima et al., 2011; Gonzalez et al., 2007). Upon binding to its target cell via ADAM10, α-toxin oligomerizes to a pre-pore a heptameric mushroom-shaped structure, subsequently attacking the cell membrane by extrusion the β-barrel of through the lipid bilayer to form a hydrophilic transmembrane channel (Gouaux, 1998). One of the earliest and most important signalling following pore formation is the influx of extracellular calcium into the cells. This increased in intracellular calcium has been suggested to stimulate cytokines and release nitric oxide, as well as modulate host cellular processes. These inflammatory stimuli as well as triggers of pyroptosis (lytic programmed cell death) (Bantel et al., 2001), leads to immune cell recruitment(Craven et al. 2009), increased reactivity of vasculature (Seeger et al., 1990), and modulating host immunity (Buerke et al., 2002). Overall, α-toxin is among the most potent virulence factors in *S. aureus* that allows it to play multiple roles in pathogenesis.

1.2.2 Establishment of infection and tissue invasion

After epithelial breach, *S. aureus* can establish systemic infection through active attack and elimination of immune cells such as neutrophils, the most prominent leukocyte in the blood, via cytolytic toxins. In the liver, the bacteria encounter Kupffer cells (macrophages of the liver) that have phagocytic activity allowing the liver to act as a bottleneck for subsequent bacteremia and establishment of infection in other organs (Surewaard et al., 2016). If the bacteria survive this stage, they can further disseminate through the bloodstream, attach to and invade tissues using MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) surface proteins that adhere to host extracellular matrix factors (Patti et al., 2003). These proteins are secreted by the general secretion pathway and are tethered to the peptidoglycan via reactions catalyzed by sortase A that links the threonine of a conserved N-terminal structure (LPXTG motif) to the amino acid group of the terminal glycine residue in the peptidoglycan (Mazmanian et al., 2000).

MSCRAMMs are defined by the two adjacent IgG-like folded domains that promote binding to ligands. In *S. aureus*, the two families of MSCRAMMs are those related to clumping factor A (ClfA) and those similar to the collagen-binding protein (Cna family). The two mechanisms in which MSRAMMs bind to ligands are the dock-lock-latch or the collagen-hug mechanisms, both of which require conformational changes and can only be separated by

very strong forces(Foster et al. 2014). Interestingly, data from our lab have shown that an *S. aureus* strain with aberrant up-regulation in FnBPs caused accelerated mortality in mice (Goncheva et al., 2019), as well as enhanced invasion of epithelial cells (Goncheva, Flannagan, and Heinrichs, 2020).

1.2.3 Avoidance of killing by phagocytes

Once distributed in the blood, the bacterium faces host innate immune cells with the most predominant and most important in clearing *S. aureus* infections are neutrophils (Cheung, Bae, and Otto, 2021). The bacteria avoid phagocytosis via many levels that include 1. Inhibition of neutrophil extravasation from the bloodstream into tissues, activation, and chemotaxis (El-Benna et al., 2016), 2. Inhibition of phagocytosis by aggregation, protective surface structures and biofilm formation (Skurnik, Cywes-Bentley, and Pier, 2016), 3. Inhibition of opsonization (Lambris, Ricklin, and Geisbrecht, 2008), 4. Inhibition of neutrophil killing mechanisms (Kobayashi, Malachowa, and DeLeo 2018) and 5. Direct elimination of neutrophils by cytolytic toxins (such as α-toxin discussed above) or triggering apoptosis (Wang et al., 2007).

Despite possessing anti-phagocytic effectors, *S. aureus* can still be captured and ingested by professional phagocytes including neutrophils. Neutrophils have potent anti-microbial effectors that attack phagocytosed bacteria through oxygen dependent and oxygen independent mechanisms (Kobayashi, Malachowa, and DeLeo, 2018). Oxygen-dependent killing is driven through activation of NADPH oxidase (Nox2) that catalyzes the formation of superoxide (O_2^-) which spontaneously dismutates into hydrogen peroxide (H_2O_2). Subsequently, the enzyme myeloperoxidase (MPO) utilizes H_2O_2 as a substrate in the catalysis of hypochlorous acid (HOCl) which is a major reactive oxygen species (ROS) effector molecule (Klebanoff et al. 2013). Simultaneously, oxygen-independent effectors also act to intoxicate phagocytosed bacteria and these effectors include molecules such as defensins, specifically the human neutrophil peptides (HNPs). These molecules have also been found to increase phagocytosis of *S. aureus* by human and murine macrophages by induction of TNF-α and IFN-γ release (Soehnlein et al., 2008).

To counter host immune defenses, *S. aureus* expresses several factors that promote bacterial survival. This includes enzymes that can detoxify phagocyte formed ROS or enzymes that can altogether inhibit ROS production (Fig.1). For example, staphyloxanthin, the

carotenoid pigment produced by *S. aureus*, scavenges oxygen radicals owing to a series of conjugated double bonds that comprise its molecular structure (Clauditz et al., 2006). Additionally, *S. aureus* produces superoxide dismutases that convert superoxide to the less toxic H_2O_2, catalase (KatA) and alkyl hydroperoxide reductase C (AhpC) which further detoxify H_2O_2 into oxygen and water (Cosgrove et al., 2007). As well, a lactate dehydrogenase is produced by induction of nitric oxide (NO) and contributes to maintaining redox homeostasis in the phagosomes (Richardson, Libby, and Fang, 2008). *S. aureus* can also inhibit oxidative burst by converting ADP and AMP to adenosine via the adenosine synthase A (AdsA) (Thammavongsa et al., 2009) and directly inhibit the activity of MPO via the staphylococcal peroxidase inhibitor (SPIN) that block the H_2O_2 substrate from the active site of MPO.

Regarding the oxygen-independent mechanism, neutrophils secrete defensins and other anti-microbial peptides that are commonly positively-charged; and thus, are attracted to the bacterial surface by electrostatic interaction. *S. aureus* has mechanisms that reduce the anionic character of cell membrane and cell wall – namely the *dlt* operon that incorporate D-alanine into lipoteichoic acid (Perego et al., 1995) and MprF (multiple peptide resistance factor) that catalyzes the synthesis and flipping of the positively charged phospholipid lysyl-phosphatidylglycerol within the cell membrane, overall increasing positive charge and changing cell membrane fluidity (Mishra et al., 2009).

Lastly, *S. aureus* is among one of the few bacteria that are completely insensitive to lysozyme. As a muramidase that cleaves peptidoglycan, lyzozyme is secreted by various host cells(Keshav et al., 1991) (monocytes, macrophages and colonic epithelial cells) and is present in body fluids such as serum, saliva, sweat and tears. The activity of lysozyme is reduced by alteration of peptidoglycan catalyzed by O-acetyltransferase (OatA), an integral membrane enzyme that acetylates the muramic acid parts of peptidoglycan (A. Bera et al., 2005).

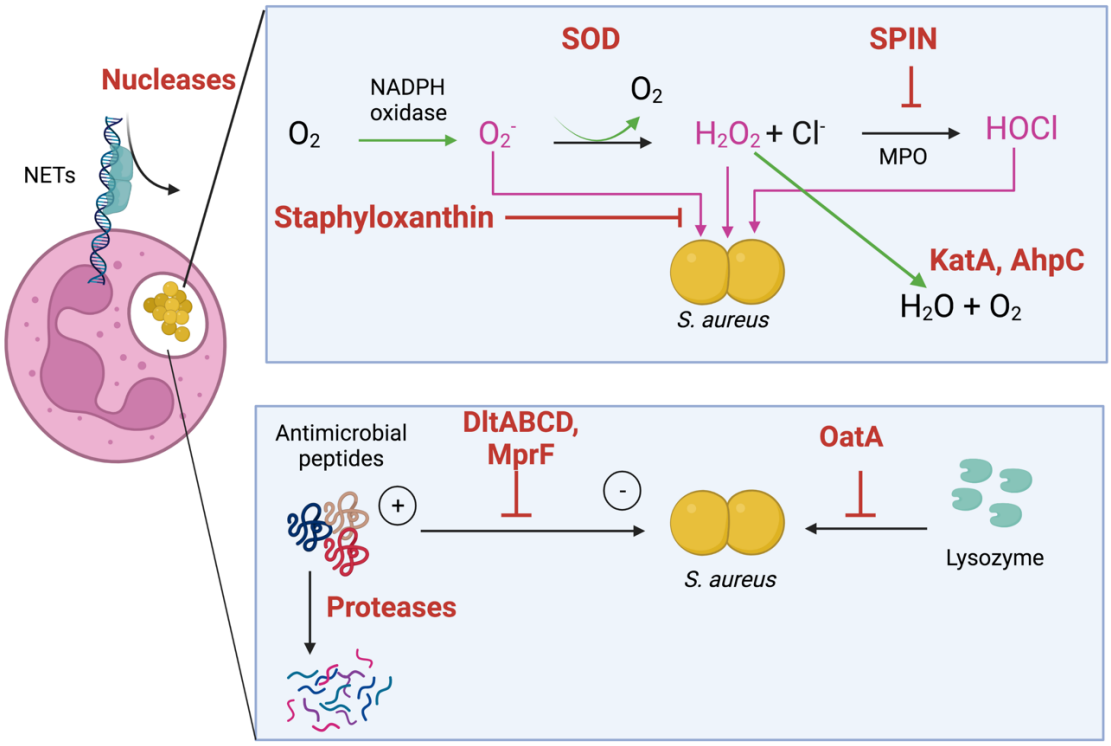


Fig. 1: ***S. aureus*** **subverts both oxygen-dependent and -independent bactericidal mechanisms of neutrophil phagosome.** In the oxygen-dependent mechanisms, superoxide dismutases (SOD) convert harmful superoxide and catalase as well as AhpC convert hydrogen peroxide to oxygen and water. The SPIN protein inhibits myeloperoxidase (MPO), which produces the most potent reactive oxygen species (ROS), hypochlorite. The yellow pigment staphyloxanthin generally protects *S. aureus* cells from ROS. In the oxygen-independent mechanisms, the activity of antimicrobial peptides (AMPs) is diminished by secreted proteases and reducing the anionic character of the bacterial cell surface. Lysozyme activity is reduced by OatA-catalyzed alteration of peptidoglycan. Finally, *S. aureus* secretes nuclease that can digest neutrophil extracellular traps (NETs). Modified fromg Cheung, Bae, and Otto, 2021.

1.2.4 Maintenance of infection

As bacterial density increases, nutrients availability decreases. To scavenge nutrient macromolecules from host cells, *S. aureus* produces cytolytic peptides such as α-toxin, phenol-soluble modulins (PSM) and leucocidins. As discussed above, α-toxin can lyse red blood cells, platelets, neutrophils, monocytes, T cells, pneumocytes, and keratinocyte(Berube and Wardenburg, 2013). PSMs have been found to have at least two main functions related to their detergent-like feature that may allow *S. aureus* to persist on epithelial surfaces of humans and other mammals or on medical devices such as catheters (Cheung, Bae, and Otto, 2021). Specifically, PSMs have strong surfactant activities with relatively low hydropathicity that facilitates colonization of wet surfaces, starting from planktonic or biofilm-associated states

(Tsompanidou et al., 2013; Periasamy et al., 2012). As cytolysins, PSMs promote cell lysis via a receptor-independent mechanism (unlike α-toxin) which includes membrane attachment, disintegration and followed by pore formation (Kretschmer et al., 2010). Lastly, *S. aureus* also produces a series of the bicomponent toxins that have two separate water-soluble monomeric subunits. These toxins can kill immune cells via recognition of cellular receptor on the surface of host cells, dimerization and oligomerization into an assembly of an octomeric pre-pore structure, leading to membrane insertion and forming β-barrel pores that span the phospholipid bilayer (Alonzo, III, and Torres, 2014). These bicomponent toxins can further be characterized into: the Panton-Valentine leukocydin family (PVL, consisting of LukS and LukF proteins), the leukocidins LukDE and LukAB, and gamma-toxin (gamma δ-hemolysin, HlgA, HlgB and HlgC)(Otto 2014). Like other pore-forming toxins, pore formation leads to ion influx and efflux, initiation of a variety of apoptotic and necrotic processes and ultimately cell death (Aman and Adhikari, 2014).

1.2.5 Survival within host cells

To further hide from attacks of immune cells, *S. aureus* can also hide inside a wide variety of host cells (Fraunholz and Sinha, 2012) – including phagocytes such as neutrophils and monocytes as well as epithelial and endothelial cells such as keratinocytes and osteoblasts. To invade non-professional phagocytes, *S. aureus* uses the zipper type mechanism that involves bridging of the fibronectin (Fn)-binding proteins A and B (FnBPA and FnBPB) to $\alpha_5\beta_1$ integrins on host cell surfaces in a Fn-dependent manner (Sinha et al., 1999).

As an intracellular pathogen, *S. aureus* is able to replicate in the phagosome or freely in the cytoplasm of host cells. *S. aureus* has been shown in a cystic fibrosis cell line to employ α-toxin to form pores and disrupt the phagosomal membrane, then to translocate into the cytoplasm where it can replicate (Jarry, Memmi, and Cheung, 2008). Macrophage-phagocytosed *S. aureus* can survive within the mature phagosomes (Lâm et al., 2010), cause disseminated of infection within the monocyte-derive macrophages (Kubica et al., 2008) and use various strategies to impeded macrophage recruitment, phagocytosis and degrative abilities. In fact, our lab has characterized the replication of *S. aureus* within the phagolysosomes of macrophages and developed a fluorescence based-proliferation assay that can identify replicating bacteria within host cells (Flannagan et al., 2018; Flannagan, Heit, and Heinrichs, 2016). Moreover, *S. aureus* can subvert autophagy (sequestering cytoplasmic

content via usage of isolation membrane and eventually delivering them to lysosomes), a mechanism host cells use to dispose leaky vesicles or intracellular bacteria. To do so, *S. aureus* inhibits fusion of phagosome with lysosomes (Schnaith et al., 2007). Finally, *S. aureus* induces cell death mechanisms such as apoptosis or pyronecrosis (pro-inflammatory cell death) of phagocytes via the use of three types of toxins discussed above (Fraunholz and Sinha, 2012).

1.2.6 Intracellular persistence and growth

Not all bacterial cells are killed by the phagolysosomes. As such, *S. aureus* has been reported to persist inside phagocytes or endothelial cells for prolonged periods (Sendi and Proctor, 2009). Persistent *S. aureus* has been associated as small-colony variants (SCVs) which are slow-growing cells with reduced metabolism that has been associated increased antibiotics resistance and persistence within host cells (Proctor et al., 2014). Interestingly, compared to "normal" cells, SCVs have been shown to have increased uptake by host cells and *S. aureus* SCVs isolated from clinical specimen have been consistently associated with deficiency in electron transport or in thymidine biosynthesis (Proctor et al., 2014). Furthermore, SCVs have been found to display a thick cell wall (Bulger and Bulger, 1967) and an upregulation of the alternative sigma factor σB that allows it to cope with various environmental stressors (Moisan et al., 2006). Overall, there has been increasing evidence that *S. aureus* can persist and retain its infectivity due to its extreme durability and thus, can serve as a source of recurrent infections (Fraunholz and Sinha, 2012).

In summary, *S. aureus* possess a large arsenal of virulence factors that allow it to initiate infection, evade killing by innate immune cells, survive within host cells and contribute to persistence infections.

1.3 Nutritional Immunity: a mechanism at which host cells restrict transition metal access from pathogens

Transition metals are important micronutrients critical for survival of all life forms. They are involved in many biological processes such as incorporation into metalloenzymes, storage proteins and transcription factors. As a way to limit proliferation of pathogens, vertebrates employ a strategy termed "nutritional immunity" to restrict free transition metals from bacteria or direct the toxicity of these metals against them (Hood and Skaar, 2012). Four metals including iron, manganese, zinc and copper, are the most well-studied transition metals

in the context of host nutritional immunity and their roles in bacterial physiological processes. For the purpose of this study, I will focus on the mechanism in which host sequester free Mn and how *S. aureus* competes for Mn (and Zn, as these metal have overlapping mechanisms).

1.3.1 Calprotectin withholds Mn and Zn from pathogens

The bioavailability of Mn in the host can vary widely – with different tissues exhibiting differences in Mn levels. In humans, Mn concentration in blood and serum range from 20 to 200 nM, 20-50 nmol/g in liver and kidneys and less than 20 nmol/g in other organs such as brain, heart and lungs (Hurley, Keen, and Baly, 1984). The S100 family of calcium binding proteins found in vertebrates have been implicated in defense against infection. Importantly, the S100A8 and S100A9 function as a heterodimer called calprotectin. With high antimicrobial properties against bacterial and fungal pathogens, calprotectin makes up of approximately 40% of protein composition of the neutrophil cytoplasm and leads to an excess concentration of 1 mg/mL within tissue abscesses (Gebhardt et al., 2006). It protects against infection by chelating Mn^{2+} and Zn^{2+} through two high-affinity binding sites. Interestingly, both sites can bind to Zn^{2+} at nanomolar affinity while only one site has such affinity for Mn^{2+} (Kehl-Fie et al., 2011). A study by Corbin *et al.*, 2008 was the first publication that showed the protective function of calprotectin. They also found that neutrophil-derived calprotectin can inhibit *S. aureus* growth through metal chelation and results in re-programming of the bacterial transcriptome. Moreover, abscesses of calprotectin-deficient mice were found to be enriched in metal as well as to have increased bacterial proliferation. Overall, these findings demonstrate that calprotectin is critical in innate immune response to infection and define metal chelation as a strategy to inhibit microbial growth abscessed tissue.

1.4 Manganese

1.4.1 Manganese is an essential nutrient

Virtually all biological systems require Mn ions as they play vital roles for living organisms, including bacterial pathogens. Bacteria use Mn as cofactors in diverse range of biochemical processes (Table 1), such as carbon transformation, nucleic acid and protein synthesis, DNA replication, regulation of virulence factor regulation and metabolism of ROS (Jakubovics and Jenkinson, 2001). This thesis will focus on Mn which *S. aureus* utilizes as a cofactor for various enzymes in many biological processes. In *S. aureus*, Mn^{2+} is mostly known

to be the cofactor for superoxide dimutases and plays important roles in defending the bacterial cells against the oxidative bursts of phagocytes during infection (Cassat and Skaar, 2011). Paradoxically, excess of Mn is toxic to the cells; therefore, maintenance of intracellular Mn is tightly regulated.

Table 1. Selected functions of Mn within bacterial cells (from Jakubovic and Jenkinson, 2001)

Process or pathway	**Enzyme or protein**
Photosynthesis	Mn-stabilizing protein (PSII-O)
Gluconeogenesis	PEP synthase, Pyruvate carboxylase
Glycolysis	3-Phosphoglycerate mutase
Sugar metabolism	L-Fucose isomerase
	6-Phospho-beta-glucosidase
Amino acid metabolism	Arginase
	Glutamine synthetase
	Threonine 3-dehydrogenase
Aromatic acid metabolism	Muconate cycloisomerase
Peptide cleavage	Aminopeptidase P
Nucleic acid degradation	Ribonuclease HII
	Endonuclease IV
Signal transduction	Serine/threonine protein phosphatase 1 and 2
Stringent response	(p)ppGpp 3'-pyrophosphaohydrolase
Oxidative stress defence	Mangani-catalase
	MnSOD

1.4.2 Manganese homeostasis in *S. aureus*

The two high affinity staphylococcal systems that import Mn ions from the environment are the MntABC ATP-binding cassette-type transporter and MntH which belongs to the NRAMP (natural resistance-associated macrophage protein) family of transporters (Fig. 2, Horsburgh et al., 2001). During tissue infections, both transport systems compete with the vertebrate host protein calprotectin, for Mn acquisition (Kehl-Fie et al., 2013). In the 3-

protein ABC transporter, *mntA* encodes the ATP-binding domain, *mntB* the transmembrane permease and *mntC* the substrate-binding lipoprotein.

Notably, MntC has been found to be an important virulence factor of *S. aureus* as well as a moonlighting protein that performs multiple independent functions in different cell compartments. Other than binding to metal ions, MntC was found to bind extracellular matrix and plasminogen, suggesting a role in staphylococcal mucosal colonization and establishment of invasive disease (Salazar et al., 2014). In fact, MntC is one of the most abundant lipoproteins in *S. aureus* (Stoll et al., 2005) where strains lacking *mntC* were shown to be more susceptible to oxidative burst by neutrophils (Coady et al., 2015) and concurrent lack of *mntC* and *mntH* resulted in decreased bacterial burdens in a staphylococcal skin abscess model of infection (Kehl-Fie et al., 2011).

As a critical component for pathogenesis that was found to be expressed during early stage of infection, MntC has been included in passive and active *S. aureus* immunization study by Pfizer as well as four other important virulence factors; clumping factor A (ClfA), fibronectin binding protein B (FnbB) and capsular polysaccharides (Anderson et al., 2012; Rauch et al. 2014). Immunization studies found that MntC can induce a protective immune response and was effective in reducing the bacterial load of *S. aureus* in the blood in a mouse bacteremia model (Anderson et al., 2012). Additionally, anti-MntC monoclonal antibodies that can bind to both *S. aureus* and *S. epidermidis* cells were found to be protective in an infant rat passive protection model (Yu et al., 2018).

Transcription of MntABC is regulated by MntR, the DtxR family metalloregulator manganese transport regulator (Horsburgh et al., 2002a). The MntR transcriptional regulator binds intracellular Mn and represses MntABC transcription. It is unclear whether transcription of MntH is Mn-dependent and/or regulated by MntR. To prevent cytoxicity whereby intracellular Mn concentration is high, excess Mn is effluxed through MntE which belongs to the cation diffusion facilitator transporter family. Interestingly, presence of MntR and MntE was required for *S. aureus* to express full virulence in murine models of infection (Grunenwald et al., 2019), suggesting the importance of the regulation of intracellular Mn for pathogenesis.

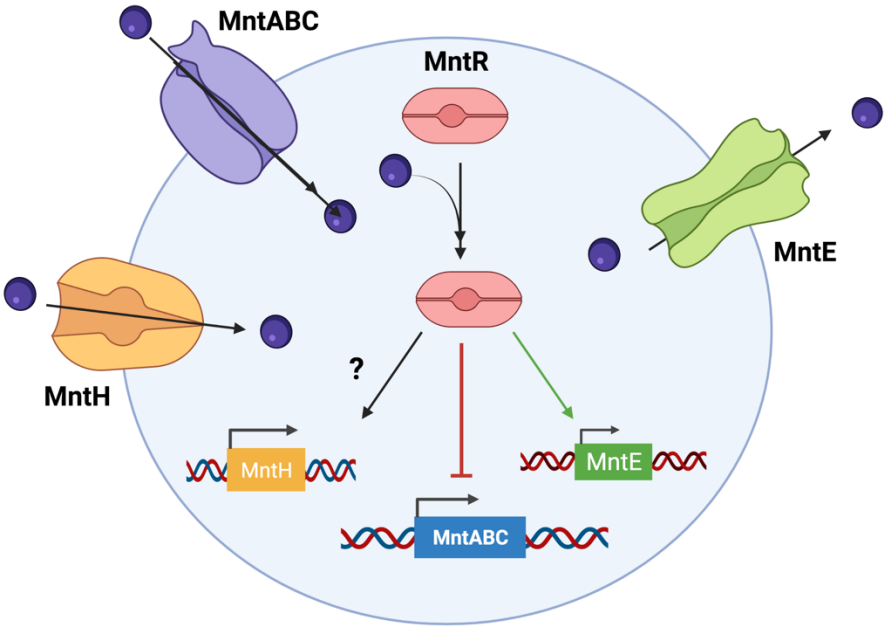


Fig. 2: Manganese homeostasis in *S. aureus*. Mn^{2+} ions are imported into the cell by the MntABC and MntH transporters while MntE export excess Mn^{2+} to prevent cytotoxicity. The transcriptional regulator MntR binds to intracellular Mn and represses *mntABC* transcription. Modified from Price and Boyd, 2020.

1.4.3 Manganese and iron are essential cofactors for superoxide dismutases

One of the prime colonizing sites in *S. aureus* is in the nasopharynx which is rich in metal ions including manganese (Gupta et al., 2013). Here, Mn is taken up via the MntABC transporter to protect the bacterial cells from oxidative burst mechanism employed by the host. Mn taken up by MntABC is then incorporated as a co-factor into the ROS-detoxification enzymes, superoxide dismutase (SodA and SodM). SodA and SodM are metalloproteins that catalyze the dismutation of O_2^-, the first ROS produced upon reduction of oxygen, by converting it to H_2O_2 and O_2. These dismutases have been shown to be important for full virulence of *S. aureus* (Karavolos et al., 2003b) and for repair following oxidative damages to establish growth after phagocytic oxidative bursts by host cells (Horsburgh et al., 2002b). Interestingly. *S. aureus* isolated from the inflammatory and oxidized sputum of cystic fibrosis (CF) patients have increased transcription of *sodA* and *sodM*, the latter having higher transcriptional level than in bacterial *in vitro* cultures (Treffon et al., 2020). These findings further provide evidence that these superoxide dismutases contribute to *S. aureus* virulence and allow the bacteria to persist within the hostile environments of the host, such as the CF airways.

While all staphylococcal spp. encode SodA, *S. aureus* is unique in that it encodes an additional superoxide dismutase, SodM, which presumably enhances the ability of *S. aureus* to resist superoxide dependent intoxication as compared to other staphylococcal spp. SodA strictly binds Mn as a co-factor and was found to play an important role for ROS resistance under Mn replete conditions (Garcia et al., 2017a). In contrast, SodM was found to be important under Mn-deplete conditions suggesting that *S. aureus* employs different dismutase depending on Mn concentration. Conceivably, this is due to the fact that SodM can function using Mn or Fe as a metal cofactor *in vitro*, and can be activated with both metals *in vivo* (Garcia et al., 2017b). This finding suggests that cambialistic SOD may represent a strategy for *S. aureus* to maintain defense against superoxide in niches where Fe and Mn can fluctuate.

1.5 Brief overview of *S. aureus* metabolism

The ability to synthesize or acquire nutrients is critical for pathogen to survive and replicate. As well, to advance disease progression, *S. aureus* needs to express its virulence factors; however, this activity is driven by the metabolic state of the cells (Somerville and Proctor, 2009a). Unlike the metal micronutrients discussed above, *S. aureus* can synthesize some of these macromolecules on its own. To give an overview of its metabolism, *S. aureus* can synthesize the 12 basic metabolic precursors used to build macromolecules. To metabolize carbohydrates, it utilizes glycolysis and the pentose phosphate pathways (PPP), the latter also produces phosphoribosyl pyrophosphate (PRPP). PRPP is an important metabolite because it is the starting compound in the production of purines and pyrimidines (Fig. 3). To produce reductants for respiration and other essential precursors, *S. aureus* can complete the tricarboxylic acid (TCA) cycle that is repressed in exponential phase when nutrients such as amino acids and glucose are abundant and when glucose is limited the repression is released. *S. aureus* can also synthesize the majority of amino acids, including branched-chain amino acids (BCAAs), although work from our lab found that it prefers to acquire BCAAs from the environment (Kaiser et al., 2016). In this thesis, I will only focus on purine metabolism which involves *de novo* synthesis, the purine salvage pathway and extracellular import.

1.5.1 Metabolism and virulence control

To infect and survive in the host, *S. aureus* requires a sufficient level of important metabolites and co-factors to maintain cellular integrity and permit replication. Regulation of

metabolites allows *S. aureus* to utilize variable energy storage to control its virulence, especially during its transfer from a colonization to infection state (Goncheva et al., 2019a; Sause et al., 2019). *S. aureus* does so by activating transcription of virulence genes in a manner that is regulated by environmental and nutritional signals (Lakhundi and Zhang, 2018). Altered metabolic status results in the transduction of intracellular signals that can be sensed by regulatory proteins (Somerville and Proctor, 2009b). These include CodY that responds to intracellular BCAAs and GTP, catabolite-control protein A (CcpA) that responses to glycolytic intermediates to avoid carbon-wasting and energy-synthesizing macromolecules (Henkin et al.,1991), GlnR that is responsive to key intracellular nitrogen donors that are glutamate, glutamine and ammonium in growth medium (Guédon et al., 2001), and most important to this study, PurR (transcriptional regulator of purine biosynthetic genes) which has also recently been demonstrated by our lab to have roles in regulating production of FnbA and FnbB (Goncheva et al., 2019b). In summary, the ability for *S. aureus* to cause disease by expressing virulence factors is tightly regulated by metabolic cues and other environmental factors.

1.5.2 Purine Metabolism

Purine metabolism is a key process that allows the cell to acquire building blocks of DNA as well as energy storage molecules both of which are essential for bacterial survival and replication. Purines are heterocyclic aromatic organic compounds that consist of two rings. They are essential components involved in various cellular functions, such as nucleic acid synthesis, cellular potential energy storage and transfer, cellular metabolism and signal transduction. Several reports have described the importance of nucleotide biosynthesis to cause bacterial infection as certain purine auxotroph mutants of *Salmonella* (Stocker, Hoiseth, and Smith, 1983), *S. aureus* (Mei et al., 1997) and *S. pnuemoniae* (Brooks and Mias, 2018) have been shown to be avirulent in murine infection. These findings suggest that purines and some amino acids are scarce *in vivo*, therefore, pathogens must rely on their ability to synthesize these compounds *de novo* or acquire them extracellularly to survive and cause disease in the host.

The first component of purine metabolism is *de novo* purine synthesis which *S. aureus* can accomplish, as evidenced by growth of WT strain in purine-free media. In summary, the *de novo* purine biosynthesis produces inosine 5-monophosphate (IMP) as the base purine

molecule that can be converted via two separate pathways into adenosine triphosphate (ATP) or guanosine triphosphate (GTP), both of which are energy storage molecules (Fig. 3). Through subsequent modifications, ATP and GTP can be converted into nucleotides used in DNA replication as well as generation of signalling molecules such as cyclic-di GMP(Goncheva, Chin, and Heinrichs, 2022a). Therefore, the reliance of the purine synthesis pathway for growth and proliferation is widely observed in bacteria (Kilstrup, Hammer, Jensen, et al., 2005). Indeed, our lab has already demonstrated that *de novo* purine synthesis is required for *S. aureus* intracellular growth (Goncheva, Flannagan, and Heinrichs, 2020a).

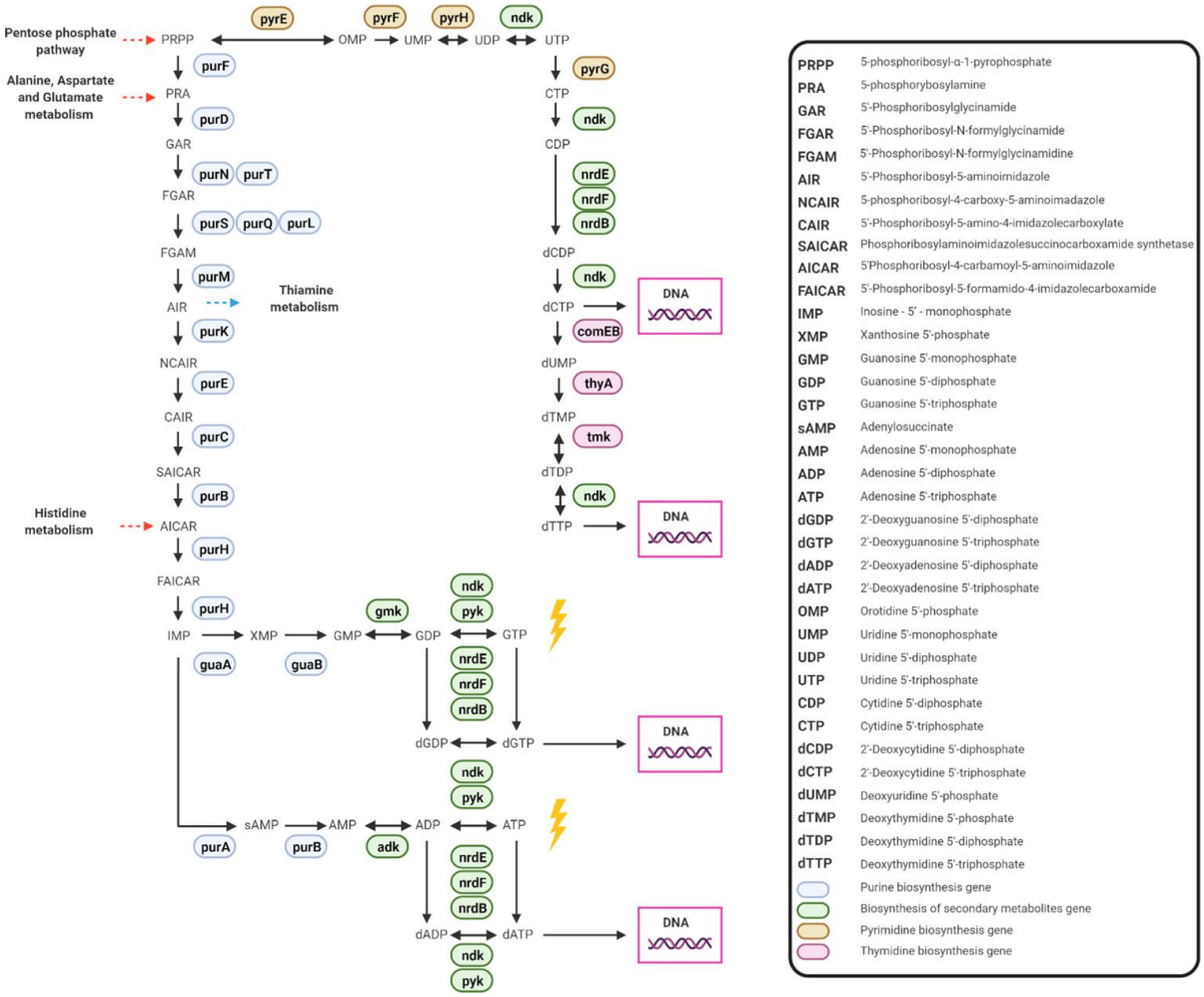


Fig.3: *De novo* purine and pyrimidine synthesis pathways of bacteria. PRPP generated from the pentose phosphate pathway is used in pyrimidine synthesis to produce pyrimidine (thymine, uracil and cytosine) nucleotides and in purine synthesis to generate energy storage compounds, ATP and GTP, as well as their dinucleotides via a series of biochemical reactions. Diagram from Goncheva, Chin, and Heinrichs 2022a.

In *S. aureus*, *de novo* purine biosynthesis is regulated by PurR which acts as a transcriptional repressor by binding to an operator called a "pur box" within the promoters of PurR regulated genes. The purine biosynthetic precursor, PRPP, binds to PurR and alters its affinity for DNA. Recently, our lab and another research group have linked PurR-dependent gene regulation to virulence in *S. aureus* (Goncheva et al., 2019a). The *purR* mutant with upregulated expression of purine biosynthesis genes was demonstrated to be hypervirulent by our lab as *purR*-infected mice displayed increased disease severity and mortality in a systemic model of infection. The *purR* mutants also had increased bacterial burdens in the heart, blood and kidneys of infected mice (Goncheva et al., 2019a). Another group demonstrated that *purR* was acutely virulent in bloodstream infection of mice and in primary human neutrophils (Sause et al., 2019). Furthermore, two recent studies proposed an important clinical relationship between purine biosynthesis and outcomes of MRSA bacteremia (Li et al., 2018) and endovascular infection (Li et al., 2020). MRSA strains isolated from patients with persistent bacteremia (PB) are associated with higher expression of purine biosynthesis genes, earlier activation of key virulence factors and faster growth rates compared to isolates from patients with resolving bacteremia (RB). Taken together, the significant coregulation of purine biosynthesis and virulence factors suggest that *S. aureus* increases its pathogenic potential when faced with nucleotide shortage and that nutrient acquisition is critical for pathogenesis and survival (Goncheva, Chin, and Heinrichs, 2022).

1.5.3 Transport of exogenous purines in *S. aureus* is not well-characterized

In the absence of *de novo* purine biosynthesis, exogenous purines can be imported into the bacterial cells through transporters (Fukuda and Schuetz, 2012). In *Lactococcus lactis*, another Gram-positive bacterium, common nucleosides are transported by an ATP-binding cassette (ABC) transporter called BmpA-NupABC, while uridine and deoxyuridine are transported by the UriP transporter (Martinussen et al., 2010). Similarly, *nupNOPQ* and *nupG* genes in *Bacillus subtilis* have also been identified to code for an ABC transporter that imports guanosine and other purine nucleosides (Belitsky and Sonenshein, 2011). In *E. coli*, the broad-specificity transporter, NupG, transports purine and pyrimidine nucleosides while the more selective transporter, NupC, transports mainly guanosine and inosine (Craig, Zhang, and Gallagher, 1994; Westh Hansen, Jensen and Munch-Petersen, 1987; Patching et al., 2005). Interestingly, various studies of nucleotide import in *Borrelia burgdorferi*, *Salmonella typhi*

and *Streptococcus pneumoniae* have reported that extracellular import is the main method of nucleobase and nucleotide acquisition in some instances and that the host restriction of these nutrients can control bacterial infections (Bucarey et al., 2005; Cuellar et al., 2020; Saxena et al., 2015).

Although *S. aureus* has been extensively studied, not much is known about the roles of specific proteins in its purine biosynthesis pathway, or the role of the intracellular purines pool for growth and virulence. Most bacteria can produce nucleotides *de novo* while others, such as some lactic acid bacteria, require addition of either purines or pyrimidines in the media to grow. In turn, these bacteria utilize salvage pathways for conversions to nucleotides (Kilstrup, Hammer, Ruhdal Jensen, et al., 2005). On the other hand, many bacteria such as *B. subtilis* and *E. coli* can utilize nucleotides as purine or pyrimidine sources but they need to be dephosphorylated by extracellular nucleotidases before being imported into the cells (Zakataeva, 2021; Terakawa et al., 2016).

In abscess lesions, staphylococci replicate within the fibrin-encapsulated communities and attract phagocytes, such as neutrophils, to implement purulent destruction of host tissues (Cheng et al., 2010). Here, activated neutrophils release traps (neutrophil extracellular traps, NETs) of extracellular matrix composed of nuclear and mitochondrial DNA, antimicrobial peptides and granular proteins (Thammavongsa, Missiakas, and Schneewind, 2013). Although many pathogens are susceptible to killing by NETs, *S. aureus* can pivot this extracellular (e)DNA-rich environment to its advantage by using secreted nucleases (Nuc) and the cell surface-attached nucleotidase adenosine synthase A (AdsA) to convert host eDNA into dAMP and subsequently deoxyadenosine (dAdo), a compound that exquisitely kills macrophages by attacking the mammalian purine salvage pathway (Thammavongsa et al., 2009). Clearly, the abscess represents an environment that is nucleotide-rich and *S. aureus* has the ability to break down host nucleotides via the nucleases; however, it has not been demonstrated whether *S. aureus* can import these purines into the cells within the NETs-populated abscesses. An example of how bacteria can utilize host eDNA as purine source and increase fitness is demonstrated in *V. cholerae* where extracellular nuclease degrade eDNA into nucleotides which are transported though the outer membrane via the pore-forming outer membrane OmpK, and subsequently dephosphorylated nucleosides via three periplasmic phosphatase (nucleotidase). As free nucleosides, they are then taken up by the NupC nucleoside systems and used as a source of carbon and nitrogen (Gumpenberger et al., 2016a). Nonetheless, our

lab has demonstrated *in vitro* that *S. aureus* can import exogenous purines when it cannot synthesize purines *de novo*, either from inactivation of purine biosynthetic genes or from the use of pharmacologic inhibitors of purine synthesis (Goncheva, Flannagan, and Heinrichs, 2020b; Flannagan and Heinrichs, 2020). The complete growth rescue of purine auxotrophic *S. aureus* by exogenous purines suggests that there exists at least one purine importer that uptakes purines from the environment.

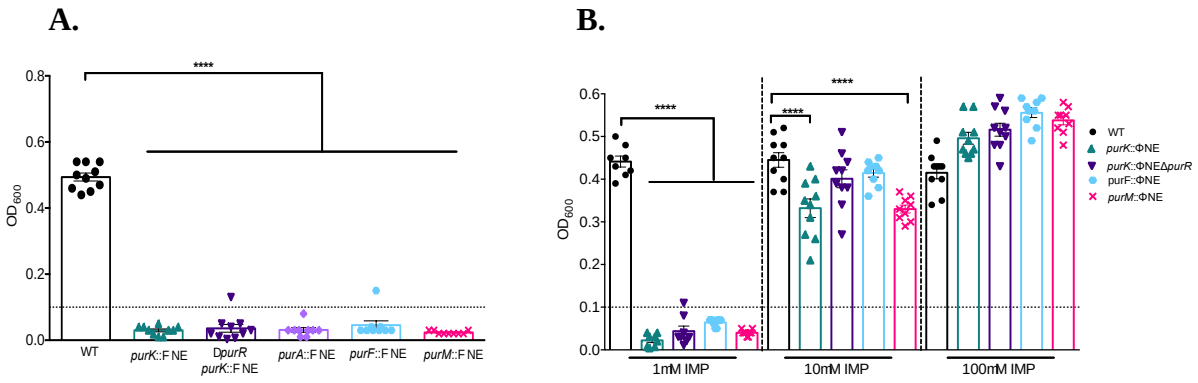


Fig. 4: The growth defect of purine biosynthetic *S. aureus* mutants in purine-free media can be rescued by exogenous IMP. Growth after 24 hours of WT and mutants of purine biosynthesis in purine-free media (A) and IMP-rich media (B). Graphs modified from Goncheva *et al.*(Goncheva, Flannagan, and Heinrichs 2020a)

1.5.4 Known purine transporters in *S. aureus*

In *S. aureus*, some of the characterized transporters of purine nucleotides and nucleosides can be found in the purine salvage pathway. First, the xanthine permease (encoded by *pbuX*) imports xanthine and, together with *xpt* (xanthine phosphoribosyltransferase), *guaA* (IMP dehydrogenase) and *guaB* (GMP synthetase), makes up the riboswitch that has been found to regulate both purine biosynthesis and purine salvage pathways, in response to guanine concentration (Fig. 4). Bacterial riboswitches such as this one that regulate *de novo* guanine synthesis have gained attention as a novel class of antimicrobial drug targets because they have high affinity for small molecules, are absent in humans, and regulate expression of multiple genes, many of which are essential for cell viability. This riboswitch functions as a negative feedback loop where the end product of the biosynthetic or nutrient salvage operon, guanine, directly inhibits its own expression. In short, binding of guanine to its riboswitch causes transcriptional attenuation by formation of an intrinsic terminator, shutting down the

expression of the cognate biosynthetic operon (Serganov and Nudler, 2013). Within the purine salvage pathway of *S. aureus*, PbuX functions to recycle nucleosides and nucleobases by importing hypoxanthine and xanthine into the cells to be converted into IMP (by Hpt) or XMP or GMP (by Xpt) which can be further modified to generate nucleotides (Kofoed et al., 2016a).

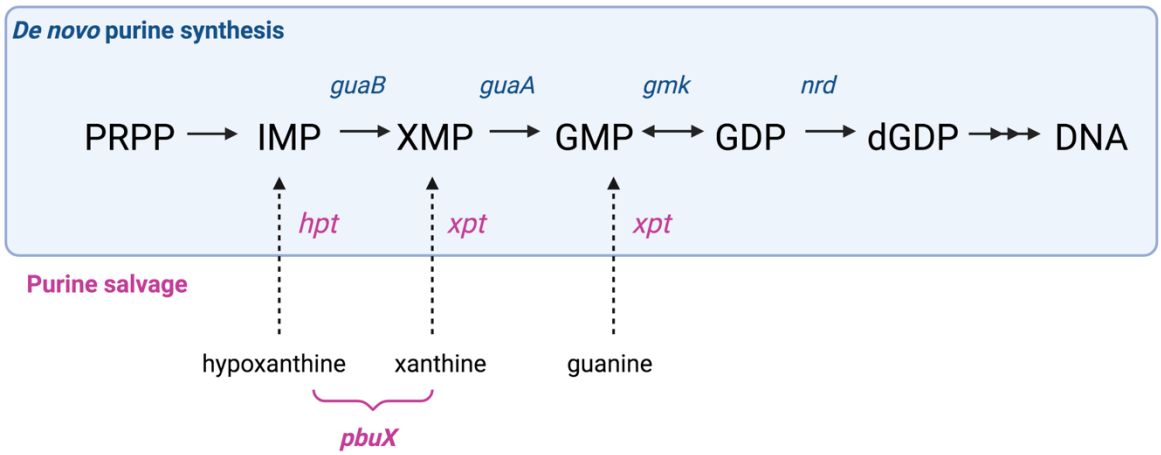


Fig.4: Xanthine permease (PbuX) functions by importing hypoxanthine and xanthine to enter the purine salvage pathway. PbuX salvages nucleosides and nucleobases and convert them into IMP, XMP or GMP. IMP is the first branch point for guanine and adenine de novo biosynthesis. GuaB converts IMP to XMP as the first committed step to generate dGTP used in DNA synthesis. Modified from Kofoed *et al*., 2016.

Other than PbuX, *S. aureus* also encodes for NupC transporter. In *E. coli*, NupC is was found to transport both pyrimidine and adenine nucleosides (Xie et al., 2004) while it was found to mainly transport purines in *H. pylori* (Miller, Vaish, and Maier, 2012). In *S. aureus*, NupC has been annotated as a nucleoside permease in the UniProt database (NupC - Nucleoside Permease NupC - *Staphylococcus aureus* (Strain COL)). Although recent work have shown that it transports thymidine to support the growth of thymidine-dependent small colony variants (TD-SCVs) (Kriegeskorte et al., 2014), it is possible that NupC may import purines as well.

Lastly, recent works by our lab has found that mutations in the xanthine-uracil permease (*stgP;* also annotated guanine permease *(pbuG)*) and the *hpt* gene from the purine salvage pathway lead to resistance to a purine analog anti-virulence compound, 6-thioguanine (6-TG)(Chin, Goncheva, Flannagan, and Heinrichs, 2021). This work suggests that StgP is likely the major transporter of 6-TG into the cell. Other than its annotation as a xanthine-uracil transporter and the resistance to a purine analog of this mutant, the substrates of StgP have not

yet been investigated. Overall, the three transporters of purines described above may potentially be involved in the transport of exogenous purines that are responsible for the growth rescue of purine auxotrophic mutants that cannot synthesize purines *de novo*.

1.6 Methotrexate and 6-mercaptopurines as purine biosynthesis inhibitors

Considering the importance of purine biosynthesis in bacterial survival, several reports have outlined the use of nucleoside analogs in treatment of bacterial infections and highlighted a few compounds that have effects *in vivo* (Goncheva, Chin, and Heinrichs, 2022b). Among these compounds, our lab has recently demonstrated that multiple staphylococcal species naturally secrete the purine analog, 6-TG, that has the ability to inhibit *S. aureus* expression of virulence factors (Chin, Goncheva, Flannagan, Deecker, et al., 2021). A large number of nucleotide synthesis inhibitors that are used clinically have been developed for treatment of various cancers but can be adapted for use as antimicrobials. (Goncheva, Chin, and Heinrichs, 2022).

In this study, I have used two inhibitors of *de novo* purine biosynthesis – methotrexate (MTX) and 6-mercaptopurine (6MP), two anti-cancer drugs that both interfere with human and bacterial DNA replication. MTX is a folate antagonist that interferes with folate synthesis, an important co-factor needed for synthesis of nucleotides (Baggott and Tamura, 2015). In eukaryotic cells, 6MP has shown antiproliferative effects through functioning as a purine analog and thereby incorporating into nucleic acid chains, leading to DNA damage and apoptosis, and blocking the *de novo* purine synthesis (Taylor, Watson, and Bradley, 2005; Chouchana et al., 2012). Interestingly, both compounds were found to be potent antibiotics at high doses for *Mycobacterium avium* subspecies *paratuberculosis* (MAP), the causative agent of some cases of inflammatory bowel disease (Greenstein et al., 2007). At low doses, they have been associated with clinical improvement in inflammatory and autoimmune diseases such as Crohn's and inflammatory bowel diseases (IBD) due to its ability to reduce the production of pro-inflammatory cytokines (Bernstein et al., 1994; Rian et al., 2009). Overall, both of these compounds have the potentials to inhibit *S. aureus* growth via nucleotide synthesis and to be used as tools to study extracellular purine transport.

1.7 Project rationale and hypothesis

S. aureus infections can be challenging to treat because of its complex pathogenesis and the emerging antibiotic resistance. The ability for *S. aureus* to cause disease can be attributed to its prolific ability to acquire nutrients such as purines in the nutrient-starved environment of the host (Goncheva, Flannagan, and Heinrichs, 2020a). To date, the mechanism of purine import in *S. aureus* has not been investigated, despite the abundance of knowledge of purine transporters in other bacteria. Numerous studies, including those published by our lab, have established the intimate link between purine metabolism, and expression of virulence genes, highlighting importance of purines in *S. aureus* pathogenesis, virulence and persistence. Considering the importance of purines in cell proliferation, energy storage and virulence control, this knowledge gap represents an interesting avenue to explore to gain a better understanding of how *S. aureus* acquire purines from the environment.

Our lab has demonstrated that *S. aureus* with mutations in purine biosynthesis genes (such as *purK, purA, purM* and *purF)* cannot grow in purine-free media (Fig. 5A) but upon addition of IMP, they can grow to WT levels (Fig. 5B) (Goncheva, Flannagan, and Heinrichs, 2020a). The complete growth rescue of purine biosynthetic mutants with exogenous purines indicates that *S. aureus* must be transporting exogenous purines into the cells. Other than the three purine transporters mentioned above, it is possible that other purine transporters exist and have not been characterized. Therefore, **I hypothesize that *S. aureus* possess at least one purine transporter that imports exogenous purines.** To do so, we used two selective conditions that both consist of a drug that inhibits the purine biosynthesis and a purine, MTX+guanine and 6MP+IMP to screen for library of individual transposon mutants for the mutant of the purine transporter. I reasoned that purine biosynthesis mutants would grow normally in rich and minimal media as they can synthesize purines *de novo.* However, when inhibitor drugs are present in selective conditions, the purine transporter mutants will not grow since *de novo* purine biosynthesis is inhibited and the transposon mutation disrupts the transporter, which will result in absence of growth.

1.8 Research objectives

To address my hypothesis, I pursued the following research objectives:

Objective 1: Characterize how *S. aureus* transport purines

- Identify which types of purines are transported by *S. aureus*
- Test known purine transporter mutants for growth defects in conditions where *de novo* synthesis is inhibited

Objective 2: Develop screening conditions to identify putative mutants of the purine transporters

- Define concentrations at which MTX and 6MP (pharmacologic inhibitors of *de novo* synthesis) that can inhibit WT growth in purine-free media
- Define concentrations of IMP and guanine that can rescue the growth defect of a purine biosynthetic mutant in purine free-media
- Optimize the conditions of MTX+ guanine and 6MP+IMP that can rescue WT growth but would impose a growth defect in putative purine transporter mutants

Objective 3: Identify a mutant of the purine transporter using the Nebraska Transposon Mutant Library, a collection of 1,952 *S. aureus* mutants with known transposon insertions in non-essential genes (Fey et al., 2013)

- Characterize the growth of each of these mutants in rich media, purine-free media, 6MP+IMP and MTX+guanine
- Identify mutants with growth defect in 6MP+IMP and MTX+guanine
- Confirm the identity of putative purine transporter mutant using PCR
- Evaluate whether the growth defect in the screening condition can be rescued by providing the gene *in trans* via plasmid complementation of the mutant

Chapter 2 – Materials and Methods

2.1 Bacterial strains, plasmids and growth media

All bacterial strains used in this study can be found in Table 2. *S. aureus* USA300 LAC cured of its endogenous antibiotic resistance plasmid served as the WT *S. aureus* strain for this study. *S. aureus* strains were grown in tryptic soy broth (TSB; Wisent), Roswell Park Memorial Institute-1640 (RPMI-1640; Wisent) or Chemically Defined Media (CDM) and *E. coli* in Luria-Bertani (LB) broth at 37˚C shaken at 200 rpm overnight, unless otherwise stated..

CDM was made as previously described in Horsburgh *et al*.(M. J. Horsburgh, Ingham, and Foster 2001) with components in mg/L as follows: Na_2HPO_4 (7,000), KH_2PO_4 (300), adenine sulfate (20), guanine-HCl (20), l-glutamic acid (2,220), l-aspartic acid (2,220), l-proline (2,220), glycine (2,220), l-threonine (2,220), l-serine (2,220), l-alanine (2,220), l-lysine-HCl (560), l-isoleucine (560), l-leucine (560), l-histidine (440), l-valine (440), l-arginine (330), l-cystine (220), l-phenylalanine (190), l-tyrosine (170), l-methionine (170), l-tryptophan (60), pyridoxal (0.8), pyridoxamine-2-HCl (0.8), d-pantothenic acid (0.4), riboflavin (0.4), nicotinic acid (0.4), thiamine-2HCl (0.4), and biotin (0.02). When specified, CDM and RPMI were treated with 20 g of Chelex-100 (Biorad) per liter and with stirring overnight at 4°C to remove any trace metals. When appropriate, cultures were supplemented with erythromycin (3 µg/mL), lincomycin (10 µg/mL), tetracycline (12 µg/mL), kanamycin (50 µg/mL) or chloramphenicol (12 µg/mL). Solid media was prepared with 1.5% (w/v) Bacto agar.

2.2 Bacterial growth curves

Growth kinetics assessment of the Δ*mntC mntH* mutant relative to WT was performed in metals-free CDM (with acid-washed flasks) while that of *sodA::Tn*, *sodM::Tn* and *sodAM::Tn* were performed in TSB and CDM. In all cases, single-isolated colonies were grown overnight in TSB and sub-cultured into the flasks with 25 mL of suitable media at an initial OD_{600} of 0.01 before allowing to grow at 37˚C. The OD_{600} values were measured using the spectrophotometer every 2 hours for 12 hours (TSB) or 24 hours (CDM) using the respective media as a blank.

2.3 Screening of the Nebraska Transposon Mutant Library

Strains from the Nebraska Transposon Mutant Library containing individual 1,952 gene mutants of *S. aureus* were inoculated from frozen stock into 200 µL of TSB with erythromycin and lincomycin in 96-well plates which were grown at 37˚C and shaken at 200 rpm overnight. Each culture was then diluted to 1:100, followed by two 10-fold dilutions in saline. Drops of 10 µL from each culture and each dilution from overnight culture (10^{-2}, 10^{-3} and 10^{-4}) were dropped onto three sets of plates; tryptic soy agar (TSA), Roswell Park Memorial Institute-1640 (RPMI-1640) and RPMI-1640 plates containing an inhibitor or a purine, either 0.01 mg/mL of MTX with 400 µM guanine or 25 mM of 6MP with 100 µm inosine monophosphate (IMP). TSA and RPMI-1640 plates were incubated for 24 hours while the selective RPMI-1640 plates were incubated for 72 hours at 37˚C and number of colonies were counted at a dilution with 10-40 visible colonies. Candidates were grown in tubes of 5 mL TSB overnight, diluted to the OD_{600} of 0.1, and re-screened as described in Results section. Number of colonies in each media condition were enumerated as CFU/mL.

2.4 General molecular genetic techniques

2.4.1 Chromosomal DNA extraction of *S. aureus*

For each strain, 500 µL of its overnight culture were diluted or allowed to grow to an OD_{600} of 10 and pelleted. The pellet was resuspended in 200 µL of STE (5 M sodium chloride, 1 M Tris, 0.2 M EDTA (ethylenediaminetetraacetic acid)), 50 µL lysostaphin in STE (0.25 µg/µL) and incubated at 37˚C for at least one hour. After cell lysis, 20 µL of 10% sodium dodecyl sulfate (SDS) and 20 µL of 20 mg/mL Proteinase K were added and samples incubated overnight at 55˚C. On the next day, 80 µL of 5M sodium chloride were added to each tube which was mixed by inversion. Next, each tube was supplied with 320 µL of 25:24:1 phenol: chloroform: isoamyl alcohol (IAA), mixed well by inversion and centrifuged at 13,000 rpm for 15 minutes. The aqueous layer was transferred and supplied with 300 µL 24:1 chloroform: IAA, mixed by inversion and centrifuged at 19,000 x g for 15 minutes. This step was repeated but with 400 µL isopropanol. After centrifugation, pellets were washed with 750 µL of 70% ethanol, centrifuged at 19,000g for 10 minutes and allowed to dry after ethanol removal. DNA were re-suspended in 10-100 µL of ddH_2O. DNA concentration was measured using Nanodrop® ND-1000 UV-Vis spectrophotometer.

2.4.2 Purification of plasmid DNA

Plasmid DNA was purified according to manufacturer's instruction using an E.Z.N.A. Plasmid Mini Kit (Omega Bio-tek). To prepare plasmid DNA from *S. aureus*, 500 µL of an overnight culture in TSB were pelleted, followed by addition of 250 µL of Solution I-lysostaphin (50 mg of lysostaphin was added to 1 mL of Solution I) to each sample and incubation at 37°C for a minimum of 45 minutes until the suspension looked clear and viscous, indicative of lysis.

2.4.3 Polymerase chain reaction (PCR) and validation of genes and plasmid inserts

All oligonucleotides used in this study are listed in Table 3. PCR for cloning was conducted at a volume of 10 µL per reaction using Phusion DNA polymerase (0.02 U/µl), 5X Phusion High-Fidelity buffer (1X), forward and reverse primers (0.5 µM), dNTPs (200 µM). PCR for screening of mutants was conducted at a volume of 12.5 µl per reaction using of *Taq* Polymerase (0.0625 U), 10X *Taq* polymerase buffer (1X), dNTPs (200 µM), forward and reverse primers (0.2 µM) and 0.5 µl of template DNA. PCR products were visualize via gel electrophoresis was conducted on 1% (w/v) agarose gel in 1x TAE (40 mM Tris, 20 mM acetic acid and 1 mM EDTA (pH 8.0)) at 110 V for 22 minutes with 1 kB Plus DNA ladder (FroggaBio) to analyze sizes of amplicon.

2.4.4 Colony PCR

Colonies on selective plates with antibiotics were picked with micropipettes and resuspended in 50 µL of lysis buffer with lysostaphin (LBL; 50 µg lysostaphin resuspended in 1.25 mL of lysis buffer (25 mM Tris-HCl, 50 mM glucose, 150 mM NaCl, 10 mM EDTA; pH 8.0)). Samples were then incubated at at 37°C for 1 h before 50 µL H_2O and 2 µL of 10% (w/v) SDS were added. Samples were heated to 95°C for 10 min on a thermocycler, then cooled to room temperature (RT) for 10 min. Next, 100 µL 25:24:1 phenol:chloroform:IAA was added and samples were incubated to RT for 30 min. Samples were centrifuged at 19,000 x g for 10 min to and 10 µL was removed from the top aqueous layer, and later diluted in 90 µL water. The diluted sample was used as the template for PCR.

2.4.5 Restriction digest and ligation reactions

Restriction digests were performed using *SacI*, *KpnI* and *SacII* enzymes purchased from New England Biolabs according to manufacturer's instructions. Linear DNA fragments were purified directly from restriction reactions using the E.Z.N.A. Gel Extraction Kit (Omega

Bio-tek) as per manufacturer's instructions. Ligation of linear DNA fragments was performed using T4 DNA ligase (New England Biolabs) and the reactions were incubated at 16ºC overnight.

2.5 Phage transduction

2.5.1 Isolation of phage (Φ80α)

S. aureus RN2564 (carrying Φ80α) were grown overnight in TSB, sub-cultured the next day in 25 mL of TSB at the starting OD_{600} of 0.01 and grown to the OD_{600} of 0.3. At this point, mitomycin C was added to the culture at the final concentration of 2 μg/mL and the culture was incubated for 3 or more hours at 32ºC with slow shaking (80 rpm) until complete lysis was observed. Next, the culture was filtered through a 0.2 μm filter and the Φ80α stock was stored at 4ºC.

2.5.2 Preparation of transducing lysate

S. aureus carrying the antibiotic-marked mutation of interest was grown overnight in TSB with 0.5 mM $CaCl_2$. The next day, the culture was sub-cultured in 25 mL TSB with 0.5 mM $CaCl_2$ starting at an OD_{600} of 0.1 and the culture was grown at 37ºC with shaking to an OD_{600} of 0.7-1.0. Then, six 1 in 10 dilutions of Φ80α stock were prepared with phage buffer (0.1 M $MgSO_4$, 0.4 M $CaCl_2$, 2.5 M Tris pH 7.8, 5.9 g/L NaCl, 1.0 g/L gelatin) and 200 μL *S. aureus* was combined with 100 μL of phage for each of the phage dilutions. No bacteria (phage only) and no phage controls were run to ensure sterility of the process. After a 5 min incubation at room temperature, the phage-bacteria mix was added to melted top agar (0.8% (w/v) TSB agar) and poured on top of TSA plates containing 4 mM $CaCl_2$ for each dilution of phage. After an overnight incubation at 37°C, phage buffer was added to the plate with the phage dilution where Φ80α almost completely cleared the lawn of bacteria. The selected plate was incubated for approximately 4 h on a rocker at room temperature, then the top agar was scraped and mixed vigorously by pipetting, and the mixture was centrifuged at 3,000 x g for 15 min. Finally, the supernatant was filter sterilized twice, first with a 0.45 μm filter followed by a 0.2 μm filter and the donor phage lysate was stored at 4ºC.

2.5.3 Transduction of phage lysate in *S. aureus*

A single isolated colony of the recipient *S. aureus* strain was picked from a TSA plate and grown overnight in TSB with appropriate antibiotics and 0.5 mM $CaCl_2$. The next day the

overnight culture was sub-cultured into a flask of 15 mL TSB with 0.5 mM $CaCl_2$ at an initial OD_{600} of 0.1 and the culture was grown at 37ºC until it reaches an OD_{600} of 0.7-1.0. In the meantime, six 1:10 dilutions of phage lysate were prepared from the donor strain in phage buffer. When the recipient culture reached the appropriate $OD_{600,}$ six aliquots of 600 µL of the culture were centrifuged to pellet cells. Then, the supernatant was removed and the pellets were resuspended in 600 µL phage dilution. A phage only (no bacteria) and a bacteria only (no phage) controls were processed alongside the bacteria-phage samples to ensure sterility of the experiment. After 5 min of incubation at room temperature, 1.5 mL TSB with 0.5 mM $CaCl_2$ was added to the phage-bacteria mix and samples were incubated for 20 min at 37ºC. To stop the reaction, 1 mL of 2 mM sodium citrate was added to each sample. Next, the samples were centrifuged at 3,000 x g for 20 min to pellet the cells. After removal of the supernatant, pellets were resuspended in 100 µL 0.2 mM sodium citrate and plated on TSA plates containing 2 mM sodium citrate and the appropriate antibiotics markers for the desired mutation. Plates where then incubated 37ºC for at least 24 hours or until colonies appear after which the colonies can be screened for mutation with colony PCR.

2.6 Cloning and mutagenesis of *S. aureus*

2.6.1 Mutagenesis of *ΔmntC* using pKOR1

Oligonucleotides used in generating and *ΔmntC* mutants via allelic replacement can be found in Table 3. The plasmid pKOR1 was used to create in-frame, markerless deletion of *mntC* following methods described in Bae and Schneewind(Bae and Schneewind 2006). In summary, approximately 1 kb region upstream and downstream of *mntC* were amplified using 2 pairs of primers: attB1-mntC-D and *SacII*-mntC-C and attB2-mntC-A and *SacII*-mntC-B. The two fragments were sticky-ends ligated using *SacII*. The fusion of these fragment represents a 1 kB deletion of the *mntC* gene. This linear construct was introduced into pKOR1 temperature-sensitive allelic replacement plasmid through site-specific recombination using BP-Clonase-II as described, generating *pKOR1ΔmntC*. The vector was constructed in *E. coli* DH5α and passaged through *S. aureus* RN4220 grown at 30ºC before electroporation into USA300. Mutagenesis was performed by temperature shifting a growing a colony of USA300 carrying *pKOR1ΔmntC* from 30ºC to 42ºC, plated on chloramphenicol-TSA, and further plated on anhydrotetracycline (aTc). *ΔmntC* colonies were confirmed by colony PCR with primers

amplifying outside of *mntC*. Colonies positive for *ΔmntC* display a downward shift in PCR band size compared to WT control reflecting that the gene has been deleted.

2.7 Generation of recombinant MntC

A plasmid containing nucleotide sequence encoding for MntC omitting the signal peptide at the N-terminus. p*mntC*, was obtained commercially and cloned into *E. coli* DH5α (see nucleotide sequence in Table 3). p*mntC* was purified, digested with *Ndel* and *BamHI*, and ligated into a pET28a+ expression vector to generate pET28a+-*mntC* which was transformed into *E. coli* DH5α . pET28a+-*mntC* was then transformed into *E. coli* BL-21. To produce MntC, a single *E. coli* BL-21 carrying pET28a+-*mntC* was grown overnight, sub-cultured the next day to at an initial OD_{600} of 0.1 and grown at 37 ºC to an OD_{600} of 0.6 where 1 mM of isopropyl ß-D-1-thiogalactopyranoside (IPTG) was added to induce overexpression of MntC. After 4 hours, the culture was centrifuged at 6,500 × g for 15 min at 4°C. After discarding the supernatant, the pellet was resuspended in 5 mL of protein binding buffer (50 mM NaH_2PO_4, 10 mM imidazole and 300 mM NaCl, pH = 8). The sample was process through the One-shot cell disruptor (Pressure Biosciences Inc) twice and centrifuged 6,500 × g for 20 min 4°C. Then, the supernatant was transferred to ultracentrifuge tubes and ultra-centrifuged (Beckman Cloutier Life Sciences) at 300,000 × g for 45 min. The supernatant from the ultracentrifuged sample was then processed using a peristatic pump P-1 (Pharmacia Biotech) and a Ni-NTA column (HisTrap HP Cytiva) to purify His-tagged MntC. The wash buffer was composed of 50 mM NaH_2PO_4, 20 mM imidazole and 300 mM NaCl, pH = 8 and the elution buffer was composed of 50 mM NaH_2PO_4, 125 mM imidazole and 300 mM NaCl. Different fractions were run on SDS-PAGE and the fraction with a single band at 33 kDa were dialysed to suspend the purified protein in Hepes buffer (pH 7).

2.8 Western blot

To examine MntC expression of in different *S. aureus* strains, western blot of whole-cell lysates were performed. *S. aureus* strains were grown overnight in the specified media and conditions. Overnight cultures were normalized to an OD_{600} of 1.0, pelleted by centrifugation (19,000 × g for 2 min) and washed 0.9% (w/v) saline. Then, the whole-cell lysates were prepared by resuspending the pellets in 75 µL LBL and incubated at 37˚C for 1 h. After lysis, 25 µL 4 x Laemmli buffer (240 mM Tris-HCl, pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol,

0.04% (w/v) bromophenol blue) was added to each sample and *S. aureus* samples were boiled for 10 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% polyacrylamide gel. After electrophoresis, the proteins were transferred to a nitrocellulose membrane following standard protocol. The membrane was blocked with 8% (w/v) skim milk overnight. The next day, primary antibody (rabbit anti-MntC, diluted 1:1000) was added and left to incubate for 2 hours at room temperature. After washing of the membrane with PBS, secondary antibody (donkey anti-rabbit IgG antibody, DyLight 800 conjugated (diluted 1:20,000); Rockland Immunochemicals, Inc., Limerick, PA) was added. To image the membranes, An Odyssey CLx Imaging System and LI-COR Image Studio 4.0 software (LI-COR Biosciences) were used.

Table 2: Bacterial strains used in this study

Strain name	Description	Source
S. aureus		
USA300 LAC	Community-associated Methicillin Resistant *S. aureus* (CA-MRSA) used as "wildtype"; cured of antibiotic resistance plasmid	Lab Stock
RN4220	Strain used to uptake foreign DNA; r_k^- m_k^+	Lab Stock
RN2564	Strain used to store phage Φ80α	Lab Stock
purK::Tn	Transposon mutant of *purK* gene; a representative mutant unable to synthesize purines	Fey et al., 2013
WT USA300 pALC	Strain of CA-MRSA with pALC2073 plasmid insert used as a vehicle control	This study
0618::Tn (mntC::Tn)	Transposon mutant of manganese ABC transporter (MntABC) substrate-binding protein	Fey et al., 2013
0619::Tn (mntB::Tn)	Transposon mutant of manganese ABC transporter (MntABC) permease	Fey et al., 2013
0620::Tn (mntA::Tn)	Transposon mutant of manganese ABC transporter (MntABC) ATP-binding protein	Fey et al., 2013
pbuX::Tn	Transposon mutant of xanthine permease	Fey et al., 2013
nupC::Tn	Transposon mutant of nucleoside transporter	Fey et al., 2013
stg::Tn	Transposon mutant of six thioguanine permease	Fey et al., 2013
mntH::Tn	Transposon mutant of the NRAMP manganese transporter	Fey et al., 2013
ΔmntC	Markerless deletion of manganese ABC transporter substrate-binding protein	This study
ΔmntC purK::Tn	Markerless deletion of manganese ABC transporter substrate-binding protein transduced with transposon insertion in *purK*	This study

ΔmntC mntH::Tn	Markerless deletion of manganese ABC transporter substrate-binding protein transduced with transposon insertion in *mntH*; Mn-deficient mutant	This study
sodA::tet Newman	Transposon mutant of superoxide dismutase A in Newman	Garcia et al., 2017
sodM::ery Newman	Transposon mutant of superoxide dismutase M in Newman	Garcia et al., 2017
sodAM::tet::ery Newman	Transposon mutant of superoxide dismtase A and M in Newman	Garcia et al., 2017
sodA::Tn USA300	Transposon mutant of superoxide dismutase A in USA300	This study
sodM::Tn USA300	Transposon mutant of superoxide dismutase M in USA300	This study
sodAM::Tn USA300	Transposon mutant of superoxide dismutase A and M in USA300	This study
E. coli		
DH5α	Strain used for cloning; F- Φ_{80} dLacZΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17;* (r_k^-, m_k^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)U169phoA	Promega
BL-21	Strain used for expression of recombinant MntC	Lab Stock
Plasmids		
pALC2073	Shuttle vector; Amp^R in *E. coli*; Cm^R in *S. aureus*	Bateman et al., 2001
p:0618-0620	pALC2017 derivative for expression of ABC manganese transporter (MntABC)	This study
pET28a+-mntC	pET28a+ derivative for expression of MntC	This study
pKOR1-*Δ*mntC	pKOR1 derivative for in-frame, markerless deletion of *Δ*mntC	This study

Table 3: Oligonucleotides used in this study

Primer name	**Function**	**Sequence (5' → 3')**
pALC MCS F	Validate pALC2073 plasmid vehicle insertion	ATACCGCACAGATGCGTAAGG
pALC MCS R		CGATGACTTAGTAAAGCACATCTAA
0618-0620 pALC F	Validate transposon insertion of the entire *0618-0620 (mntABC)* operon	TTTGGTACCCTTGACATGATAAATATTCTCAG
0618-0620 pALC R		TTTGAGCTCGTGTTATTTCATGCTTCCGTGTACA
0618 F		ACTTCTAGTTGCTGCATGTGGT

0618 R	Validate the transposon insertion of *mntC* gene	GCGTGTTATTTCATGCTTCCGT
0619F	Validate the transposon insertion of *mntB* gene	TGACCCTAGTCAAAGGAGCA
0619 R		ACCACCAGTACCACATGCAG
0620 F	Validate the transposon insertion of *mntA* gene	TATCGATACCAGTACGCGGC
0620 R		TATCGATACCAGTACGCGGC
attB2-mntC D	Generation of a markerless allelic replacement in-frame deletion of *mntC* gene	GGGGACCACTTTGTACAAGAAAGCTGGGTCAATCACATAAGAACCTCTAACTTCG
SacII- mntC C		GGACCTCCGCGGGTAAGATGAATTGATGTTGATGCAAC
SacII-mntC B		CCGCGGACTGTACACGGAAGCATGAAATAAC
attB1-mntC A		TTTTTTGGGGACAAGTTTGTACAAAAAAGCAGGCCTTTGGTGATAGTGAAGAGGCTATGAG
mntH F	Validate the transposon insertion of *mntH* gene	CCAACATTTTAATGGTTTTATTTTTAACTTTGT
mntH R		TAAGAAGTAGAGGTGAGTCAAAATGAATAA
sodA F	Validate the transposon insertion of *sodA* (SAUSA300_1513) gene	TTTAAGAGACCGAACAAGTAATCATACTT
sodA R		AGACATTTTAGGAGGATGATTATT
sodM F	Validate the transposon insertion of *sodM (*SAUSA300_0135) gene	AGTATATTCATAAAAGGAGGAATATACTT
sodM R		TCCACCTCAATTATATTAAGTTAT
Nucleotide sequence for *mntC* to be expressed from amino acids: 33-309		
CATATGTTAAAAGTAGTAACGACGAATTCAATTTTATATGATATGGCTAAAAATGTTGGTGGAGACAACGTCGATATTCATAGTATTGTACCTGTTGGTCAAGATCC		

TCATGAATATGAAGTTAAACCTAAAGATATTAAAAAGTTAACTGACGCTGACG
TTATTTTATACAACGGATTAAATTTAGAGACTGGTAACGGTTGGTTTGAAAAA
GCCTTAGAACAGGCTGGTAAATCATTAAAAGATAAAAAAGTTATCGCAGTATC
AAAAGATGTTAAACCTATCTATTTAAACGGTGAAGAAGGCAACAAAGATAAA
CAAGATCCACACGCATGGTTAAGTTTAGATAACGGTATTAAATACGTAAAAAC
AATTCAACAAACATTTATCGATAACGACAAAAAACATAAAGCAGATTATGAA
AAGCAAGGTAACAAATACATTGCTCAATTGGAAAAATTAAATAATGACAGTA
AAGACAAATTTAATGACATTCCAAAAGAACAACGTGCCATGATTACAAGTGAA
GGTGCCTTCAAGTACTTCTCAAAACAATACGGTATTACACCAGGTTATATTTGG
GAAATTAACACTGAAAAACAAGGTACACCTGAACAAATGAGACAAGCTATTG
AGTTTGTTAAAAAGCACAAATTAAAACACTTATTAGTAGAAACAAGTGTTGAT
AAGAAAGCAATGGAAAGTTTATCTGAAGAAACGAAGAAAGATATCTTTGGTG
AAGTGTACACAGATTCAATCGGTAAAGAAGGCACTAAAGGTGACTCTTACTAC
AAAATGATGAAATCAAATATTGAAACTGTACACGGAAGCATGAAATAACACG
CTGGGATCC

Chapter 3: Results

3.1 Investigating purine transport of *S. aureus*

To grow in chemically defined media such as RPMI-1640 without purines, *S. aureus* must synthesize purines *de novo*. Moreover, mutants with defects in purine biosynthesis cannot grow without provision of exogenous purines such as inosine monophosphate (IMP) (Goncheva et al., 2019a). These data suggest that *S. aureus* can import purines from the environment, ostensibly through one or more transporters (Fig. 5). To study purine transport, I needed to find a condition where growth of *S. aureus* is dependent only on the transport of exogenous purines and not *de novo* synthesis.

As a first step toward this goal I tested the effects of MTX, a known inhibitor of *de novo* purine biosynthesis could impeded *S. aureus* growth in purine restricted RPMI. To this end I assessed bacterial growth in liquid culture in the presence of MTX and used a range of concentrations to establish the minimum inhibitory concentration (MIC) for the MTX inhibitor. This experiment revealed that after 24 hours of growth, WT *S. aureus* grew to an OD_{600} of approximately 1.0-1.2 in RPMI alone without MTX. In contrast in the presence of MTX bacterial growth was inhibited in a dose-dependent manner (Fig.6A) where 0.8 mM MTX completely blocked growth. Therefore, 0.8 mM MTX was used at this concentration moving forward in liquid RPMI.

Next, I sought to find the concentration of IMP that can completely rescue purine dependent growth of a purine biosynthesis mutant or WT bacteria in the presence of MTX. Here, we used a *purK*::Tn that cannot synthesize N-5-carboxyaminoimizadazole ribonucleic acid (NCAIR) which is an intermediate that is necessary for the generation of IMP and growth of this mutant has been shown to be rescued by IMP (Goncheva, Flannagan, and Heinrichs, 2020b) (Fig. 5). These experiments showed that *purK*::Tn *S. aureus* cannot grow in RPMI alone which differed significantly from WT bacteria (Fig 6B). In contrast, the addition of IMP, in a dose dependent manner, restored growth of the *purK* mutant. Indeed, IMP at a concentration of 20 to 100 μM fully restored *purK* growth to WT levels (Fig. 1B). In parallel, I tested the ability of IMP to rescue the growth of WT bacteria grown in RPMI with 0.8 mM MTX. These experiments again revealed that WT growth is inhibited in the presence of MTX however supplementation of the growth medium with

100 μM IMP rescued the growth of WT *S. aureus* (Fig. 6C). Of note, in the presence of IMP and MTX, WT growth is modestly reduced relative to the RPMI alone condition suggesting MTX may have additional effects on the bacteria in liquid culture. Nevertheless, these experiments established a condition in liquid medium where WT *S. aureus* could be made to be dependent on an exogenously supplied purine (*i.e.* IMP).

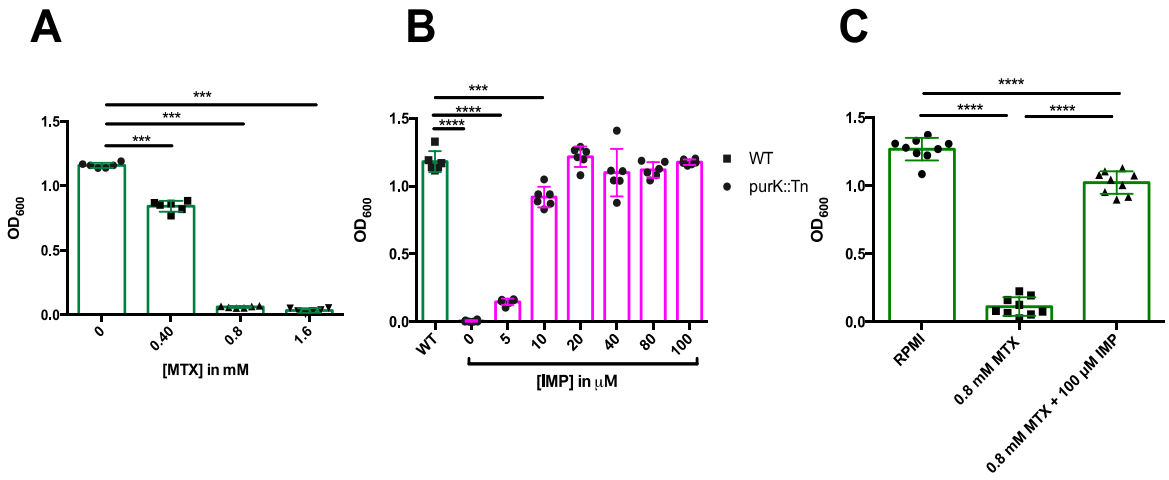


Fig.6: Addition of IMP into purine-free media can rescue growth defect of WT *S. aureus* inhibited by MTX. (A**)** Growth of WT in RPMI after 24 hr with different concentrations of MTX. (B) Growth of *purK*::Tn in RPMI with different concentrations of IMP after 24 hr with WT in RPMI as reference. (C). Growth of WT in RPMI after 24 hr in presence of MTX at inhibitory concentration and IMP at concentration that can rescue *purK*::Tn growth. Data are plotted as mean ± SEM, three or more biological replicates. Non-significant bars are omitted in (B). **** $p \leq 0.001$, ***$p \leq 0.001$ one-way ANOVA multiple comparisons.

3.2 *S. aureus* can transport IMP and guanosine phosphates when *de novo* purine synthesis is inhibited by MTX.

3.2.1 Other purines and pyrimidines were tested for the ability to restore WT growth in presence of MTX.

While the preceding experiments showed that *S. aureus* can utilize IMP to support purine-dependent growth, I also sought to determine whether *S. aureus* could utilize additional purine- or pyrimidine-related compounds for growth. Indeed, in some bacterial species nucleoside transporters have been shown to transport both purines and pyrimidines (Westh Hansen, Jensen and Munch-Petersen, 1987; Martinussen et al., 2010; Gumpenberger et al., 2016b). In *S. aureus*, there are known nucleoside transporters such as NupC (Kriegeskorte et al., 2014) that transports thymidine and PbuX that transports xanthine in the

purine salvage pathway (Kofoed et al., 2016a). To assess whether *S. aureus* can transport other purines, the ability of WT *S. aureus* to grow in RPMI with MTX and different concentrations of adenine (Fig. 7A), hypoxanthine (7B), thymine (7C) and uracil (7D) were assessed. As a control growth was also analyzed in RPMI with MTX alone. In the presence of MTX the addition of adenine at any concentration tested did not rescue the growth of WT *S. aureus*. In contrast, hypoxanthine, thymine, and uracil all rescued bacterial growth to some extent however levels were not restored to that seen for bacteria grown in the absence of MTX. Interestingly, increasing the concentration of hypoxanthine or thymine to as much as 4 mM impaired growth of *S. aureus* in presence of MTX as compared to conditions with lower concentrations of the metabolites that could promote growth. These data suggest that hypoxanthine and thymine at high concentrations are toxic to *S. aureus*, at least in the presence of MTX. Moreover, these experiments demonstrated that none of the purines or pyrimidines tested can restore growth as well as IMP (compare Fig. 6C and Fig. 7).

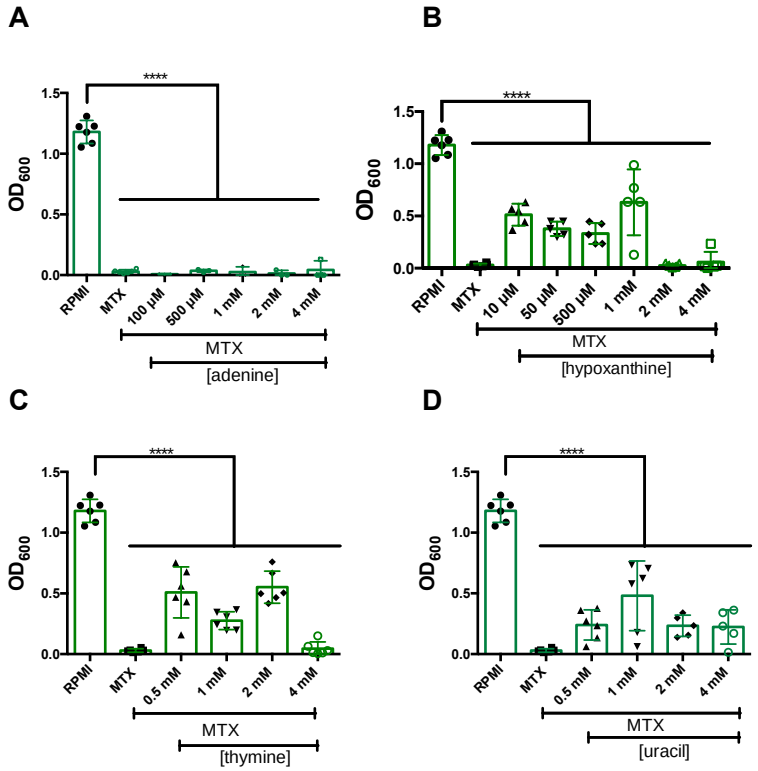


Fig. 7: Adenine cannot restore WT growth when *de novo* purine synthesis is inhibited by MTX and hypoxanthine, thymine and uracil only partially restore growth. WT *S. aureus* were grown in RPMI for 24 hours with MTX and adenine (A), hypoxanthine (B), thymine (C) and uracil (D) added at different concentrations. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *** $p\leq0.001$ one-way ANOVA multiple comparisons.

3.2.2 Guanosine phosphates are preferentially imported by *S. aureus* compared to adenosine phosphates when *de novo* purine synthesis is inhibited by MTX

Given I did not see robust growth of *S. aureus* when grown in RPMI and MTX in the presence of purine/pyrimidine bases we sought to investigate whether purine phosphates would allow for improved bacterial growth under the same conditions. *De novo* purine synthesis functions to generate IMP as a base purine molecule that then is shuttled into two separate pathways that ultimately generate adenosine triphosphate (ATP) and guanosine triphosphate (GTP) (Goncheva, Chin, and Heinrichs, 2022a). Therefore, I speculated that providing the end products of the *de novo* synthesis (i.e. ATP and/or GTP) could rescue MTX-induced growth impairment of WT *S. aureus*. These experiments showed that supplementation of RPMI with GMP, GDP and GTP, in the presence of MTX, partially restored growth to WT *S. aureus* (Fig. 8A). In contrast the addition of AMP, ADP and ATP did not improve bacterial growth (Fig. 8A). To determine whether combining guanosine and adenosine compounds could have an additive effect we grew *S. aureus* in the presence of MTX with combinations of purines. Interestingly, these experiments revealed an additive effect on growth where the addition of AMP + GMP (Fig. 8B) increased growth as compared to that seen in presence of either AMP or GMP alone (Fig. 8A). However, this was not observed for diphosphates and triphosphates where growth in GDP and GTP were higher than that observed when grown with GDP + ADP or GTP + ATP.

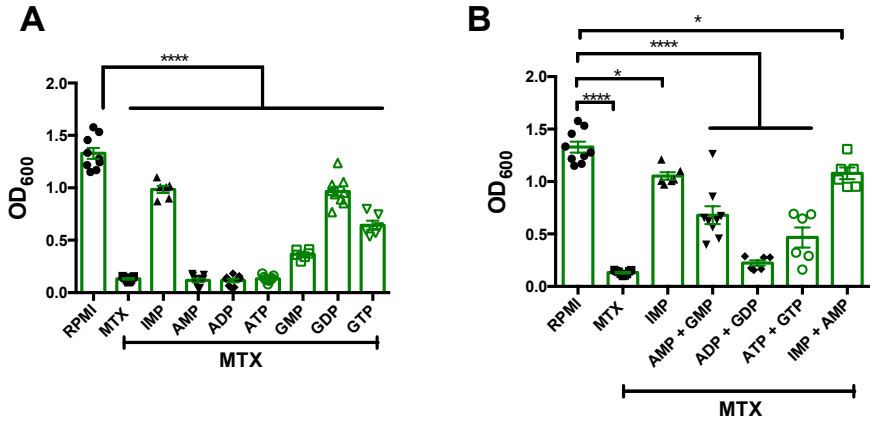


Fig.8: Guanosine phosphates are preferentially imported by *S. aureus* compared to adenosine phosphates when *de novo* purine synthesis is inhibited by MTX. WT *S. aureus* were grown in RPMI for 24 hours with MTX and single adenosine and guanine phosphates in (A), and combined adenosine and guanine phosphates in (B). All purine sources had a final concentration of 100 µM. Data are plotted as mean ± SEM, at least six biological replicates. **** $p<0.001$, *** $p\leq0.001$ one-way ANOVA multiple comparisons.

3.2.3 Testing known purine transporter mutants for growth defects in MTX and IMP

To further characterize how *S. aureus* transports purines, I pursued our next aim which was to test known purine transporter mutants for growth defects in the conditions where *de novo* synthesis is inhibited. As mentioned, *S. aureus* has been reported to encode putative purine transporters that are encoded by the *pbuX*, *stgP* and *nupC* genes respectively (McClure and Zhang, 2017). PbuX transports hypoxanthine, a precursor of IMP, and is encoded in an operon with a xanthine phosphoribosyltransferase (*xpt*). Interestingly, the *pbuX-xpt* operon are organized in a cluster together with essential *de novo* guanine biosynthetic genes, *guaA* (GMP synthase) and *guaB* (IMP dehydrogenase), suggesting that guanine biosynthesis and purine salvage function together (Fig 4A). StgP is a xanthine/uracil permease (also called six-thioguanine permease). Recently, our lab has found that mutations in *stgP* leads to resistance to 6-thioguanine that inhibits purine biosynthesis (Chin, Goncheva, Flannagan, and Heinrichs, 2021). NupC is known to transport thymidine into the cells and it was shown that when *S. aureus* is challenged with trimethoprim-sulfamethoxazole (SMX-TMP), an antifolate that acts to inhibit the *de novo* thymidine synthesis, it can bypass *de novo* synthesis via NupC dependent thymidine import.

To determine whether any of these genes play a role in IMP transport and growth of *S. aureus* in the presence of MTX, I utilized these mutants from the Nebraska Transposon Mutant Library where each of these genes were individually inactivated through transposon insertion. If any of these genes were indeed involved in IMP transport then we would expect a growth defect in the presence of MTX and IMP. Growth analysis of *pbuX*::Tn, *stgP*::Tn and *nupC*::Tn mutants in either RPMI, RPMI+IMP or MTX+IMP revealed that each of these strains grew equally well under all the conditions tested suggesting that either these genes are not dedicated IMP transporters or that there exist more than one transporters; in that case, a functional transporter would mask phenotype associated with the mutant understudy (Fig. 9). Although these mutants did not show growth defects under the conditions tested, it is conceivable that they are redundant for IMP transport and therefore a double or triple mutant may be required to observe a phenotype. It is also possible that there could be other purine transporter(s) in addition to PbuX, StgP and NupC that allow(s) for IMP-dependent growth in the presence of MTX.

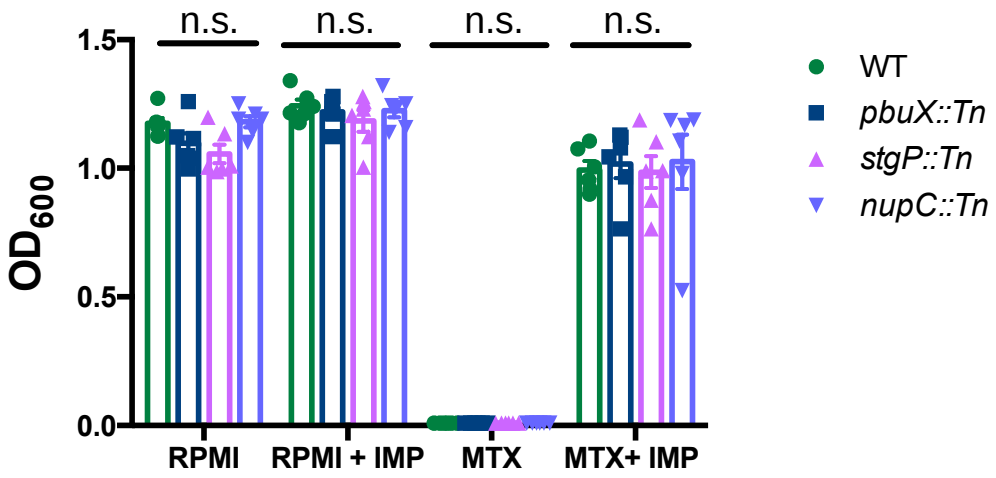


Fig.9: Known purine and pyrimidine transporter mutants do not show a growth defect in MTX+IMP. WT, *pbuX*::Tn, *stgP*::Tn and *nupC*::Tn *S. aureus* were grown in RPMI for 24 hours with combinations of MTX and IMP. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *** $p\leq0.001$ one-way ANOVA multiple comparisons.

3.3 Screening the Nebraska Transposon Mutant Library identifies *mntC::Tn* as a putative mutant of the purine transporter

As previously stated, I hypothesized that *S. aureus* must possesses one or more purine transporters because a mutant that cannot synthesize purine such as *purK::Tn* can grow if purines are provided exogenously. I then sought to investigate if the purine transporter of our interest were indeed PbuX, StgP or NupC. To this end, I did not see any difference in growth compared to WT of *pbuX::Tn, stg::Tn* and *nupC::Tn*, I hypothesized that there must be other transporters of purines (other than these three) that are allowing for growth when *de novo* synthesis is inhibited. Therefore, I decided to utilize the Nebraska Transposon Mutant Library (NTML) (Fey et al., 2013) that consisted of approximately 2,000 individual transposon mutants as a tool in identifying the novel purine transporter. Before I conduct the screen, I needed to develop screening conditions to screen for putative mutants of the purine transporters.

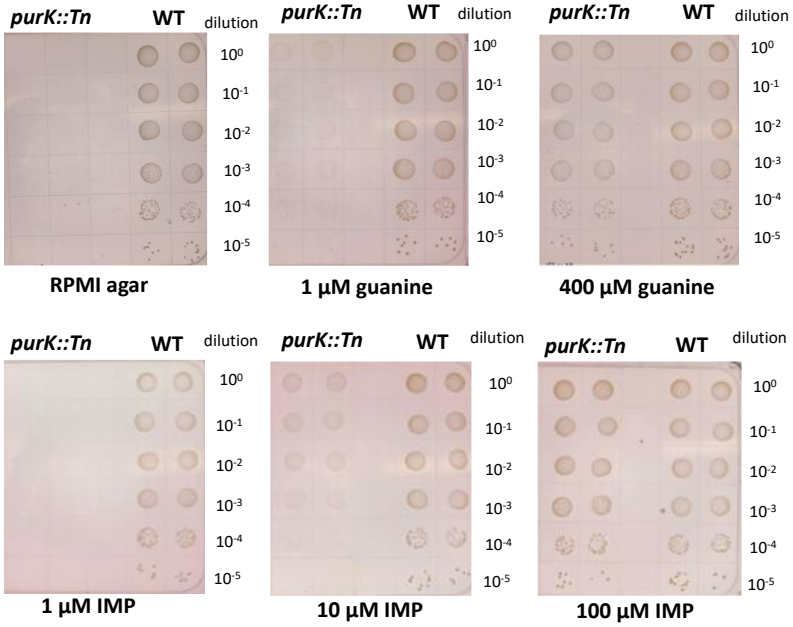
3.3.1 Establishing screening condition for the purine transporter on agar plates

As a first step in establishing screening conditions, I decided to assess the growth of each mutant on agar plates because this method would allow us to drop-plate several strains simultaneously; thus, improving work-flow efficiency. Subsequently, I then needed to identify the concentrations of purines (IMP and guanine) that would successfully restore *purK*::Tn growth in a chemically defined medium (Fig.10A). These purine concentrations represent the

baseline amount of exogenous purines the cells needed to grow when they cannot synthesize purines *de novo*.

Next, I sought to determine the concentrations of MTX and 6MP that eliminate growth of WT *S. aureus* in this medium. The absence of WT growth in these conditions represented total inhibition of *de novo* purine synthesis because the bacteria could not synthesize purines due to the inhibitors and there were no purines in the media that would allow for growth (Fig.10B). And ultimately, I combined the concentrations of purines (IMP and guanine) with the inhibitors (MTX and 6MP) to establish two conditions that would be used as screening conditions for the mutant(s) the purine transporter(s). In other words, I predicted that our mutants of interest would exhibit a growth defect in these conditions because they could not synthesize purines due to presence of MTX or 6MP nor transport exogenous IMP or guanine into the cells due to the transposon-inactivated gene. I found that combinations of 2 mM of MTX with 400 µM of guanine and 25 mM of 6MP with 100 µM IMP restored the growth of WT *S. aureus* eliminated by the inhibitors (Fig. 11). As such, these conditions can be used to identify mutants that are unable to transport purines because growth under these conditions only occur if the bacteria have a functional purine transporter. Thus, these two conditions were used to screen a transposon *S. aureus* library for mutants that displayed poor growth under these culture conditions as these mutants could have defects in purine import.

A.



B.

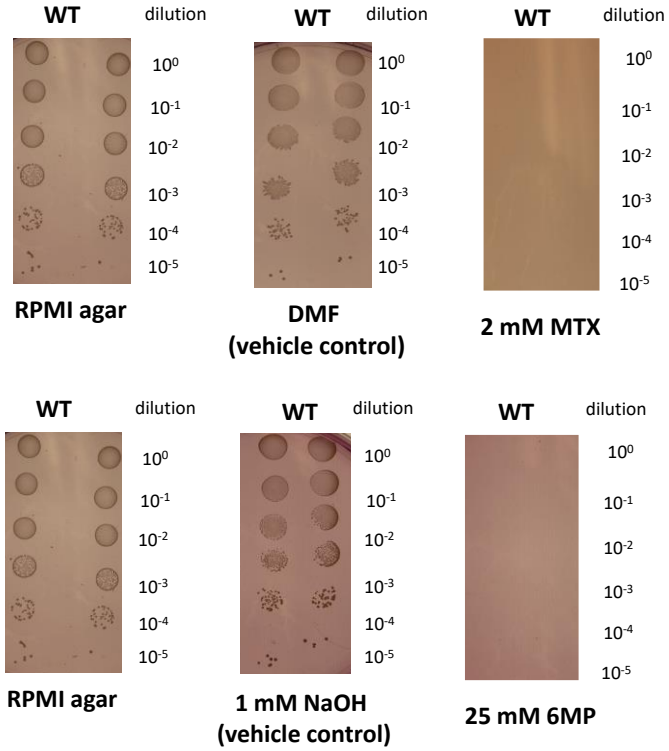


Fig.10: Exogenous IMP can rescue growth defect of a purine-deficient mutant and *de novo* purine synthesis of *S. aureus* can be inhibited with MTX or 6MP. A. The growth WT *S. aureus* USA300 and a purine biosynthesis mutant, *purK*::Tn, was assessed on RPMI-1640 plate alone and in presence of 400 µM of guanine and 100 µM of IMP **B.** Growth of WT *S. aureus* was assessed in presence of two inhibitors of purine biosynthesis – MTX at 0.01 mg/mL and 6MP at 25 mM. Growth of each strains is presented in two biological replicates.

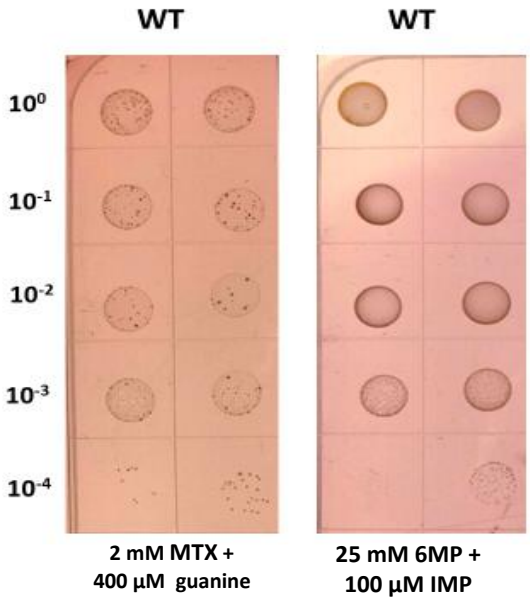


Fig.11: Establishing a screening condition for *S. aureus* purine transporter. Growth of WT *S. aureus* (presented in 2 biological replicates) was assessed in two conditions- MTX at 2 mM with guanine at 400 µM and 6MP at 25 mM with IMP at 100 µM.

3.4 Screening of the Nebraska Transposon Mutant Library for purine transport mutants in *S. aureus.*

Now that I have established two screening conditions to screen for putative mutants of the purine transporters, I proceeded to our third aim which was to identify a mutant of a purine transporter using the NTML. The 1,952 individual transposon mutants in the library each contains a single transposon insertion in a nonessential gene (Fey et al., 2013). Because I can trace back the identity of each mutant in the library using the genome annotation database if any interesting growth phenotypes are observed, I reason that the library is a suitable genetic tool for this study.

For this process, I have organized the screening into the following steps:

1. **Initial screen**
2. **Re-screen**
3. **Bioinformatics analysis**
4. **Final screen**

3.4.1. Initial screen: To begin, I screened the 1,952 mutants on 3 plates, as shown in Fig. 12A. TSA is a rich medium, thus all mutants are expected to display normal growth. In chemically defined medium supplemented with inhibitors, the established inhibitor concentration ensures growth of WT *S. aureus* is inhibited; therefore, I expected no growth on these plates. When growth inhibitory medium is supplemented with exogenous purines, I observed restored growth for WT, therefore, mutants with reduced growth represent strains of interest that could contain a mutation in a purine transporter. Mutants of interest were selected if they exhibited the following phenotypes: normal growth on TSA (positive control), no growth on MTX (negative control), and decreased growth on MTX + guanine relative to WT (Fig.12B). This first screen using MTX+ guanine resulted in a list of 97 candidate mutants that were selected for further analysis.

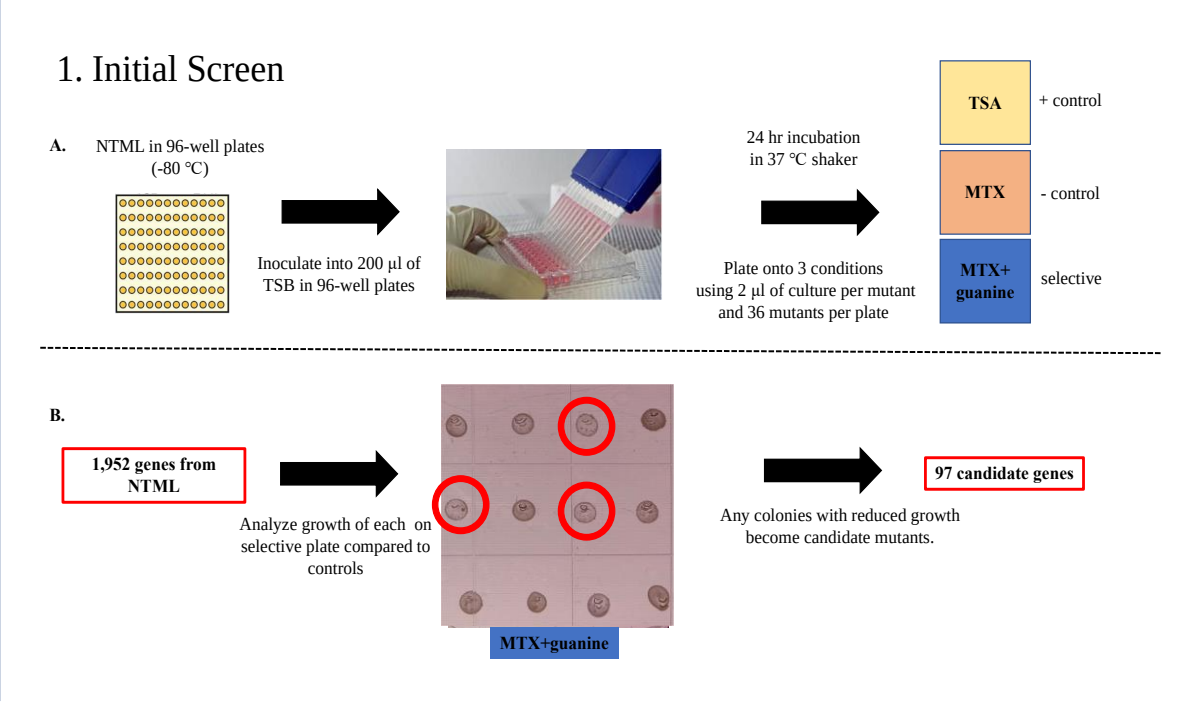


Fig.12: Initial screen of the Nebraska Transposon Mutants Library (NTML) for candidate *S. aureus* mutants of the purine transporter using MTX+ guanine as a selective condition. A. In the first step, 1,952 mutants from the frozen library organized in 96-well plates (-80 ℃) were grown in 200 µL of TSB supplemented with erythromycin and lincomycin overnight. Then, 2 µL of culture from each mutant were plated on 3 conditions; TSA, MTX and MTX+ guanine and incubated at 37℃. **B.** Growth of each colony on MTX+ guanine plates were observed and compared to colonies on TSA and MTX plates. Representative colonies with reduced growth are circled in red.

The entire transposon mutant library was also screened on medium containing 6MP with IMP along with the control conditions described above. The screen using 6MP+IMP generated a list containing 123 mutants of interest. Because the initial screen could be error-prone due to the large number of mutants screened, strains that showed ambiguous growth in both conditions were also included in the subsequent screen in addition to the mutants that clearly showed reduced growth.

3.4.2 Re-screen: I re-screened the identified candidate mutants using the culture conditions from the initial screen, which included rich medium (TSA), inhibitor control (MTX and 6MP) and selective (MTX+ guanine and 6MP+IMP) plates, as described in Fig.13. However, with a manageable number of mutants to screen, I plated 3 different dilutions of each mutant to more accurately and quantitatively assess growth defects. Mutants that show normal growth on TSA, no growth on inhibitor plates and reduced growth on inhibitor plates supplemented with exogenous purine relative to WT were considered as candidate mutants that lacked the ability to transport purines. Upon completion of this screen, I had identified 13 candidate mutants with distinguished growth defect on MTX+ guanine (Table 4) and a total of 26 candidate mutants from 6MP+IMP (Table 5).

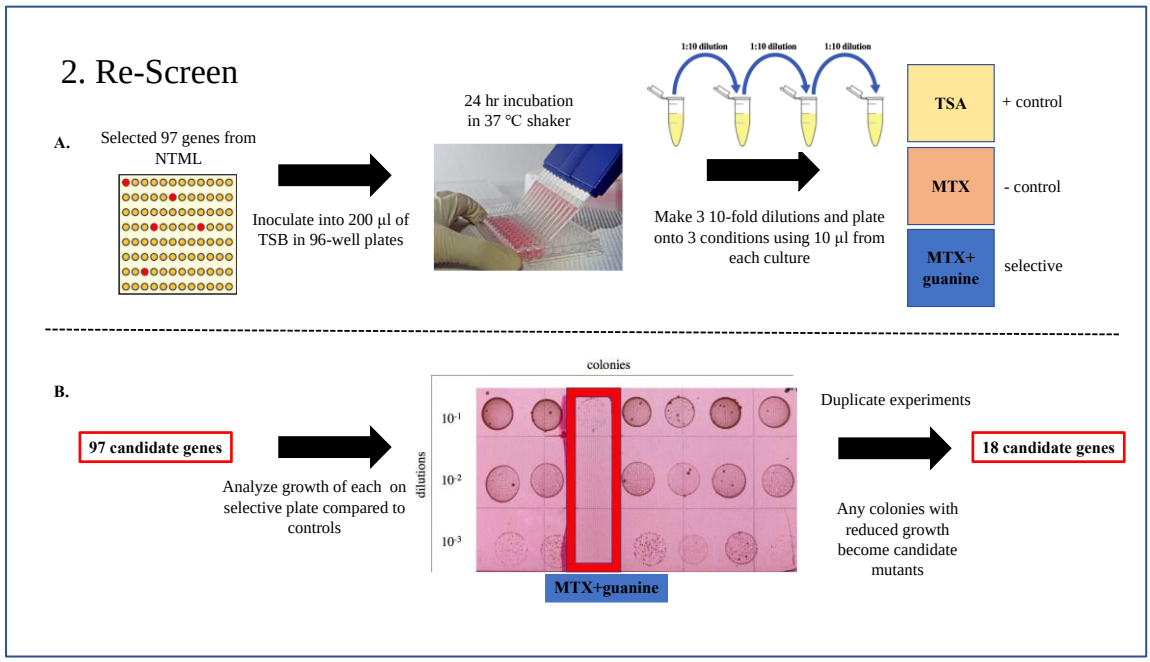


Fig 13. Re-screening of the NTML for candidate *S. aureus* mutants of the purine transporter using MTX+ guanine as a selective condition. A. Selected 97 candidate mutants that showed reduced growth from the initial screen were re-screened by inoculating from frozen NTML plates into 200 µL of TSB supplemented with erythromycin and lincomycin and grown overnight in a 96 well plate. Next day, 3 10-fold dilutions from each mutant were made before plating drops of 10 µL onto on 3 conditions; TSA, MTX and MTX+ guanine, and incubated at 37 ℃. **B.** Growth of colonies, especially at 10^{-3} dilution, was observed on MTX+ guanine plate and compared to TSA and MTX. A representative colony in 3 dilutions with reduced growth are boxed in red.

Table 4: Candidate mutants of the purine transporter from re-screen of NTML with growth defect on MTX + guanine. Blue highlights corresponds to putative mutants with growth defects in the selective conditions

	Gene	Gene description	Accession Number
1		putative lipoprotein/ nitrite reductase	SAUSA300_2354
2		ABC transporter ATP-binding protein	SAUSA300_0620
3	opp-1A	oligopeptide permease, peptide-binding protein	SAUSA300_2411
4		conserved hypothetical protein	SAUSA300_1003
5	pfoR	perfringolysin O regulator protein	SAUSA300_0310
6		transcription regulatory protein	SAUSA300_2326
7		hypothetical protein	SAUSA300_1692
8	dat	D-alanine aminotransferase	SAUSA300_1696
9		ABC transporter substrate-binding protein	SAUSA300_0618
10		ABC transporter permease	SAUSA300_0619
11		hypothetical protein	SAUSA300_1899
12	aroC	chorismate synthase (SCV)	SAUSA300_1357
13	alr	alanine racemase	SAUSA300_2027

Table 5: **Candidate mutants of the purine transporter from re-screen of NTML with growth defect on 6MP+IMP**

	Gene name	gene description	Accession Number
1	qoxA	quinol oxidase, subunit II	SAUSA300_0963
2	galM	aldose 1-epimerase	SAUSA300_2285
3		conserved hypothetical protein	SAUSA300_2311
4		putative transporter (Glucarate/carbohydrate)	SAUSA300_2449
5	tcaA	teicoplanin resistance associated membrane protein TcaA protein	SAUSA300_2302
6		conserved hypothetical protein	SAUSA300_1560
7		amino acid carrier protein (alanine/glycine symporter)	SAUSA300_1252
8		conserved hypothetical protein	SAUSA300_1301
9		methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase	SAUSA300_0965
10	rrfA	5S ribosomal RNA	SAUSA300_0457
11		phosphosugar-binding transcriptional regulator	SAUSA300_2271
12		conserved hypothetical protein	SAUSA300_0465
13		conserved hypothetical protein	SAUSA300_0128
14		transcriptional regulator, AraC family	SAUSA300_0104
15	rex	redox-sensing transcriptional repressor Rex	SAUSA300_1999
16		oligopeptide ABC transporter, permease protein	SAUSA300_2410
17		transposase	SAUSA300_0267
18		hypothetical protein	SAUSA300_1494
19	dat	D-alanine aminotransferase	SAUSA300_1696
20	carB	carbamoyl phosphate synthase large subunit	SAUSA300_1096
21	gcvH	glycine cleavage system protein H	SAUSA300_0791
22	_	ABC transporter substrate-binding protein	SAUSA300_0618
23	aroC	chorismate synthase	SAUSA300_1357
24	alr	alanine racemase	SAUSA300_2027
25	argR	arginine repressor	SAUSA300_1469
26	lpdA	dihydrolipoamide dehydrogenase	SAUSA300_1467

3.4.3. Bioinformatics analysis: The genes corresponding to the putative mutants from steps depicted in Fig. 13 were further confirmed with the NCBI database using the accession number of each mutant, as described in Fig. 14. Mutants were eliminated from the list if the disrupted gene had a function that was associated with one of the following: *de novo* purine biosynthesis, metabolic pathways and regulation of transcription. This was done because:

- First, mutants of *de novo* purine biosynthesis, such as *purK*::Tn which was used to establish screening conditions, are unable to synthesize purines and must import exogenous purines into the cells. Therefore, it is expected that these mutants grow poorly on minimal media lacking purines.
- Second, mutants of metabolic pathways such as branched-chain amino acid (BCAAs) biosynthesis mutants, will grow poorly in minimal media because BCAAs are vital for growth and for the physiology of the bacterium (Kaiser et al., 2016), regardless of ability to import purines.
- Third, mutants of transcriptional regulators, such as a CodY mutant, have defects in metabolic pathways that are also linked to control of virulence factors (Pohl et al., 2009), and thus; show poor growth as a large number of genes are affected (Kaiser et al., 2018). Following the analysis, mutants of transcriptional regulators were eliminated from the list of candidate mutants in both conditions.

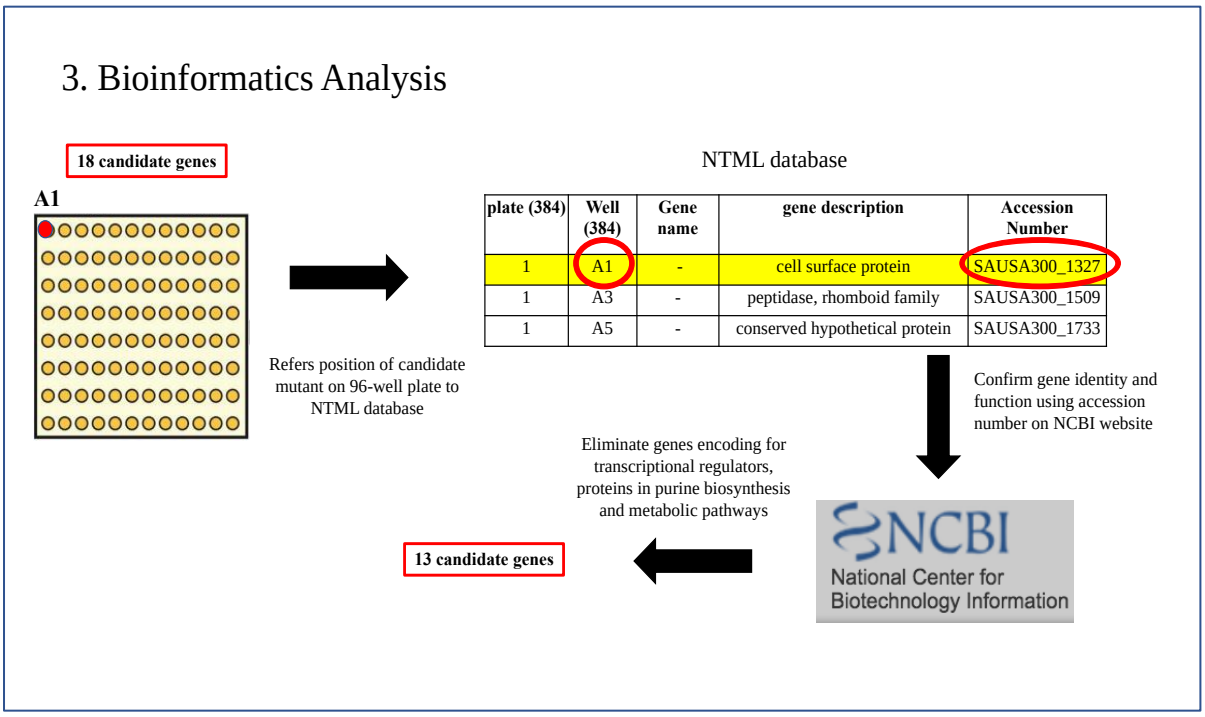


Fig.14: Bioinformatics analysis of candidate *S. aureus* purine transporter gene mutants. The position (A1 in this example) in the 96-well plate of the frozen NTML was identified for each of the 18 candidate mutants and the accession number for each gene was noted. Using the acquired accession number from the NTML database, the identity and function of each gene was determined using NCBI.

3.4.4. Final screen: In the final screen, each mutant was grown overnight, diluted, and plated onto 3 conditions:

1. Rich medium (TSA) as a positive control as all mutants should display normal growth in this condition;

2. Purine-free chemically defined medium (RPMI-1640) to eliminate mutants with growth defects that do not allow for growth in this medium;

3. Selective plates (MTX+ guanine and 6MP+IMP) where purine import mutants would be unable to grow.

Candidate purine transporter mutants were selected based on the following growth characteristics: normal growth in TSA and the defined medium, and reduced growth in selective plates. The 13 candidate mutants from the MTX+ guanine condition and the 26 mutants from the 6MP+IMP condition were grown as 2 independent biological replicates, standardized to a starting a OD_{600} of 1.0, serially diluted, and plated onto the 3 plates to accurately assess growth by enumerating colony forming units (CFUs) (Fig.15).

Mutants showing a 2-log growth reduction on the selective condition while displaying normal growth on TSA and RPMI-1640 as compared to WT were selected for further analysis (Fig. 16A, B). As a result, 6 final candidate mutants were chosen from the MTX+ guanine condition (Table 3) and 4 candidate mutants were selected from the 6MP+IMP condition (Table 4), as highlighted in blue. Interestingly, mutants with an insertion in alanine racemase (SAUSA300_2027) and ABC transporter substrate-binding protein (SAUSA300_0618) exhibited a robust growth defect in both conditions. An alanine racemase mutant is considered a small colony variant (SCV) which cannot synthesize its own nutrients and shows characteristic auxotrophic growth deficiency for alanine (Melter and Radojevič, 2010). Because this mutant was expected to have a growth defect, I chose to further investigate the ABC transporter substrate-binding protein mutant, along with mutants carrying a transposon in the two other genes in the ABC transporter operon (Fig.17). These genes encoded an ABC transporter permease (SAUSA300_0619), and an ATP transporter ATP-binding protein (SAUSA300_0620).

Overall, I identified a putative mutant of the purine transporter from the screening of over 2,000 transposon mutants. The robust growth defect of SAUSA300_0618 (thereafter *0618::Tn*) mutant in both MTX+guanine and 6MP+IMP as well as its annotation from the genome sequence as a substrate-binding protein of an ABC-transporter were key evidence that led me to hypothesize that this ABC transporter may indeed be the purine transporter I was attempting to characterize. Therefore, I decided to confirm the identity of these transporter mutants before proceeding with further analysis.

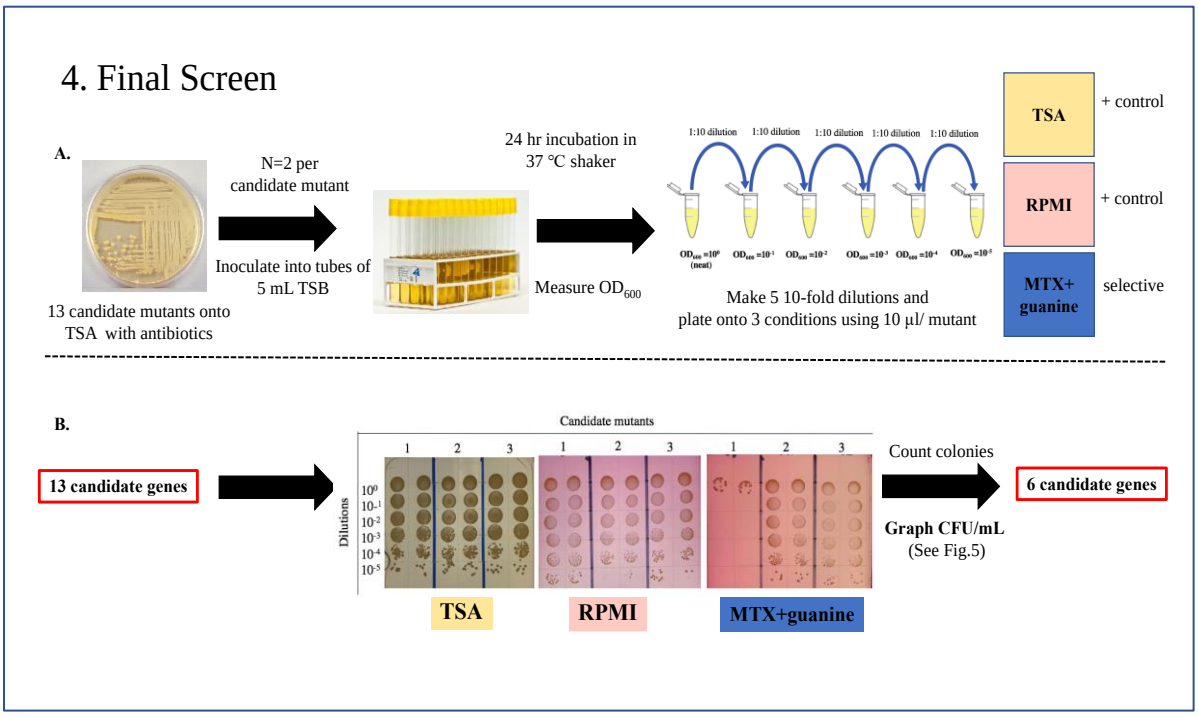


Fig.15: CFU/mL-based analysis in the final screen of candidate *S. aureus* mutants of the purine transporter using MTX+ guanine as a selective condition. The OD_{600} value was measured from each culture and standardized to a starting OD_{600} of 1.0, followed by 5 10-fold serial dilutions for each culture. Then, 10 µL of each dilution was dropped onto three plates (TSA, RPMI and MTX+ guanine). Colonies were counted to construct a CFU/mL graph of each strain (Fig.5). The final screen was conducted in triplicate experiments with 2 biological replicates per experiment and resulted in 6 final candidate genes.

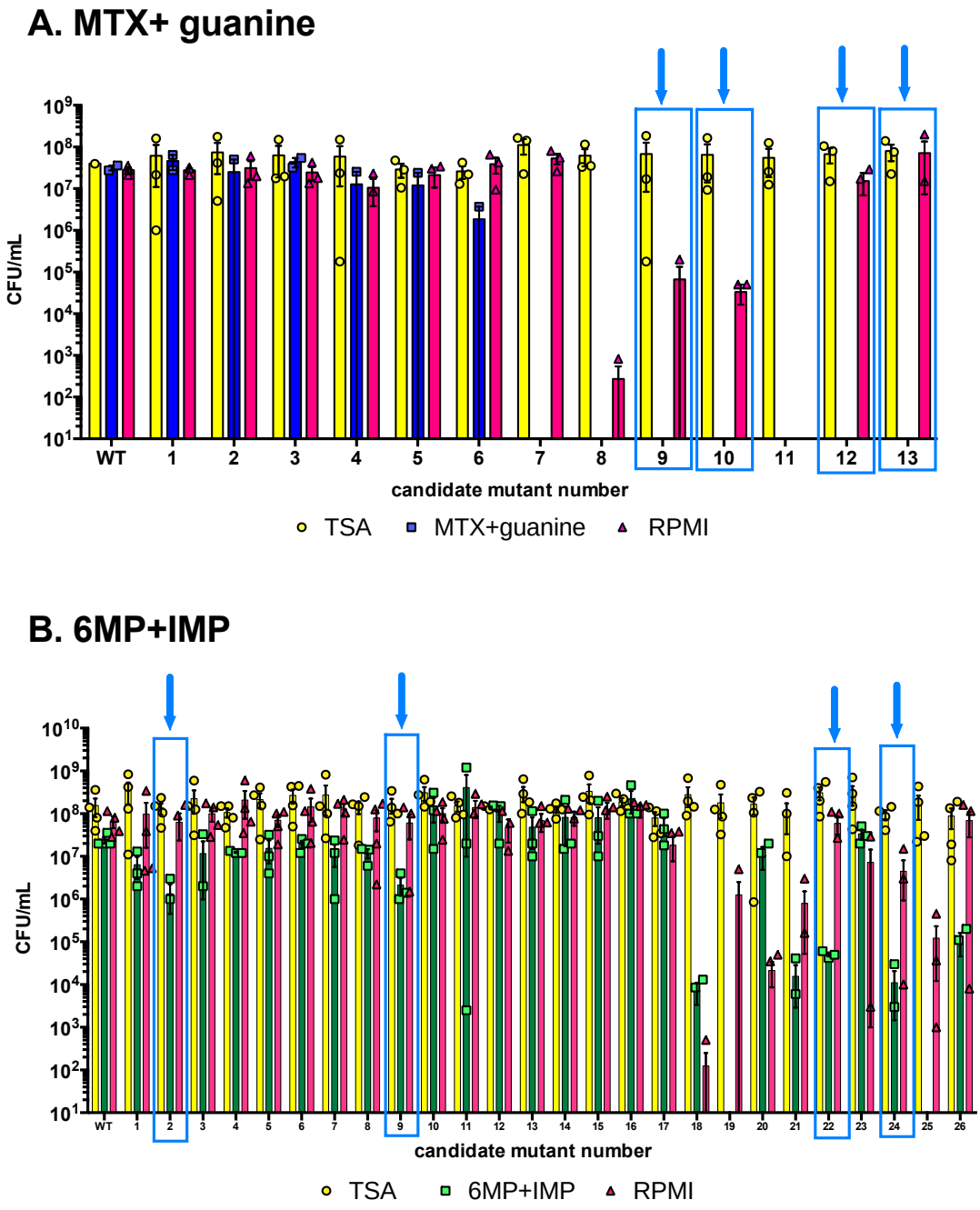


Fig. 16: CFU/mL of *S. aureus* mutants following the final screen for candidate mutants of purine transporter on grown in TSA, RPMI and selective plates. Each strain was grown in TSB, shaken overnight at 37˚C, diluted to an OD_{600} of 0.1 and further diluted to 10^{-5}. 10 µl drops of each dilution were plated in the 3 types of plates, as indicated in the legend. TSA plates were incubated for 24 hours while the rest were incubated for 72 hours. Graph shows the average CFU/mL from three independent experiments. **A.** In MTX+ guanine selective condition, arrows indicate 4 final candidate mutants (Table 3A.) out of 13 mutants isolated from MTX+ guanine. **B.** In 6MP+IMP selective condition, arrows indicate 4 final candidate mutants (Table 3B) from 6MP+IMP.

Table 6. Final 6 candidate mutants from screening of the Nebraska Transposon Mutant library using MTX+ guanine selective condition.

Candidate mutant from MTX+guanine	Gene name	Gene description	Accession number
6	-	transcription regulatory protein	SAUSA300_2326
7	-	hypothetical protein	SAUSA300_1692
9	-	ABC transporter substrate-binding protein	SAUSA300_0618
10	-	ABC transporter permease	SAUSA300_0619
12	*aroC*	chorismate synthase (SCV)	SAUSA300_1357
13	*alr*	alanine racemase (SCV)	SAUSA300_2027

Table 7. Final 4 candidate genes from screening of the Nebraska Transposon Mutant library using 6MP+IMP selective condition

Candidate mutant from 6MP+IMP	Gene name	Gene description	Accession number
2	-	aldose 1-epimerase	SAUSA300_2285
9	-	Methylenetetrahydrofolatedehydrogenase/ methenyltetrahydrofolate cyclohydrolase	SAUSA300_0965
22	-	ABC transporter substrate-binding protein	SAUSA300_0618
24	*alr*	alanine racemase (SCV)	SAUSA300_2027

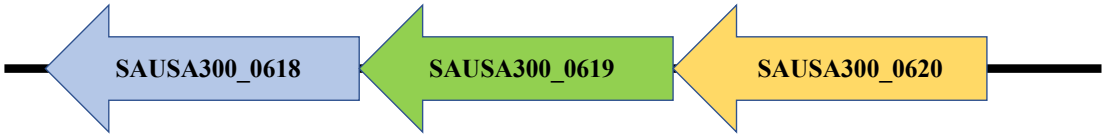


Fig. 17: The ATP-Binding Cassette (ABC) transporter operon of *S. aureus*. The operon is approximately 3 kb in size and consists of three genes; SAUSA300_0618 encoding for the substrate-binding protein, SAUSA300_619 encoding for permease and SAUSA300_620 encoding for ATP-binding protein of the ABC transporter.

3.5 The growth deficiency of *0618*::Tn mutant can be partially complemented.

As the next step in identifying the purine transporter, I sought to confirm the identity of our mutants of interest using PCR. Since I could identify the location of the transposon in the three mutants of interest that I identified from our screen, I performed a diagnostic PCR to ensure that the expected gene indeed carried an inserted transposon (data not shown).

Upon confirmation of the mutated genes, I next sought to evaluate whether the growth defect in MTX+guanine and 6MP+IMP of the *0618::Tn* mutant could be rescued by providing the gene *in trans* via plasmid complementation of the mutant. To do so, I PCR amplified the entire ABC transporter operon from WT *S. aureus* USA300 and cloned the resulting amplicon into the *S. aureus* expression plasmid pALC2073 (or pALC thereafter). The resulting plasmid named *p0618-0620* encoding the ABC transporter operon was introduced into the transposon mutants of interest along with the empty parental plasmid as a vector control.

To quantify the growth of each strain, WT carrying pALC and the mutants carrying either the empty plasmid or the complementation plasmid *p0618-0620* were grown in biological duplicates overnight in TSB , standardized to an OD_{600} of 1.0, serially diluted and drop plated onto MTX+guanine so that growth of each strain could be enumerated. As demonstrated in Fig. 18A, *0618::Tn pALC* cannot grow on MTX+ guanine; however, *0618*::Tn p0618-0620 significantly improved growth however growth was not restored to WT levels. This indicates that providing the operon *in trans* partially complements the mutant. In contrast to *0618::Tn,* the strains *0619::Tn* (pALC) and *0620::Tn*(pALC) nor the complemented strains, showed any growth defect in MTX+guanine as compared to WT pALC (Fig. 18B and C). These data indicate that these two genes are dispensible under growth in the selective conditions. Altogether, these data indicate that the *0618::Tn* mutant has a defect in presence of MTX or 6MP, and growth can be partially complemented by the addition of the operon *in trans*.

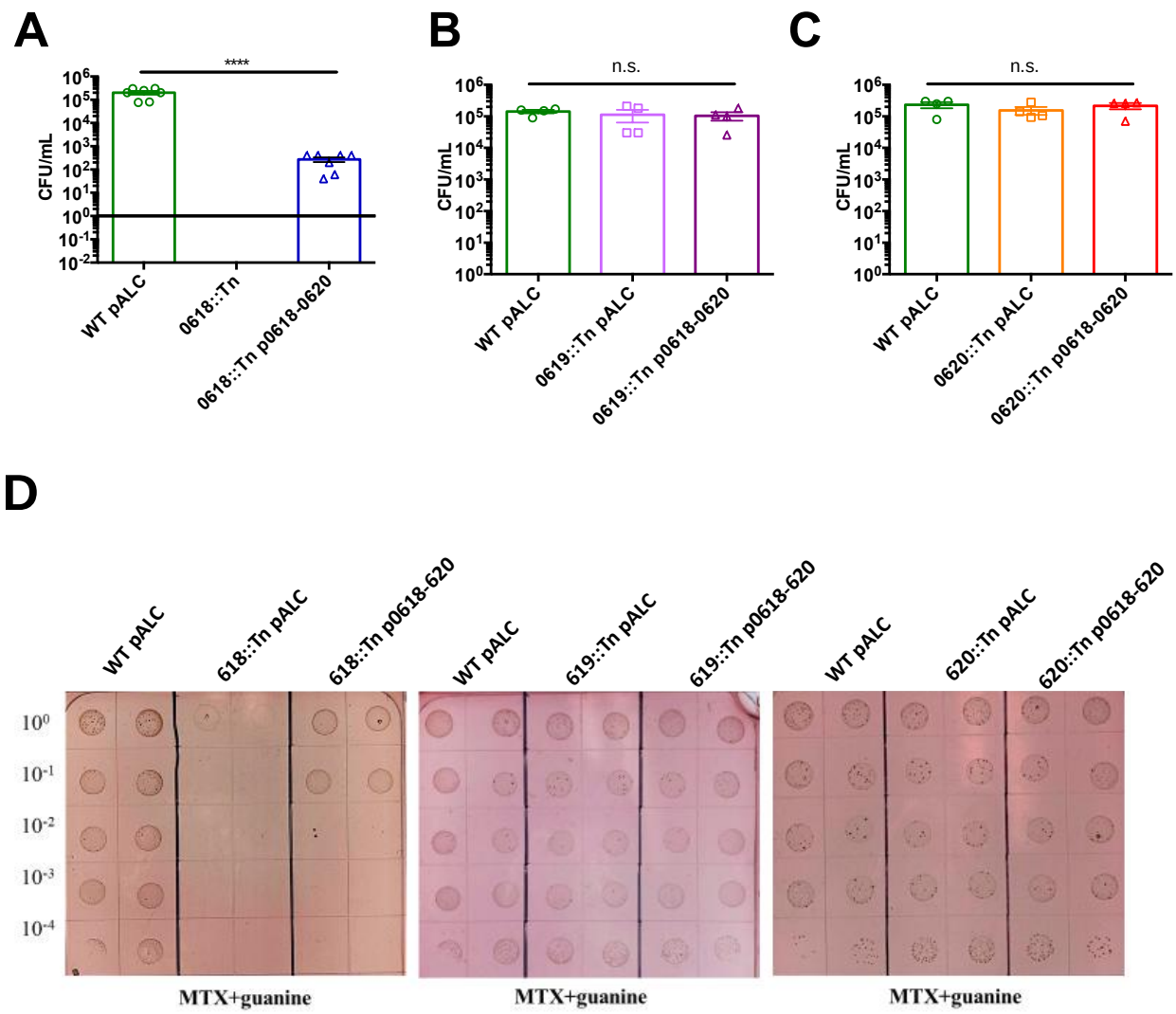


Fig. 18: Growth quantification by CFU/mL of each *S. aureus* mutant and their complemented strains. WT pALC and mutants *0618::Tn* pALC (**A.**), *0619::Tn* pALC (**B.**) and *0620::Tn* pALC **(C.)** and respective complemented mutants *0618::Tn* p*0618-0620*, *0619::Tn p0618-0620* and *0620::Tn p0618-0620* were grown in TSB in duplicates, shaken overnight at 37˚C, diluted to an OD_{600} of 1 and further diluted to 10^{-4}. **(D.)** From two biological replicate cultures, 10 µl drops of each dilution were plated on MTX+guanine plates corresponding to each strains below.

3.6. Partial complementation of *0618::Tn p0618-0620* is not due to plasmid instability.

Because I observed partial complementation of growth under MTX+ guanine in the complemented strain of *0618::Tn p0618-0620,* I wondered if this incomplete growth rescue was due to plasmid instability. Specifically, the presence of an inhibitory drug such as MTX, plasmid loss may occur due to stress imparted on the bacteria when there is no selective pressure. To investigate whether partial complementation in Fig. 18A was due to plasmid instability, I conducted a plasmid stability check by scraping WT (pALC) colonies from a MTX+ guanine plate, serially diluting the bacteria in saline solution and plating the bacteria onto TSA with or without chloramphenicol as the plasmid pALC2073 carries a *cat* (chloramphenicol acetyltransferase) gene. As shown in Fig. 19, MTX has no effect in plasmid stability as WT (pALC) grew to the same extent in in the presence and absence of chloramphenicol.

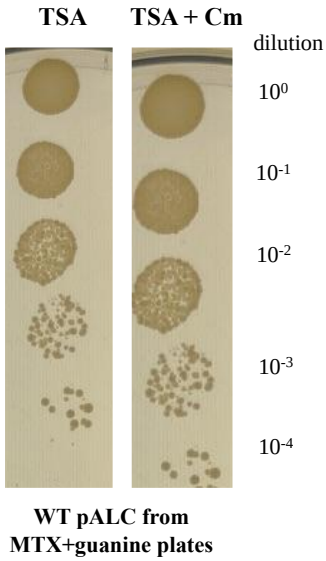


Fig. 19: Partial growth rescue is not due to plasmid instability. A streak of WT pALC from MTX+ guanine plates were deposited in 200 µl normal saline solution. Five 10-fold dilutions were made and 10 µl of each dilution were plated on a TSA and a TSA+ chloramphenicol plates. Following 24 hours incubation at 37 ℃, colony growth on each plate are displayed above.

3.7 The identity of SAUSA300_0618 gene is *mntC* which encodes a Mn-binding lipoprotein of the ATP-binding cassette transporter, MntABC

3.7.1 MntC does not play a role in purine transport

At this point of my study, I confirmed that the mutant of interest, SAUSA300_0618 or *0618::Tn*, has a mutation in the *mntC* gene which encodes the Mn-binding lipoprotein that is part the ATP-binding cassette type transporter for Mn called MntABC. Although the original objective was to identify a purine transporter in *S. aureus*, we ended up identifying a gene that has been classified as a Mn transporter instead. Nonetheless, it is possible that MntC is still playing an indirect role in purine import because we observed a robust growth defect of *mntC*::Tn mutant from both inhibitor + purine conditions and the growth defect could be rescued partially by providing the gene *in trans*. Furthermore, since MntC has been established as a prominent Mn transporter and an important virulence factor in *S. aureus*, we wondered if Mn imported from MntC is needed for the import or utilization of purines. To answer this question, we next investigated the effect of Mn and Fe (also a substrate of MntC) for growth of the *mntC*::Tn mutant in MTX + IMP.

When grown in Mn-rich conditions, *mntC::Tn* is able to grow in MTX+IMP in a dose-dependent manner while the addition of Mn to MTX alone does not rescue *mntC*::Tn growth (due to inhibited *de novo* purine synthesis and lack of exogeneous purines, Fig. 20A). In the presence of 10 µM Mn, growth of *mntC*::Tn in RPMI with MTX+IMP is close to the uninhibited condition. In contrast, the addition of iron only partially rescued the growth defect of the *mntC*::Tn mutant in the presence of MTX+IMP (Fig. 20B). At 100 µM of Fe, *mntC*::Tn still exhibit a slight growth defect that was complemented when 10 µM of Mn was supplied. Therefore, it appears that disruption of *mntC*, which affects Mn transport, results in increased sensitivity to MTX and this can be ameliorated through provision of exogenous Mn (and to some effect Fe) .

From the prominent role MntC in Mn import and the fact that *mntC*::Tn growth defect in purine-rich condition can be corrected with addition of Mn, I hypothesize that Mn transported by MntC may have a role in purine transport and/or utilization.

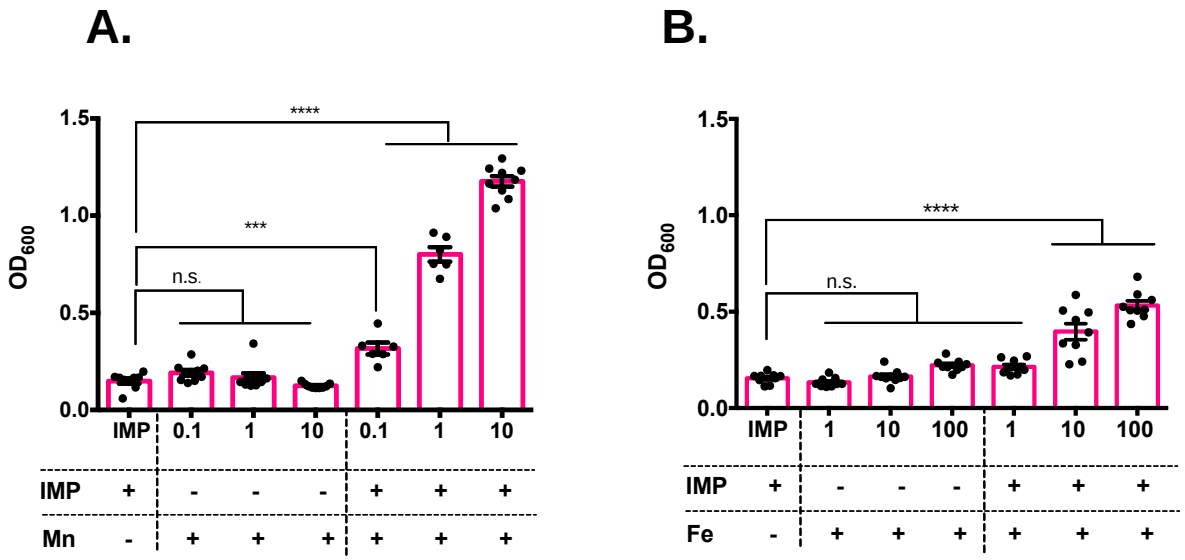


Fig. 20: MntC is not a purine transporter as growth defect in MTX+IMP can be complemented with Mn. *mntC*::Tn was grown in RPMI for 24 hours in MTX+IMP with addition of $MnCl_2$ (A) and $FeCl_3$(B) at specified final concentrations. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *$p\leq0.05$ one-way ANOVA multiple comparisons.

3.8 Purification a recombinant MntC protein and its polyclonal rabbit antibody

Since confirming the identity of our gene of interest as *mntC* and that it is not a purine transporter, I shifted the focus of my research aims in an attempt to understand the role of Mn utilization in purine dependent growth of *S. aureus*. To begin, I sought to analyze the expression of MntC in WT *S. aureus* and in purine-deficient strains such as *purK*::Tn which are reliant on exogenous purines for growth. I hypothesized that if Mn binding and/or transport by MntC participates in purine import, we may see differences in MntC expression using these strains.

At this point, I had generated a recombinant MntC protein using the steps outlined in Fig. 21 so that I could test whether it could bind to purine substrates by using surface plasmon resonance technique. However, MntC does not bind purines.

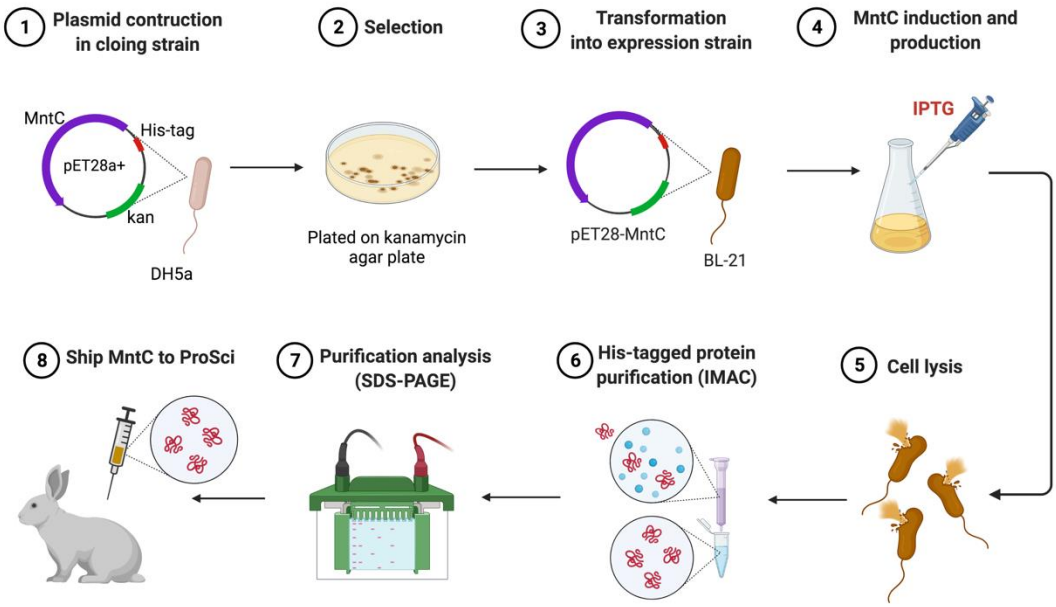


Fig. 21: Steps in purification of recombinant MntC. The nucleotide sequence of MntC (minus the signal peptide) was obtained commercially and cloned into pET28a+ expression vector. Then, the pET28a+-MntC vector was transformed into competent DH5-alpha cells and subsequently, transformed into BL-21 for overexpression of MntC under induction of IPTG. His-tagged MntC was purified with immobilized metal affinity chromatography (IMAC), analyzed with SDS-PAGE and sent to ProSci for anti-MntC rabbit polyclonal antibody generation.

To this end, I then used the purified recombinant MntC (Fig. 22A) to commercially obtain an anti-MntC rabbit polyclonal antibody as the first step to analyze expressions of MntC in Mn- and purine-rich conditions. Once I received the anti-MntC antisera from a rabbit immunized with the recombinant MntC, I sought to verify the functionality of the anti-MntC antibody. Here, western blot experiments were performed using the rabbit antisera as a primary antibody. A clear distinct band was observed at 33 kDa demonstrating that the anti-MntC recognized the recombinant MntC (Fig. 22B, lane 2). On that account, I have confirmed that our anti-MntC antibody can effectively bind to MntC.

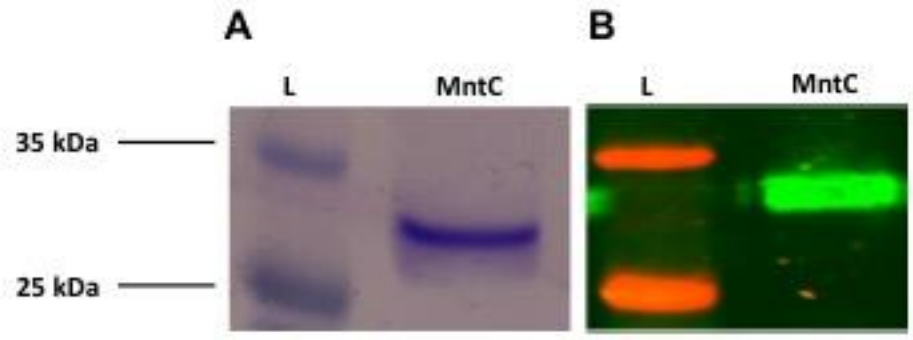


Fig. 22: Analysis of purification of recombinant MntC and verification of the generated anti-MntC polyclonal rabbit antibody. A. 5 µL of purified MntC was run on 12% SDS-PAGE gel and stained with Instant Blue dye. B. A western Blot of purified MntC was conducted using the antiserum from a rabbit immunized with MntC.

3.9 Investigating the role of MntC in purine transport and/or utilization

3.9.1 Mutant lacking *mntC* and *purK* shows similar growth to single *mntC::Tn* and *purK::Tn* in a purine-rich condition

Although I was able to rescue the growth defect of *mntC*::Tn mutant in MTX+IMP by adding Mn to the growth medium, I questioned whether Mn bound and transported through MntC would somehow affect purine import and/or utilization. In order to investigate the potential role of Mn in purine metabolism, I sought to create a double Δ*mntC purK*::Tn mutant and compare its growth to WT, *mntC*::Tn, and *purK*::Tn in different culture conditions. To do this I first created a markerless Δ*mntC* mutant through allelic replacement mutagenesis (with *pKOR1*). Then, using a phage lysate created from the *purK*::Tn mutant, the disrupted *purK* allele was transduced into the *ΔmntC* strain, creating the Δ*mntC purK*::Tn double mutant.

As expected of the purine-auxotrophic mutants, *purK*::Tn and Δ*mntC purK*::Tn failed to grow in RPMI unless exogenous IMP was provided (Fig. 23A). Moreover, in IMP-rich culture conditions all of the strains grew to the same level suggesting that IMP was transported into the cells irrespective of MntC expression. Interestingly, I observed a reproducible growth defect for the strains that were MntC deficient in the presence of MTX. This MntC-specific MTX sensitivity could suggest that MntC plays a role overcoming potential stresses imposed by MTX. Once again, I observed that Mn supplementation of the medium containing MTX+ IMP rescued the growth defect displayed the MntC-deficient strains (Fig. 23A).

Next, I wanted to determine whether there was a difference in MntC expression between WT and a strain such as *purK*::Tn that is auxotrophic for purines in MTX+IMP where growth for both strains requires purine import. To carry out this objective, I conducted a western blot on cell lysates of each strain grown in MTX+IMP and MTX+IMP+Mn. From this experiment, I indeed observed for the first time that I could in fact detect MntC expression from whole cell *S. aureus* lysates, in addition to recombinant MntC produced in *E. coli*. However, I saw no difference in MntC expression between WT and *purK*::Tn (Fig. 23B) and that MntC was downregulated in these strains in Mn-rich condition, as previously described in literature (Horsburgh et al., 2001).

Taken together, the findings from this study thus far and the established role of MntC in Mn import, suggest that MntC does not participate in purine utilization in *S. aureus* but that disruption of *mntC* leads to hypersensitivity to MTX ostensibly because of altered Mn homeostasis. Therefore, I changed our focus to try to understand the relationship between MntC and Mn transport in coping with stresses imposed by MTX.

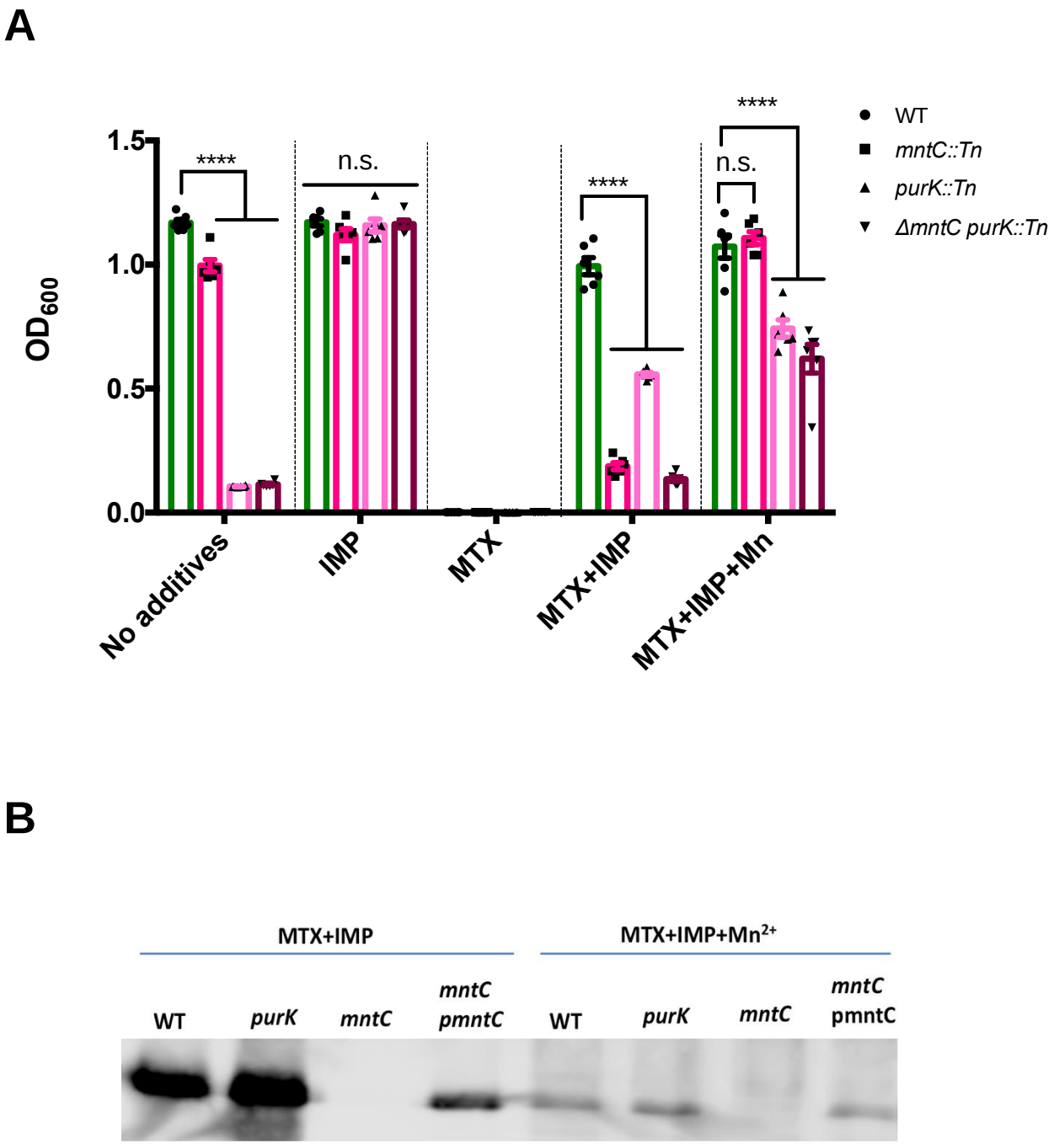


Fig. 23: MntC does not participate in purine import and/or utilization of *S. aureus*. **A.** *S. aureus* WT, *mntC*::Tn, *purK*::Tn and *ΔmntC purK*::Tn were grown in RPMI with addition of MTX, IMP and MTX as described in each condition for 24 hours. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, ANOVA multiple comparisons. **B.** Whole-cell lysates of WT, *purK*::Tn, Δ*mntC* and Δ*mntC pmntC* grown in MTX+IMP and MTX+IMP+Mn for 24 hours were normalized to an OD_{600} of 1.0 and used to performed western blot.

3.10 Defect in MntC renders *S. aureus* more sensitive to MTX as Mn homeostasis is disturbed

3.10.1 Growth of Δ*mntC mntH*::Tn is dependent on Mn availability

To study how *S. aureus* utilizes Mn transporters to cope with MTX stress, I needed to construct a strain that is completely deficient for Mn uptake. As *S. aureus* possesses two high-affinity Mn transporters – MntABC and MntH, Icreated a mutant lacking both of these genes. As I have already confirmed that Δ*mntC* created with allelic replacement mutagenesis was MntC-defective, I next generated Δ*mntC mntH*::Tn strain through phage transduction. Using this strain deficient for high affinity Mn transport I sought to demonstrate that the growth is dependent on Mn availability. To create metal free medium I used Chelex-100 resin to remove any trace metals in a chemically defined medium (CDM) that is not RPMI (Horsburgh et al., 2002a).

In metal-free CDM, WT *S. aureus* grows to an OD_{600} of about 0.7 while Δ*mntC mntH*::Tn cannot grow. Upon supplementation of this medium with increasing concentrations of Mn, I observed improved growth of WT and Δ*mntC mntH*::Tn strain in a dose-dependent manner (Fig. 24A). And at 10 μM of Mn, both strains grew to the same level after 24 hours. Interestingly, I saw that at low Mn concentrations, Δ*mntC mntH*::Tn cannot grow to WT levels. This suggests that *S. aureus* is using MntC and MntH to import Mn when there is low Mn available. However, when Mn is abundant, Δ*mntC mntH*::Tn can grow to WT levels indicating that *S. aureus* must be importing Mn through other low-affinity transporters that only function in high Mn environment.

Next, I sought to examine the growth kinetics profile of Δ*mntC mntH*::Tn compared to WT by conducting a growth experiment where strains were grown in metals-free CDM with and without Mn and the OD_{600} values were measured every 2 hours for 24 hours. This experiment allowed me to closely monitor the growth kinetics of these strains in a manner that cannot be accomplished by measuring end-point values. In metal-free CDM, I observed a distinct and significant growth defect of Δ*mntC mntH*::Tn compared to WT (Fig. 24B) throughout the 24-hour growth.

When 10 μM of Mn was supplied, I saw that the growth defect of Δ*mntC mntH*::Tn had diminished and that it reached the same level as WT growth for all time points (Fig. 24C). This suggests that Δ*mntC mntH*::Tn does not experience a growth delay compared to WT in Mn-

rich conditions. Additionally, the end-point OD_{600} in the growth experiment also reflected similar values as observed in the last condition of Fig. 24A. In addition to the fact that Δ*mntC mntH*::Tn does not grow in metals-free CDM, I observed that WT grew at a faster rate in Mn-rich condition than in Mn-deplete. Therefore, I also observed that Mn had a positive effect on the doubling time of *S. aureus*. Taken together, I established that the growth defect of Δ*mntC mntH*::Tn is Mn-dependent and its growth can be rescued by exogenous Mn.

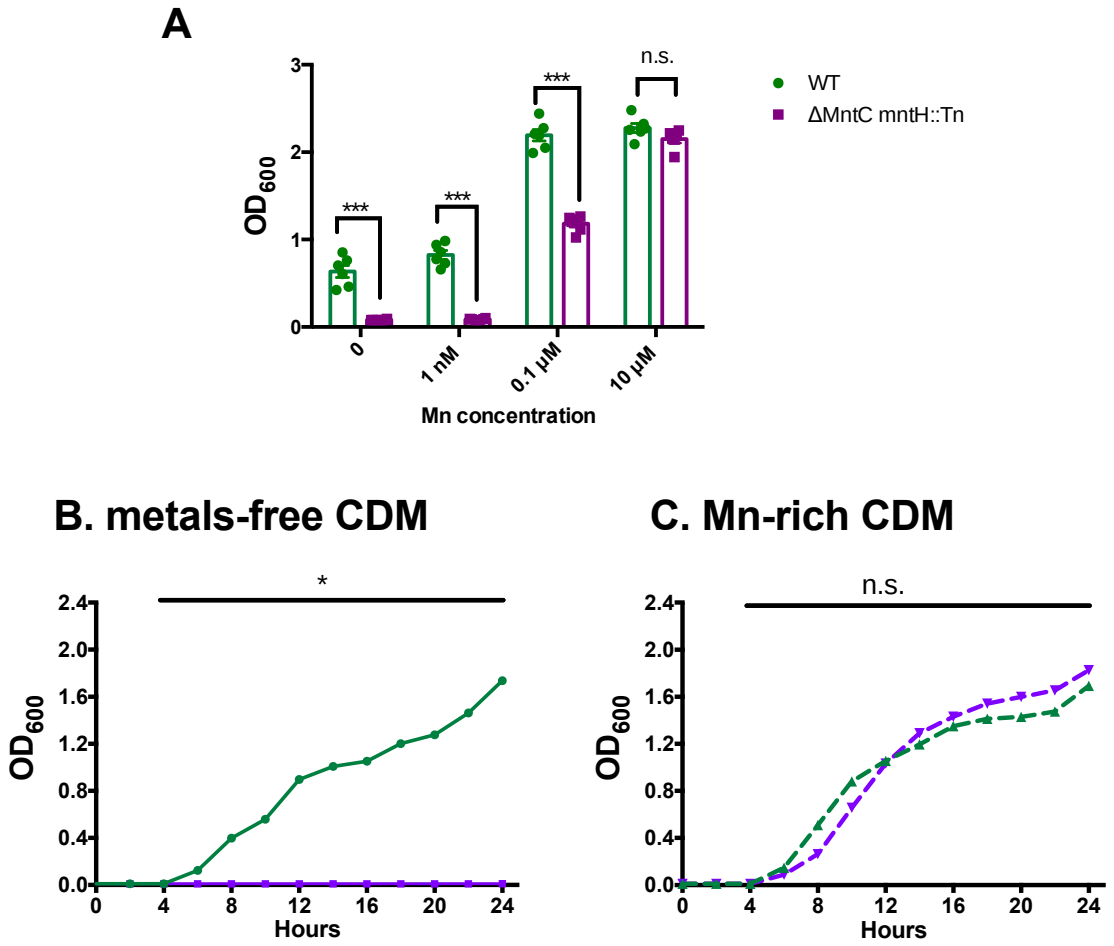


Fig. 24: Growth defect of Δ*mntC mntH*::Tn in metal-free media can be rescued to WT level with addition of Mn in media. *S. aureus* WT, and Δ*mntC mntH*::Tn were grown in metals-free CDM with $MnCl_2$ at specified concentrations for 24 hours (pre-cultured in CDM). A. Growth assay was conducted and end point OD_{600} were taken in with 6 biological replicates. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *$p\leq0.05$ one-way ANOVA multiple comparisons. B. and C. Growth curve was conducted and OD_{600} values were measured every 2 hours for 24 hours. Data are plotted as mean, 3 biological replicates. *** $p<0.001$, *$p\leq0.05$ Multiple t-tests.

3.10.2 Function of MntC, but not MntH, is important for coping with MTX

Up until this point, I had speculated that sensitivity to MTX of *mntC*::Tn was due to disrupted Mn homeostasis and demonstrated that chelexed-CDM can be used as a medium to analyze growth in different Mn concentrations. Next, I wondered whether only MntC or both of the Mn transporters are important for resisting MTX intoxication. In screening of the NTML for the purine transporter, I showed that *mntC*::Tn, not *mntH*::Tn, exhibited a growth defect in presence of MTX and 6MP which indicates *mntC* deficient *S. aureus* may be selectively hypersensitive to these inhibitors.

To investigate this, I conducted experiments to determine the MIC for MTX using WT, *mntC*::Tn, *ΔmntC mntH*::Tn and *mntH*::Tn in CDM. These experiments revealed that the increased sensitivity to MTX is only evident in *mntC*-deficient strains and that *mntH*::Tn mutant shows near WT growth at sub-MIC concentrations of MTX (Fig. 25). Therefore, *mntC* alone contributes to the ability of *S. aureus* to cope with MTX intoxication.

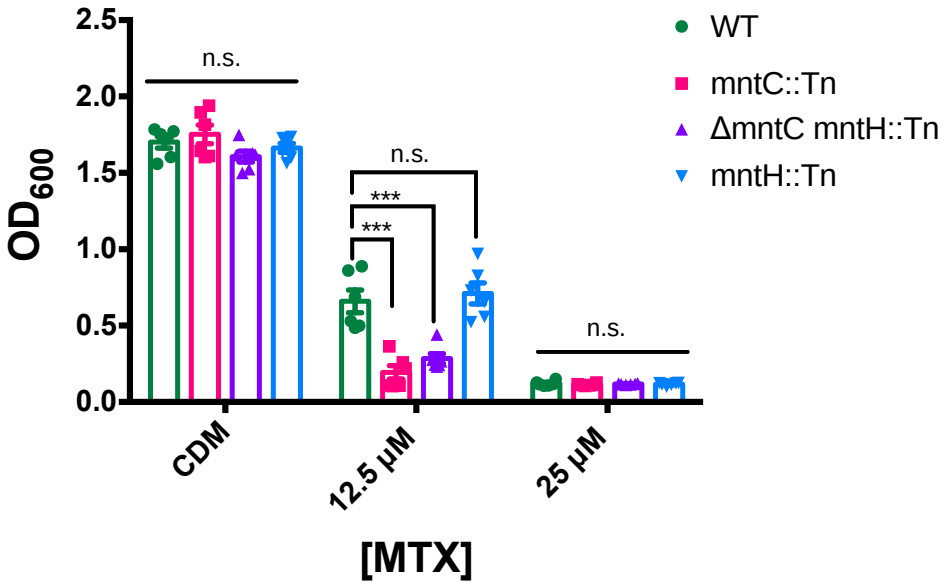


Fig. 25: *S. aureus* with defect in MntC shows increased sensitivity to MTX. *S. aureus* WT, *mntC*::Tn, *ΔmntC mntH*::Tn and *mntH*::Tn were grown in CDM in presence of MTX for 24 hours. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *$p\leq0.05$ one-way ANOVA multiple comparisons.

3.11. Exogenous Mn can quench reactive oxygen species generated by MTX, allowing for increased cell survival

3.11.1 *S. aureus* lacking both superoxide dismutases shows a growth defect in TSB and CDM

Having established that MntC is important for *S. aureus* to cope with stress imposed by MTX, I next sought to understand how MTX might be affecting *S. aureus* in such a way that Mn transport through MntC improves bacterial growth. Previous work by Gao and Horie, 2002 reported that MTX induces ROS in the intestinal mucosa of rats. Moreover, recent reports have demonstrated that MTX causes a reduction in antioxidant enzymes in eukaryotic cells which sensitizes MTX treated cells to ROS (Herman, Zurgil, and Deutsch, 2005; Phillips, Woollard, and Griffiths, 2003). Taken together these observations might suggest that MTX either induces ROS in *S. aureus* or could sensitizes the bacteria to ROS. Interestingly, it is also known that Mn, transported through MntC, is a co-factor that is used by the superoxide dismutases SodA and SodM in *S. aureus*. SODs breakdown superoxide which can be produced through the metabolic activity of cells (Clements, Watson, and Foster, 1999), the phagocyte oxidative bursts (Garcia et al., 2017b), or through exposure to chemical reagents such as methyl viologen (Kehl-Fie et al., 2013; Handke et al., 2013). Although MTX has not been shown to directly generate superoxide in bacterial cells, I hypothesized that MTX is inducing ROS in *S. aureus* and that *mntC* deficient bacteria display MTX hypersensitivity because the function of SodA and SodM is impaired due to aberrant Mn transport.

To address this hypothesis, I created superoxide dismutase mutants lacking either *sodA, sod*M or both *sodA and sodM*. Next, I analyzed the growth of these SOD mutants in rich-medium (TSB), chelex CDM and Mn-rich media (chelex-CDM supplied with 10 μM Mn). In TSB, I observed that the double mutant lacking *sodA* and *sodM* displayed a significant growth defect after 24 hours whereas endpoint growth of single SOD mutants was comparable to WT (Fig. 20A). Analysis of the growth kinetics of these mutants in TSB by performing growth curves indicated that the *sodAM*::Tn double mutant exhibited a distinct growth defect at all growth phases (Fig. 20B) while the growth of single *sodA*::Tn and *sodM*::Tn were comparable to WT.

I next characterized the growth kinetics of these SOD mutants in metal-free CDM, a condition where Mn-dependent growth of Δ*mntC mntH*::Tn is evident (see Fig. 24).

Interestingly, in chelexed-CDM *S. aureus* lacking *sodA* and *sodM* failed to grow and this defect can be rescued by Mn supplementation (Fig. 20C). Under these same condition single mutants deficient for either *sodA* or *sodM* grew similar to WT bacteria. To further investigate the growth kinetics of these strains, I also conducted a growth curve experiment in non-chelexed CDM to allow for growth of *sodAM*::Tn. Here, I observed a significant growth defect of *sodAM*::Tn in CDM compared to WT (Fig. 26D) throughout 24 hours.

These findings suggest that *sodAM*::Tn exhibits a growth defect in Mn-rich (TSB and Mn-supplied CDM), trace Mn (non-chelexed CDM) and Mn-deplete conditions (metals-free CDM). Moreover, a defect in *sodA* leads to a growth defect in trace Mn condition while defect in *sodM* allows for increased growth.

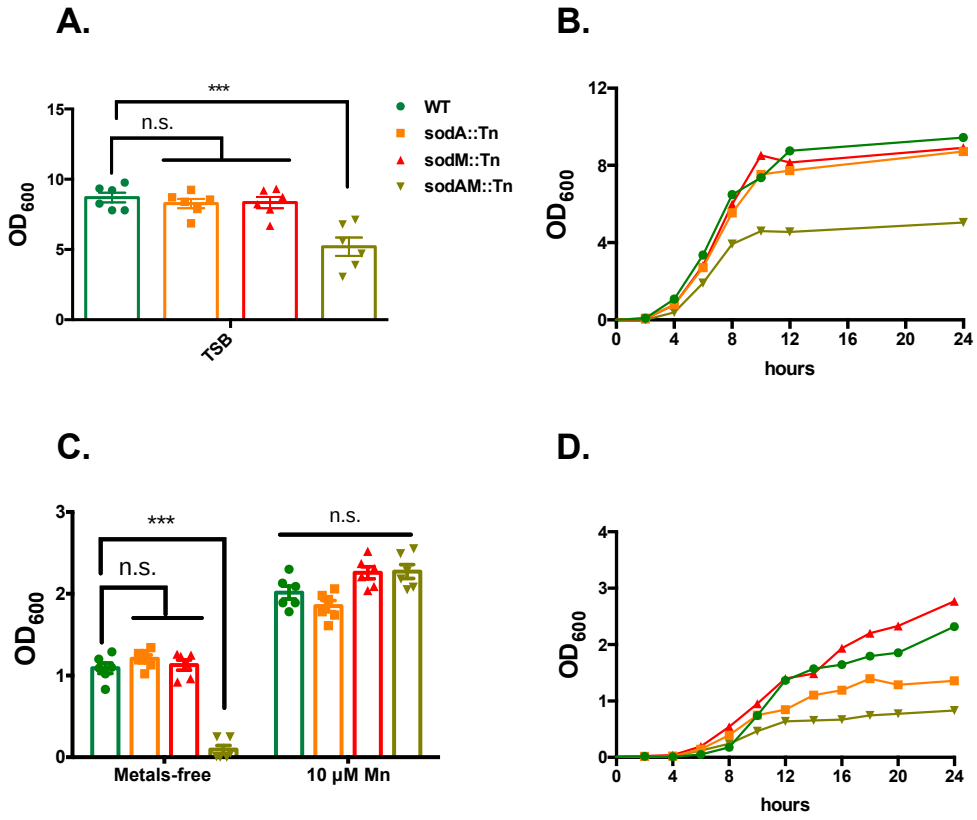


Fig. 26: Growth assessment of *sodA::Tn*, *sodM::Tn* and *sodAM::Tn* in TSB and in CDM. *S. aureus* WT, *sodA*::Tn, *sodM*::Tn and *sodAM*::Tn were grown in for 24 hours in TSB (A) and metal-free CDM (C) and end-point OD_{600} values were measured. Data are plotted as mean ± SEM, six biological replicates. **** $p<0.001$, *$p\leq0.05$ one-way ANOVA multiple comparisons. Growth curve experiments of these strains were conducted in TSB (B) and non-chelexed CDM (D) where OD_{600} values were measured every 2 hours for 24 hours. Data are plotted as mean, three biological replicates. *** $p<0.001$, *$p\leq0.05$ Multiple t-tests.

3.11.2 Exogenous Mn can correct the growth defect of *sodA::Tn* that renders cells more sensitive to reactive oxygen species caused by MTX, 6MP and methyl viologen (M.V.)

Now that I have established the baseline growth of *sodA*::Tn, *sodM*::Tn and *sodAM*::Tn in TSB and CDM, I sought to test the effect of MTX on growth of these strains. Since I also saw that disrupted Mn homeostasis causes hypersensitivity to 6MP in MntC-deficient strains, we included 6MP in subsequent experiments. Since I speculated that 6MP and MTX are not only inhibiting *de novo* purine biosynthesis, but also generating ROS to inhibit *S. aureus*, methyl viologen (M.V.) was included in this experiment as a positive control for ROS generation.

To assess the role of *sodA* and *sodM* in coping with ROS, I grew WT, *sodA::Tn*, *sodM*::Tn and *sodAM*::Tn in sub-MIC of MTX, 6MP and M.V. in CDM with and without the addition of Mn. Additionally, I included Δ*mntC mntH*::Tn as a control for *S. aureus* lacking high-affinity Mn transporters. When subjected to MTX (Fig. 27A), I observed a robust growth defect for *sodA*::Tn, *sodAM*::Tn and Δ*mntC mntH*::Tn bacteria as compared the no MTX condition. The *sodM::Tn* mutant also showed significant growth defect in presence of MTX. Importantly, at this MTX concentration WT was not inhibited. This finding suggests that *S. aureus* relies on SodA as an important dismutase that plays a role in the presence of MTX. Remarkably, addition of Mn rescued the defect in all strains, and growth of *sodM*::Tn, *sodAM*::Tn and Δ*mntC mntH*::Tn actually surpassed their uninhibited growth. Furthermore, this finding also recapitulates the hypersensitivity of Δ*mntC mntH*::Tn to MTX observed in RPMI (Fig. 25).

In the presence of 6MP, I did not observe a *sodA*-specific growth defect despite using high concentrations of the drug. Addition of Mn showed increased growth in all strains except for *sodAM*::Tn where its growth remained the same for uninhibited, 6MP and 6MP+Mn (Fig. 27B). These observations suggest 6MP and MTX are having different effects on *S. aureus*. Because both MTX and 6MP inhibit *de novo* purine biosynthesis, the growth defect that can be rescued by Mn we observed (in Fig. 27A and B) are not likely due to purine deficiency but rather the differences of ROS generated.

Lastly, in the presence of M.V., I observed significantly reduced growth in all strains compared to their growth in the untreated condition and that addition of Mn boost their growth to surpass untreated condition (Fig. 27C). Taken together, these findings suggest that increased

Mn availability in the media ameliorates the oxidative stress imposed by these compounds and that SodA plays an important role in resisting oxidative stress.

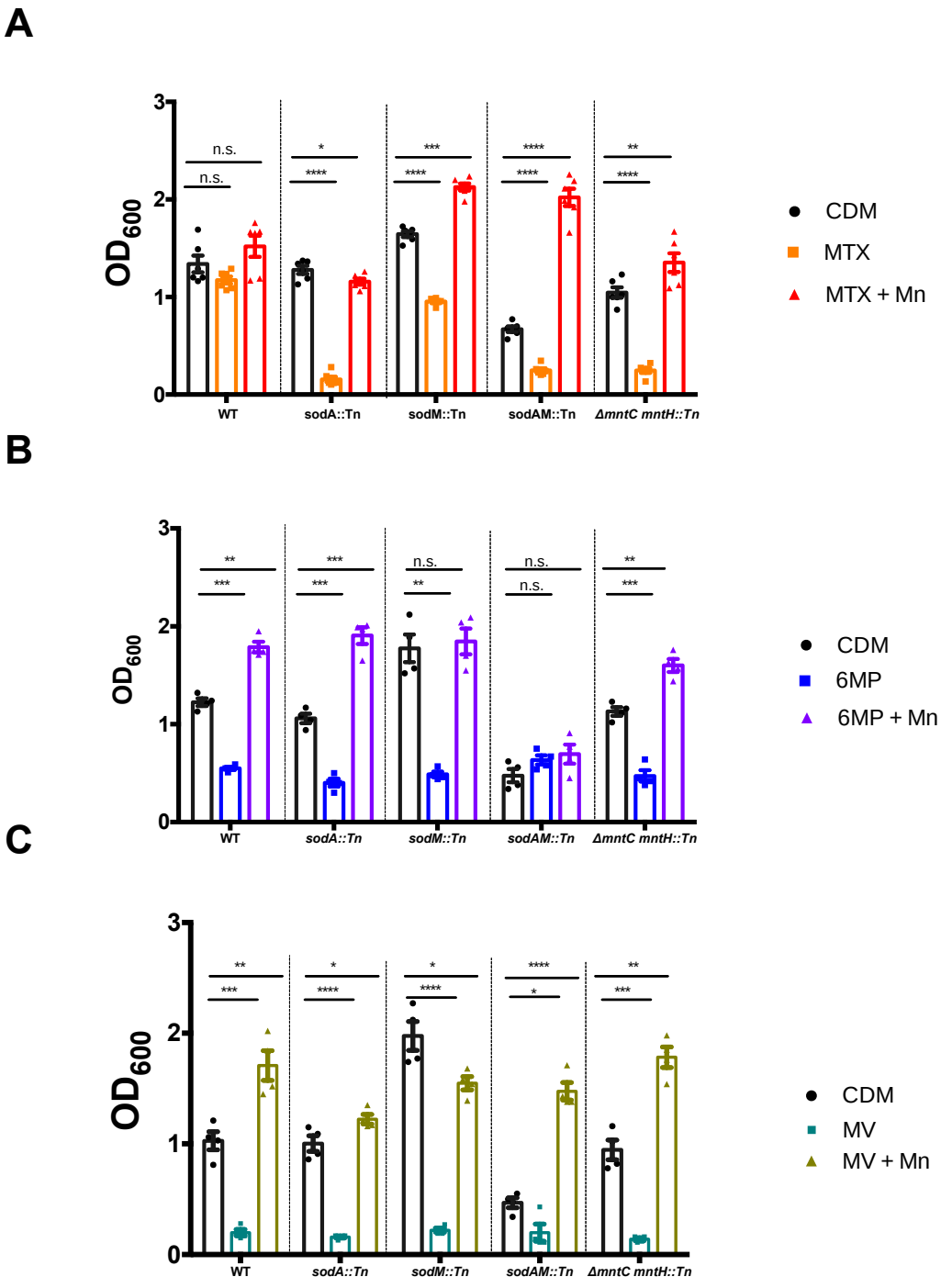


Fig. 27: Exogenous Mn can rescue growth of dismutase mutants inhibited by MTX, 6MP and M.V. *S. aureus* WT, *sodA*::Tn, *sodM*::Tn and *sodAM*::Tn were grown in non-chelexed CDM with MTX, 6MP and M.V. at specified concentrations for 24 hours. Data are plotted as mean ± SEM, four biological replicates, 2 replicate experiments. **** $p<0.001$, *$p \leq 0.05$ one-way ANOVA Multiple comparisons where end-point OD_{600} values were measured.

3.11.2 MTX and M.V. inhibit *S. aureus* growth through bactericidal mechanism while 6MP is bacteriostatic.

Now that I have established that Mn allows *S. aureus* to grow in the presence of MTX and 6MP, I next sought to determine whether these compounds inhibit *S. aureus* via bactericidal or bacteriostatic interactions. To carry out this aim, I conducted a cell viability assay where CFU/mL of each strain was enumerated (by drop-plating on TSA without any selection) at the initial inoculum and after 24 hours of growth in the presence of MTX, 6MP and M.V. and with Mn. By comparing the initial CFU/mL values to the end-point CFU/mL grown in the presence of the compounds, I could determine whether the cells are killed or simply not growing.

In the presence of MTX, I observed reduced CFU/mL in all strains, with *sodAM*::Tn and Δ*mntC mntH*::Tn exhibiting a more severe phenotype at the end of 24-hour growth. But when Mn was supplemented into the media, the CFU/mL in all strains but *sodA*::Tn and Δ*mntC mntH*::Tn did not reduce over 24 hours (Fig. 28A). This finding suggests that SodA is important in coping with stresses imposed by MTX in high Mn conditions and that absence of high-affinity Mn transporter renders cells sensitive to killing by MTX.

While I observed that 6MP can inhibit growth of *S. aureus* as measured by OD_{600} in liquid cultures, 6MP did not reduce the CFU/mL at the end of 24-hour growth (Fig. 28B). Because there were fewer viable cells recovered at the end of the survival experiment in MTX and M.V. (Fig. 28C) than the number of cells at the input, this demonstrates that MTX and M.V. are bactericidal against *S. aureus*. Since M.V. is a potent inducer of oxidative stress, it is likely that MTX can also generate ROS which would explain why the MICs for MTX is lower against MntC-deficient and dismutase-deficient *S. aureus*. On the contrary, the same number of viable cells from growth in 6MP and without 6MP suggests that 6MP is bacteriostatic against *S. aureus.*

Interestingly, I observed that supplementation with exogenous Mn would abrogate killing in media with MTX and M.V. for WT, *sodM*::Tn and *sodAM*::Tn, but not *sodA*::Tn. Specifically, *sodA*::Tn was being killed regardless of whether Mn was added into the media. This finding suggest that *sodA* is important in Mn-rich conditions to combat oxidative stress. Additionally, the fact that I observed a robust growth of *sodAM*::Tn was rescued in an Mn-dependent manner in the presence of MTX and M.V. suggests that exogenous Mn could be neutralizing the superoxide generated by these compounds (by acting as an antioxidant) and

thus, ameliorating the oxidative stress and protecting the cells from killing without needing superoxide dismutases. To this end, I have characterized that MTX inhibit *S. aureus* bactericidally while 6MP does so bacteriostatically and that addition of Mn can potentially ameliorate inhibition of these compounds.

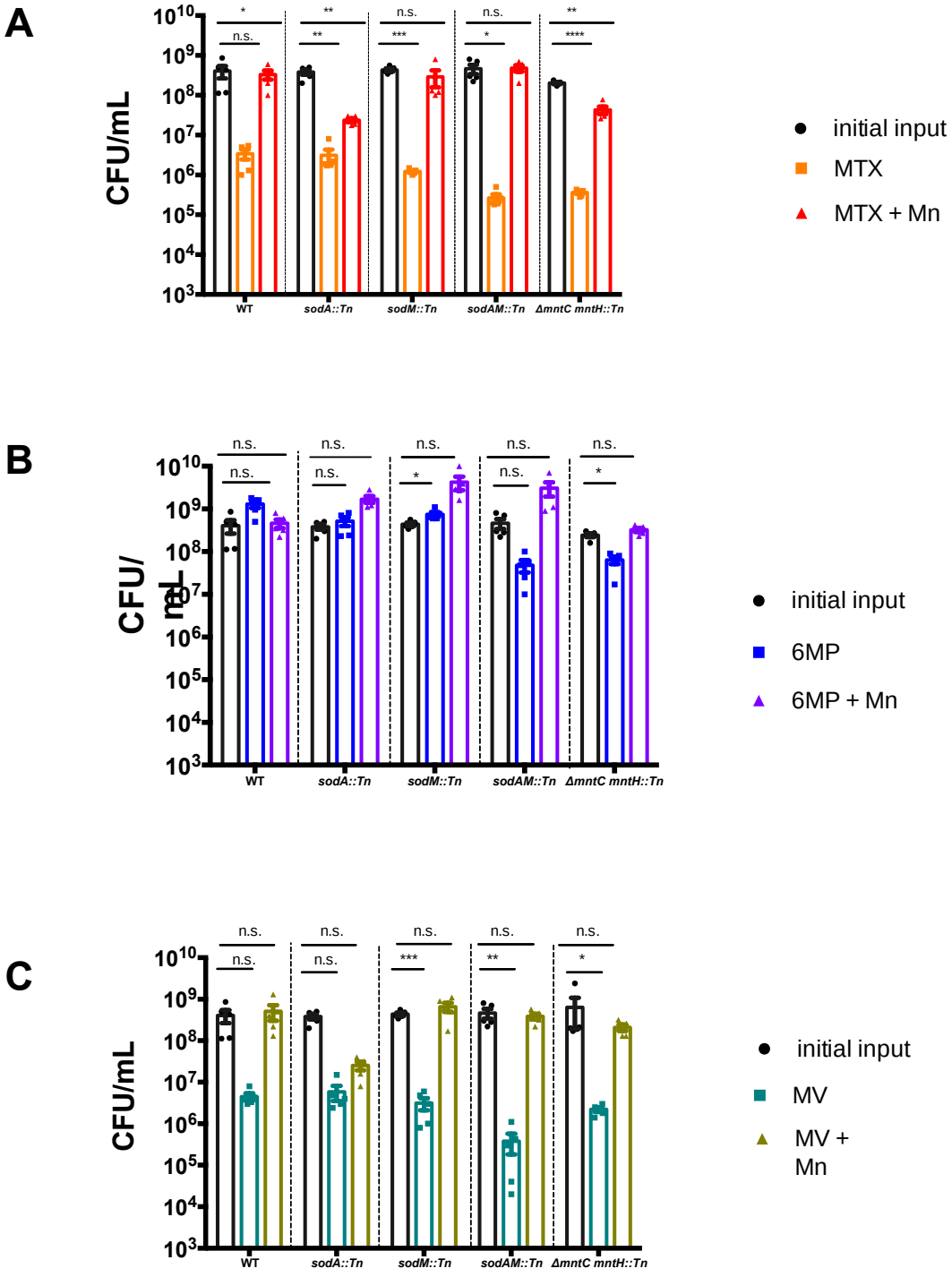


Fig. 28: MTX, 6MP and M.V. inhibits *S. aureus* by bacteriostatic mechanism which can be rescued by addition of Mn. S. aureus WT, *sodA*::Tn, *sodM*::Tn and *sodAM*::Tn were were grown in non-chelexed CDM with MTX, 6MP and M.V. at for 24 hours. Data are plotted as mean ± SEM, four biological replicates. **** $p<0.001$, *$p \leq 0.05$ one-way ANOVA multiple comparisons where end-point OD_{600} values were measured.

Chapter 4: Discussion and future directions

Infections caused by methicillin-resistant *S. aureus* (MRSA) has reached epidemic proportions (Engemann et al., 2003) and therapeutic options are increasingly limited because of antibiotic resistance. As a pathogen with a vast arsenal of virulence factors, *S. aureus* can cause invasive and life-threatening conditions. Although the host employs nutritional immunity to limit nutrient availability and the production of antimicrobial effectors such as ROS compounds to combat pathogens, *S. aureus* has various mechanisms to overcome restrictions imparted by the host. Given the significance of purines as energy-storage compounds, building blocks of nucleotide and in *S. aureus* pathogenesis, this study sought to identify genes encoding purine transporters. In doing so, I have identified a mutant lacking the gene *mntC*, which encodes a subunit of a high affinity Mn transporter, unable to grow in the presence of the inhibitors of purine biosynthesis, MTX and 6MP.

Because *mntC* has been demonstrated by many studies to acquire extracellular Mn and is important for Mn homeostasis under Mn deplete conditions, I investigated how Mn would affect growth of *S. aureus* bacteria in the presence of MTX and 6MP under Mn deplete and replete conditions. Remarkably, I found that supplementation of the growth medium with Mn rescued growth of *mntC S. aureus* in the presence of MTX or 6MP, indicating the bacteria were not growing as a result of not being able to transport purines. This prompted us to consider other ways in which purine inhibitors might be affecting the bacteria in addition to inhibiting purine biosynthesis. Given that Mn imported by MntC is known to play significant roles in *S. aureus* resisting ROS both *in vivo* generated by the host phagocytic cells[92,] or *in vitro* from experiments with ROS-inducing chemicals such as methyl viologen (Horsburgh et al., 2002b; Handke et al., 2013), it prompted me to consider that perhaps growth defects of *mntC* mutants in presence of MTX or 6MP were due to perturbed Mn homeostasis. This led me to investigate the roles of superoxide dismutases in presence of MTX and 6MP. Indeed, I found that *S. aureus* lacking SodA and both high affinity Mn transporter (*mntC* and *mntH*) showed increased killing by MTX and 6MP and that provision of extracellular Mn can abrogate the killing.

Although I did not characterize a novel purine transporter, other studies could refer to the list of putative purine transporter and further conduct growth assay using the established purine import (inhibitor + purine) conditions. However, it is important that the annotation of each mutant is taken into account. To decide whether a mutant could transport purines, it may

be worthwhile to investigate the upstream gene sequences of the putative genes whether or not it contains binding site for the repressor of the purine biosynthesis, such as a Pur box. As a purine importer, the function of the gene of interest would contribute to the intracellular purine pool. Therefore, it is not unreasonable to assume that its transcription may depend on the amount of purines in the cells, including from *de novo* synthesis. Studies in *B. subtillis* as well as *Lactococcus lactis* have identified conserved CGAA motifs in the operator sequences called "Pur boxes"(A. K. Bera et al. 2003; Jendresen, Martinussen, and Kilstrup 2012). These Pur boxes function as a binding site for PurR, a transcriptional regulator of genes that function in purine synthesis, to regulate and to bind to. Pur Boxes are a key component in regulation of purine biosynthesis as PRPP (purine biosynthetic precursor) binds to PurR and as a result, alters the affinity of PurR for Pur box DNA. Indeed, our lab has demonstrated that PurR represses transcription of the *purE* operon, one of the genes in the *de novo* purine synthesis pathway. However, it is interesting that we also identified putative Pur Boxes upstream of non-biosynthetic genes such as *fnbA* and *fnbB* (Goncheva et al., 2019b). Considering that purines imported from the environment through the putative purine transporter would contribute to the intracellular purine pool, it is possible that the putative transporter would be regulated by PurR as well, and thus, may have a Pur Box within the operator sequence that can be analyzed using a bioinformatic approach.

To avoid any off-target characterization, further studies could consider supplementing the screening condition with metals, especially Mn (i.e. MTX + IMP +Mn). This approach may be beneficial so that any putative mutants with defect in the purine import conditions would not be a result of perturbed metal homeostasis that may render cells more sensitive to oxidative stresses potentially generated by the inhibitors.

Alternatively, I could altogether avoid using pharmacologic inhibitors of *de novo* purine synthesis by generating random libraries of mutants via transposon Tn917 mutagenesis (using pLTV1 plasmid, described in Rice, 2015(Rice 2016)) under the *ΔpurK* background. With this method, mutants with growth defect in purine-rich media could be considered as "candidate mutants" of the purine transporter as it would not be able to synthesize or import purines. However, there is a possibility that these candidate mutants may not be viable due to the severe purine autotrophy (*purK* mutation) and if the purine transporter is encoded by a single gene which would severely abrogate its growth.

As inhibitors of *de novo* purine biosynthesis, MTX functions as a folate antagonist while 6MP is a thiol-substituted purine analog. Methotrexate (formerly known as amethopterin) is a chemotherapy agent and immune suppressant that is used to treat rheumatoid arthritis and other autoimmune diseases, many forms of cancer and ectopic pregnancy. It is an antifolate that competitively inhibits the dihydrofolate reductase (DHFR) enzyme in the folate synthesis pathway and blocks the conversion of dihydrofolate to the active tetrahydrofolate form. Since folic acid is necessary for *de novo* synthesis of purines and pyrimidines, MTX also inhibits synthesis of DNA, RNA, thymidylates and proteins (Cronstein and Aune, 2020) So far, most of the research on MTX has been conducted in the context of eukaryotic cells due to its mechanism to treat inflammatory diseases, especially rheumatoid arthritis. MTX has not been thoroughly used as an antibiotic nor has its mechanism as a bactericidal or bacteriostatic agent has been characterized.

6MP inhibits purine nucleotide synthesis through the inhibition of PRPP amidotransferase that is the rate-limiting step and thus, alters synthesis and functions of RNA and DNA. It has also been shown that 6MP can compete with purine derivatives such as hypoxanthine and guanine for the substrates of HGPRT, the enzyme that generates IMP in the purine salvage pathway (Bhagavan and Ha, 2015). With its ability to act as an immunomodulator through interfering nucleotide synthesis and preventing cell proliferation, 6MP has been used as a chemotherapy drug to treat certain types of cancer such as acute leukemia as well as to treat inflammatory diseases such as Crohn's disease, colitis, and IBD. Similar to MTX, 6MP was found to promote *in vitro* production of ROS, specifically superoxide, in rat vascular smooth muscle cells (Prüfer et al., 2014). More recently, it has been shown that thiopurine prodrugs such as 6MP and 6TG can generate ROS because of the strong nucleophilic nature of the sulfur-substituted nucleobases. As these drugs have been used to treat cancer, long-term medication with thiopurines have been associated with risks of cancer development through incorporation of these bases into patients' DNA (Euceda et al., 2021). Moreover, thiopurine-induced skin cancer was shown to be accelerated by exposure to sunlight, suggesting that photooxidation is involved in this adverse effect (Euvrard, Kanitakis, and Claudy, 2009). However, the use of 6MP as an antibiotic agent in bacteria has not been well characterized.

In this study, I have established that *S. aureus* lacking MntC, but not MntH, is more sensitive to growth inhibition MTX and 6MP. During pathogenesis, both MntABC and MntH

systems compete with calprotectin to acquire Mn in the vertebrate host to evade host nutritional immunity (Damo et al., 2013). The transcription of MntABC is regulated by the DtxR family metalloregulator called MntR where it acts as a transcriptional repressor to MntABC in response to high intracellular Mn concentration. We have also demonstrated by western blot that *S. aureus* MntC is downregulated in presence of excess Mn (Fig. 23B). Although the promoters of both systems contain the proposed MntR-binding sites (MntR boxes), there are conflicting evidence whether MntR regulate MntH transcription as well (Price and Boyd, 2020b). Interestingly, various *in vivo* studies have demonstrated the marked role of MntC (and to the less extent, MntH) in *S. aureus* pathogenesis. Diep et al., 2014 and Handke et al., 2018, showed that the *mntC::Tn* mutant in USA300 strain was attenuated in a mouse model of sepsis. Moreover, the latter study also showed that only *mntC::Tn* but not *mntH::Tn* mutant showed hypersensitivity to methyl viologen and demonstrated that MntC is of primary importance in conferring resistance to oxidative stress and SOD activity under low Mn concentrations (Handke et al., 2018). Taken together, the MntC-specific phenotype observed in this study agrees with the consensus in literature that is: under low Mn concentrations, Mn imported via MntABC is important for superoxide dismutases to detoxify ROS.

Additionally, we have also demonstrated a Mn-dependent growth of the mutant lacking both high-affinity Mn transporter – MntC and MntH. Indeed a mutant lacking the genes *mntC* and *mntH* that encode components of two different high affinity Mn transporter systems fails to grow in metal-depleted conditions but is rescued when Mn is replete (Fig. 24A). The rescued growth despite defects in MntC and MntH suggests that extracellular Mn is imported into the cells, potentially via other transporters – either low affinity Mn transporters that have not been characterized or other divalent metal transporters. While it remains unclear how *S. aureus* can import Mn via other transporters other than MntABC and MntH systems, the fully restored growth of the mutant lacking both high affinity Mn transport system in excess Mn suggests that Mn ions can still be imported into the cells. A study by Kehres *et al.* indeed identified a possible third Mn transporter of *S. aureus*, annotated as SA1432 from the strain N315, belonging to the family II subclass of Nramp family (Kehres and Maguire, 2003). In a follow-up study by Handke et al., the transmembrane region of SA1432 was found to be similar to that of *E. coli* MntH (Handke et al., 2018). Although this study did not find that SA1432 was

an important contributor of *S. aureus* oxidative stress resistance, other studies could still seek to further determine whether this transporter can actually transport Mn.

In other bacteria, it has been found that an avian pathogenic *E. coli* strain possesses the SitABCD (homolog of MntABC in *S. aureus*) operon, homologous to that of *Salmonella enterica*, that can transport iron and manganese and that allows increase resistance to the bactericidal effects of hydrogen peroxide (Sabri, Léveillé, and Dozois, 2006). Interestingly, SitABCD demonstrated higher affinity for Fe^{2+} than Mn^{2+} in iron transport deficient strain while for the manganese transport deficient strain, SitABCD demonstrated greater affinity for Mn^{2+} than Fe^{2+}. These dual transporter also exist in other bacteria including, the YfeABCD system of *Yersinia pestis* and its SitABCD homolog *S. Typhimurium, K. pneumoniae, P. mirabilis* and *Shigella spp* that encode the dual ABC transporter of Mn and Zn ions. (Porcheron et al., 2013). Additionally, FeoB, a transporter that has been shown to exclusively mediate ferrous ion transport in *S. aureus* was shown to also mediate Mn transport in *Porphyromonas gingivalis* (Dashper et al., 2005).

Cross-talk among different divalent metal ions where non-cognate metals such as Fe and Mn bind regulatory proteins have been demonstrated in several bacteria. For example, in *E. coli* (Mills and Marletta, 2005) and *B. subtilis* (Ma, Faulkner, and Helmann, 2012) Mn^{2+} can bind in the regulatory metal-binding site of ferric uptake regulator (Fur) which has high affinity for ferrous iron and represses the expression on the Fur-regulated proteins. Within the Fur family of *S. aureus*, PerR is a metal-dependent peroxide sensor that regulate peroxide-defenses genes. PerR contains two metal-binding site: a Zn^{2+} structural site and a regulatory metal binding site that coordinates either Mn^{2+} or Fe^{2+}(Herbig and Helmann, 2001). Works in *B. subtilis* have shown that PerR is only repressed when Mn^{2+} is bound but the most target regulons (those involved in ROS metabolism and iron regulation and storage) are repressed by both Mn and Fe forms of PerR (Price and Boyd, 2020a). For example, it has been shown that the transcription iron storage protein, ferritin, in *S. aureus* and *S. epidermidis* (FtnA and SefA) is regulated by PerR that functions as a Mn-dependent transcriptional repressor (Morrissey et al., 2004).

Additionally, findings from this study adds to the body of knowledge surrounding the function of superoxide dismutases in *S. aureus*. I found that *S. aureus* strains lacking *sodA* (both single and double *sodA sodM* mutant) shows hypersensitivity to MTX, 6MP and M.V. and that exogenous Mn can rescue these defects (Fig. 21). Interestingly, I demonstrated that

sodA::Tn mutant showed heightened sensitivity to MTX while *sodM::Tn* did not (Fig. 21). Perhaps this *sodA*-specific phenotype could be described by a finding from Karavalos et al., 2003a that SodA deficiency renders *S. aureus* more sensitive to O_2^- generated internally. Moreover, the finding that growth defects of the *sodAM* mutant can be rescued to WT with exogenous Mn suggests that Mn may be quenching ROS generated by MTX, 6MP and M.V. directly, independent of *S. aureus* dismutases. To measure if Mn can directly inhibit ROS production, a ROS quantifying assay may be optimized to measure amount of superoxide generated by these compounds in Mn-replete and Mn-deplete conditions. A luminol probe-based assay as used in Rowe et al., 2020 may be employed to detect superoxide generated from *S. aureus* that can be used to measure superoxide generated in presence of MTX, 6MP and M.V. with and without Mn. Additionally, to assess whether MTX has the bactericidal effect *in vivo* and the role of Mn transport in this context, mouse infections of *mntC::Tn* and WT *S. aureus* followed by MTX treatments could be performed. It would be interesting to see whether *in vitro* findings would be recapitulated *in vivo*, specifically if I would observe a difference in bacterial burden between the two strains and whether treatment with MTX would result in reduced viable bacteria.

Although I did not identify a novel purine transporter in *S. aureus* as intended in the project objectives, the results of this study suggests the role of Mn as an antioxidant for ROS and the role of SodA for *S. aureus* to cope with ROS generated from MTX, 6MP and M.V. To further evaluate the mechanism at which MTX and 6MP inhibit *S. aureus* growth in addition to blocking *de novo* synthesis of purines, further studies may seek to measure the amount of ROS generated by these compounds as a way to demonstrate that they are indeed generating superoxide and investigate the effect of MTX in mouse model of infection. Importantly, future projects may continue to optimize the screening for a purine transporter in *S. aureus* or further characterize potential transporters from the list of putative mutants with defects in purine import conditions using the framework and suggestions established from this study. Understanding how an antibacterial compound could be producing ROS as an off-target effect, and how the presence of metal ions such as Mn^{2+} could affect ROS is an important consideration when deciding whether a compound would be suitable for use as an *S. aureus* therapeutic. Due to the emergence of multidrug-resistant strains and persistent SCV strains, it is also important to investigate the mechanisms that *S. aureus* use to subvert the effect of antibiotics. As mentioned, purine transport is one of the mechanisms that allow the bacterium

to persist and continue to cause disease in presence of antibiotic treatment; therefore, it is important that the uncharacterized purine transporters be identified and their roles be investigated further as a way to avoid resistance or persistence of the bacterium to any antibiotic compounds.

References

Alonzo, Francis, III, and Victor J. Torres. 2014. “The Bicomponent Pore-Forming Leucocidins of *Staphylococcus aureus*.” *Microbiology and Molecular Biology Reviews : MMBR* 78 (2): 199. https://doi.org/10.1128/MMBR.00055-13.

Aman, M. Javad, and Rajan P. Adhikari. 2014. “Staphylococcal Bicomponent Pore-Forming Toxins: Targets for Prophylaxis and Immunotherapy.” *Toxins* 6 (3): 950. https://doi.org/10.3390/TOXINS6030950.

Anderson, Annaliesa S., Ingrid L. Scully, Yekaterina Timofeyeva, Ellen Murphy, Lisa K. McNeil, Terri Mininni, Lorna Nuñez, et al. 2012. “*Staphylococcus aureus* Manganese Transport Protein c Is a Highly Conserved Cell Surface Protein That Elicits Protective Immunity against s. Aureus and Staphylococcus Epidermidis.” *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jis272.

Azarian, Taj, Robert S. Daum, Lindsay A. Petty, Jenny L. Steinbeck, Zachary Yin, David Nolan, Susan Boyle-Vavra, W. P. Hanage, Marco Salemi, and Michael Z. David. 2016. “Intrahost Evolution of Methicillin-Resistant *Staphylococcus aureus* USA300 among Individuals with Reoccurring Skin and Soft-Tissue Infections.” *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jiw242.

Bae, Taeok, and Olaf Schneewind. 2006. “Allelic Replacement in *Staphylococcus aureus* with Inducible Counter-Selection.” *Plasmid* 55 (1): 58–63. https://doi.org/10.1016/J.PLASMID.2005.05.005.

Baggott, Joseph E., and Tsunenobu Tamura. 2015. “Folate-Dependent Purine Nucleotide Biosynthesis in Humans.” *Advances in Nutrition* 6 (5): 564. https://doi.org/10.3945/AN.115.008300.

Bantel, Heike, Bhanu Sinha, Wolfram Domschke, Georg Peters, Klaus Schulze-Osthoff, and Reiner U. Jänicke. 2001. “α-Toxin Is a Mediator of *Staphylococcus aureus*–Induced Cell Death and Activates Caspases via the Intrinsic Death Pathway Independently of Death Receptor Signaling.” *The Journal of Cell Biology* 155 (4): 637. https://doi.org/10.1083/JCB.200105081.

Bateman, B. T., N. P. Donegan, T. M. Jarry, M. Palma, and A. L. Cheung. 2001. “Evaluation of a Tetracycline-Inducible Promoter in *Staphylococcus aureus In Vitro* and I*n Vivo* and Its Application in Demonstrating the Role of SigB in Microcolony Formation.” *Infection*

and Immunity 69 (12): 7851. https://doi.org/10.1128/IAI.69.12.7851-7857.2001.

Belitsky, Boris R., and Abraham L. Sonenshein. 2011. "CodY-Mediated Regulation of Guanosine Uptake in *Bacillus subtilis*." *Journal of Bacteriology*. https://doi.org/10.1128/JB.05899-11.

Bera, Agnieszka, Silvia Herbert, Andreas Jakob, Waldemar Vollmer, and Friedrich Götz. 2005. "Why Are Pathogenic Staphylococci so Lysozyme Resistant? The Peptidoglycan O-Acetyltransferase OatA Is the Major Determinant for Lysozyme Resistance of Staphylococcus Aureus." *Molecular Microbiology* 55 (3): 778–87. https://doi.org/10.1111/J.1365-2958.2004.04446.X.

Bera, Aloke Kumar, Jianghai Zhu, Howard Zalkin, and Janet L. Smith. 2003. "Functional Dissection of the *Bacillus subtili*s Pur Operator Site." *Journal of Bacteriology* 185 (14): 4099–4109. https://doi.org/10.1128/JB.185.14.4099-4109.2003.

Bernstein, Charles N., Lucy Artinian, Peter A. Anton, and Fergus Shanahan. 1994. "Low-Dose 6-Mercaptopurine in Inflammatory Bowel Disease Is Associated with Minimal Hematologic Toxicity." *Digestive Diseases and Sciences 1994 39:8* 39 (8): 1638–41. https://doi.org/10.1007/BF02087769.

Berube, Bryan J., and Juliane Bubeck Wardenburg. 2013. "*Staphylococcus aureus* α-Toxin: Nearly a Century of Intrigue." *Toxins* 5 (6): 1140. https://doi.org/10.3390/TOXINS5061140.

Bhagavan, N.V., and Chung-Eun Ha. 2015. "Nucleotide Metabolism." *Essentials of Medical Biochemistry*, 465–87. https://doi.org/10.1016/B978-0-12-416687-5.00025-7.

Brooks, Lavida R.K., and George I. Mias. 2018. "*Streptococcus pneumoniae*'s Virulence and Host Immunity: Aging, Diagnostics, and Prevention." *Frontiers in Immunology* 9 (JUN). https://doi.org/10.3389/FIMMU.2018.01366/FULL.

Bucarey, Sergio A., Nicolás A. Villagra, Mara P. Martinic, A. Nicole Trombert, Carlos A. Santiviago, Nancy P. Maulén, Philip Youderian, and Guido C. Mora. 2005. "The *Salmonella Enterica Serovar Typhi* Tsx Gene, Encoding a Nucleoside-Specific Porin, Is Essential for Prototrophic Growth in the Absence of Nucleosides." *Infection and Immunity* 73 (10): 6210–19. https://doi.org/10.1128/IAI.73.10.6210-6219.2005/ASSET/3B96143A-4CA2-4B74-8811-988E46B211E4/ASSETS/GRAPHIC/ZII0100552460008.JPEG.

Buerke, Michael, Ulf Sibelius, Ulrich Grandel, Ute Buerke, Friedrich Grimminger, Werner Seeger, Jürgen Meyer, and Harald Darius. 2002. "*Staphylococcus aureus* Alpha Toxin Mediates Polymorphonuclear Leukocyte-Induced Vasocontraction and Endothelial Dysfunction." *Shock (Augusta, Ga.)* 17 (1): 30–35. https://doi.org/10.1097/00024382-200201000-00006.

Bulger, Roger J, and Ruth Ellen Bulger. 1967. "Ultrastructure of Small Colony Variants of a Methicillin-Resistant *Staphylococcus aureus*." *Journal of Bacteriology*, 1244–46. https://journals.asm.org/journal/jb.

Cassat, James E., and Eric P. Skaar. 2011. "Metal Ion Acquisition in *Staphylococcus aureus*: Overcoming Nutritional Immunity." *Seminars in Immunopathology 2011 34:2* 34 (2): 215–35. https://doi.org/10.1007/S00281-011-0294-4.

Cheng, Alice G., Molly McAdow, Hwan K. Kim, Taeok Bae, Dominique M. Missiakas, and Olaf Schneewind. 2010. "Contribution of Coagulases towards *Staphylococcus aureus* Disease and Protective Immunity." *PLoS Pathogens* 6 (8): 19–20. https://doi.org/10.1371/JOURNAL.PPAT.1001036.

Cheung, Gordon Y.C., Justin S. Bae, and Michael Otto. 2021. "Pathogenicity and Virulence of *Staphylococcus aureus.*" *Virulence* 12 (1): 547–69. https://doi.org/10.1080/21505594.2021.1878688/FORMAT/EPUB.

Chin, Denny, Mariya I. Goncheva, Ronald S. Flannagan, Shayna R. Deecker, Veronica Guariglia-Oropeza, Alexander W. Ensminger, and David E. Heinrichs. 2021. "Coagulase-Negative Staphylococci Release a Purine Analog That Inhibits *Staphylococcus aureus* Virulence." *Nature Communications 2021 12:1* 12 (1): 1–12. https://doi.org/10.1038/s41467-021-22175-3.

Chin, Denny, Mariya I. Goncheva, Ronald S. Flannagan, and David E. Heinrichs. 2021. "Mutations in a Membrane Permease or Hpt Lead to 6-Thioguanine Resistance in *Staphylococcus aureus.*" *Antimicrobial Agents and Chemotherapy* 65 (9). https://doi.org/10.1128/AAC.00760-21.

Chouchana, L, C Narjoz, P Beaune, M-a Loriot, X Roblin, Assistance publique-Hôpitaux de Paris, Hôpital Européen Georges Pompidou, and Assistance publique-. 2012. "Review Article: The Benefits of Pharmacogenetics for Improving Thiopurine Therapy in Inflammatory Bowel Disease." *Alimentary Pharmacology & Therapeutics* 35 (1): 15–36. https://doi.org/10.1111/J.1365-2036.2011.04905.X.

Clauditz, Alexandra, Alexandra Resch, Karsten Peter Wieland, Andreas Peschel, and Friedrich Götz. 2006. “Staphyloxanthin Plays a Role in the Fitness of *Staphylococcus aureus* and Its Ability to Cope with Oxidative Stress.” *Infection and Immunity* 74 (8): 4950–53. https://doi.org/10.1128/IAI.00204-06/ASSET/5F149EFF-575C-4938-8DDB-260902637EE0/ASSETS/GRAPHIC/ZII0080661110005.JPEG.

Clements, Mark O., Sean P. Watson, and Simon J. Foster. 1999. “Characterization of the Major Superoxide Dismutase of *Staphylococcus aureus* and Its Role in Starvation Survival, Stress Resistance, and Pathogenicity.” *Journal of Bacteriology* 181 (13): 3898–3903. https://doi.org/10.1128/JB.181.13.3898-3903.1999/ASSET/E8A737AF-2755-41D2-95D2-5BF7BCDF7772/ASSETS/GRAPHIC/JB1390105005.JPEG.

Coady, Alison, Min Xu, Qui Phung, Tommy K. Cheung, Corey Bakalarski, Mary Kate Alexander, Sophie M. Lehar, et al. 2015. “The *Staphylococcus aureus* ABC-Type Manganese Transporter MntABC Is Critical for Reinitiation of Bacterial Replication Following Exposure to Phagocytic Oxidative Burst.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0138350.

Corbin, Brian D., Erin H. Seeley, Andrea Raab, Joerg Feldmann, Michael R. Miller, Victor J. Torres, Kelsi L. Anderson, et al. 2008. “Metal Chelation and Inhibition of Bacterial Growth in Tissue Abscesses.” *Science*. https://doi.org/10.1126/science.1152449.

Cosgrove, Kate, Graham Coutts, Ing Marie Jonsson, Andrej Tarkowski, John F. Kokai-Kun, James J. Mond, and Simon J. Foster. 2007. “Catalase (KatA) and Alkyl Hydroperoxide Reductase (AhpC) Have Compensatory Roles in Peroxide Stress Resistance and Are Required for Survival, Persistence, and Nasal Colonization in *Staphylococcus aureus*.” *Journal of Bacteriology* 189 (3): 1025–35. https://doi.org/10.1128/JB.01524-06.

Craig, Jane E., Yianbiao Zhang, and Maurice P. Gallagher. 1994. “Cloning of the NupC Gene of *Escherichia Coli* Encoding a Nucleoside Transport System, and Identification of an Adjacent Insertion Element, IS 186.” *Molecular Microbiology*. https://doi.org/10.1111/j.1365-2958.1994.tb00392.x.

Craven, Robin R., Xi Gao, Irving C. Allen, Denis Gris, Juliane Bubeck Wardenburg, Erin McElvania-TeKippe, Jenny P. Ting, and Joseph A. Duncan. 2009. “*Staphylococcus aureus* α-Hemolysin Activates the NLRP3-Inflammasome in Human and Mouse Monocytic Cells.” *PLoS ONE* 4 (10). https://doi.org/10.1371/JOURNAL.PONE.0007446.

Cronstein, Bruce N., and Thomas M. Aune. 2020. “Methotrexate and Its Mechanisms of Action in Inflammatory Arthritis.” *Nature Reviews Rheumatology 2020 16:3* 16 (3): 145–54. https://doi.org/10.1038/s41584-020-0373-9.

Cuellar, Julia, Mia Åstrand, Heli Elovaara, Annukka Pietikäinen, Saija Sirén, Arto Liljeblad, Gabriela Guédez, Tiina A. Salminen, and Jukka Hytönen. 2020. “ Structural and Biomolecular Analyses of *Borrelia burgdorferi* BmpD Reveal a Substrate-Binding Protein of an ABC-Type Nucleoside Transporter Family. .” *Infection and Immunity*. https://doi.org/10.1128/iai.00962-19.

Damo, Steven M., Thomas E. Kehl-Fie, Norie Sugitani, Marilyn E. Holt, Subodh Rathi, Wesley J. Murphy, Yaofang Zhang, et al. 2013. “Molecular Basis for Manganese Sequestration by Calprotectin and Roles in the Innate Immune Response to Invading Bacterial Pathogens.” *Proceedings of the National Academy of Sciences of the United States of America* 110 (10): 3841–46. https://doi.org/10.1073/PNAS.1220341110.

Dashper, Stuart G., Catherine A. Butler, J. Patricia Lissel, Rita A. Paolini, Brigitte Hoffmann, Paul D. Veith, Neil M. O’Brien-Simpson, Sarah L. Snelgrove, John T. Tsiros, and Eric C. Reynolds. 2005. “A Novel *Porphyromonas gingivalis* FeoB Plays a Role in Manganese Accumulation *.” *Journal of Biological Chemistry* 280 (30): 28095–102. https://doi.org/10.1074/JBC.M503896200.

Diep, Binh An, Qui Phung, Shailesh Date, David Arnott, Corey Bakalarski, Min Xu, Gerald Nakamura, et al. 2014. “Identifying Potential Therapeutic Targets of Methicillin-Resistant *Staphylococcus aureus* through in Vivo Proteomic Analysis.” *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jit662.

DuMont, Ashley L., Tyler K. Nygaard, Robert L. Watkins, Amanda Smith, Lina Kozhaya, Barry N. Kreiswirth, Bo Shopsin, Derya Unutmaz, Jovanka M. Voyich, and Victor J. Torres. 2011. “Characterization of a New Cytotoxin That Contributes to *Staphylococcus aureus* Pathogenesis.” *Molecular Microbiology* 79 (3): 814–25. https://doi.org/10.1111/J.1365-2958.2010.07490.X.

Eiff, Christof von, Karsten Becker, Konstanze Machka, Holger Stammer, and Georg Peters. 2001. “Nasal Carriage as a Source of *Staphylococcus aureus* Bacteremia. Study Group.” *The New England Journal of Medicine* 344 (1): 11–16. https://doi.org/10.1056/NEJM200101043440102.

El-Benna, Jamel, Margarita Hurtado-Nedelec, Viviana Marzaioli, Jean Claude Marie, Marie

Anne Gougerot-Pocidalo, and Pham My Chan Dang. 2016. “Priming of the Neutrophil Respiratory Burst: Role in Host Defense and Inflammation.” *Immunological Reviews* 273 (1): 180–93. https://doi.org/10.1111/IMR.12447.

Engemann, John J., Yehuda Carmeli, Sara E. Cosgrove, Vance G. Fowler, Melissa Z. Bronstein, Sharon L. Trivette, Jane P. Briggs, Daniel J. Sexton, and Keith S. Kaye. 2003. “Adverse Clinical and Economic Outcomes Attributable to Methicillin Resistance among Patients with *Staphylococcus aureus* Surgical Site Infection.” *Clinical Infectious Diseases*. https://doi.org/10.1086/367653.

Euceda, Nelson, Joyce Jahnke, Aileen Espinal, Monique F Louis, Edan Bashkin, Patricia Roccanova, Abraham Espaillat, German V Fuentes, Fernando Nieto, and Ruomei Gao. n.d. “Thioguanine Restoration through Type I Photosensitization-Superoxide Oxidation-Glutathione Reduction Cycles †.” https://doi.org/10.1039/d1cp00101a.

Euvrard, Sylvie, Jean Kanitakis, and Alain Claudy. 2009. “Skin Cancers after Organ Transplantation.” *Http://Dx.Doi.Org/10.1056/NEJMra022137* 348 (17): 1681–91. https://doi.org/10.1056/NEJMRA022137.

Fey, Paul D., Jennifer L. Endres, Vijaya Kumar Yajjala, Todd J. Widhelm, Robert J. Boissy, Jeffrey L. Bose, and Kenneth W. Bayles. 2013. “A Genetic Resource for Rapid and Comprehensive Phenotype Screening of Nonessential *Staphylococcus aureus* Genes.” *MBio*. https://doi.org/10.1128/mBio.00537-12.

Flannagan, Ronald S., Bryan Heit, and David E. Heinrichs. 2016. “Intracellular Replication of *Staphylococcus aureus* in Mature Phagolysosomes in Macrophages Precedes Host Cell Death, and Bacterial Escape and Dissemination.” *Cellular Microbiology* 18 (4): 514–35. https://doi.org/10.1111/CMI.12527.

Flannagan, Ronald S., Robert C. Kuiack, Martin J. McGavin, and David E. Heinrichs. 2018. “*Staphylococcus aureus* Uses the GraXRS Regulatory System to Sense and Adapt to the Acidified Phagolysosome in Macrophages.” *MBio* 9 (4). https://doi.org/10.1128/MBIO.01143-18/SUPPL_FILE/MBO004183971S1.DOCX.

Flannagan, Ronald S, and David E Heinrichs. 2020. “Macrophage-Driven Nutrient Delivery to Phagosomal *Staphylococcus aureus* Supports Bacterial Growth.” *EMBO Reports* 21 (8): e50348. https://doi.org/10.15252/EMBR.202050348.

Foster, Timothy J., Joan A. Geoghegan, Vannakambadi K. Ganesh, and Magnus Höök. 2014. “Adhesion, Invasion and Evasion: The Many Functions of the Surface Proteins of

Staphylococcus aureus." *Nature Reviews. Microbiology* 12 (1): 49–62. https://doi.org/10.1038/NRMICRO3161.

Fraunholz, Martin, and Bhanu Sinha. 2012. "Intracellular *Staphylococcus aureus*: Live-in and Let Die." *Frontiers in Cellular and Infection Microbiology* 2: 43. https://doi.org/10.3389/FCIMB.2012.00043/BIBTEX.

Fukuda, Yu, and John D. Schuetz. 2012. "ABC Transporters and Their Role in Nucleoside and Nucleotide Drug Resistance." *Biochemical Pharmacology*. https://doi.org/10.1016/j.bcp.2011.12.042.

Gao, Feng, and Toshiharu Horie. 2002. "A Synthetic Analog of Prostaglandin E1 Prevents the Production of Reactive Oxygen Species in the Intestinal Mucosa of Methotrexate-Treated Rats." *Life Sciences* 71 (9): 1091–99. https://doi.org/10.1016/S0024-3205(02)01795-2.

Garcia, Yuritzi M., Anna Barwinska-Sendra, Emma Tarrant, Eric P. Skaar, Kevin J. Waldron, and Thomas E. Kehl-Fie. 2017a. "A Superoxide Dismutase Capable of Functioning with Iron or Manganese Promotes the Resistance of *Staphylococcus aureus* to Calprotectin and Nutritional Immunity." *PLoS Pathogens* 13 (1). https://doi.org/10.1371/JOURNAL.PPAT.1006125.

———. 2017b. "A Superoxide Dismutase Capable of Functioning with Iron or Manganese Promotes the Resistance of *Staphylococcus aureus* to Calprotectin and Nutritional Immunity." *PLOS Pathogens* 13 (1): e1006125. https://doi.org/10.1371/JOURNAL.PPAT.1006125.

Gebhardt, Christoffer, Julia Németh, Peter Angel, and Jochen Hess. 2006. "S100A8 and S100A9 in Inflammation and Cancer." *Biochemical Pharmacology* 72 (11): 1622–31. https://doi.org/10.1016/J.BCP.2006.05.017.

Goncheva, Mariya I., Denny Chin, and David E. Heinrichs. 2022a. "Nucleotide Biosynthesis: The Base of Bacterial Pathogenesis." *Trends in Microbiology* 0 (0). https://doi.org/10.1016/J.TIM.2021.12.007.

Goncheva, Mariya I., Ronald S. Flannagan, and David E. Heinrichs. 2020a. "De Novo Purine Biosynthesis Is Required for Intracellular Growth of *Staphylococcus aureus* and for the Hypervirulence Phenotype of a PurR Mutant." *Infection and Immunity*. https://doi.org/10.1128/IAI.00104-20.

Goncheva, Mariya I., Ronald S. Flannagan, Brigid E. Sterling, Holly A. Laakso, Nancy C.

Friedrich, Julienne C. Kaiser, David W. Watson, et al. 2019a. “Stress-Induced Inactivation of the Staphylococcus Aureus Purine Biosynthesis Repressor Leads to Hypervirulence.” *Nature Communications*. https://doi.org/10.1038/s41467-019-08724-x.

Gonzalez, M. R., M. Bischofberger, L. Pernot, F. G. Van Der Goot, and B. Frêche. 2007. “Bacterial Pore-Forming Toxins: The (w)Hole Story?” *Cellular and Molecular Life Sciences 2007 65:3* 65 (3): 493–507. https://doi.org/10.1007/S00018-007-7434-Y.

Gouaux, Eric. 1998. “α-Hemolysin From *Staphylococcus aureus*:An Archetype of β-Barrel, Channel-Forming Toxins.” *Journal of Structural Biology* 121 (2): 110–22. https://doi.org/10.1006/JSBI.1998.3959.

Greenstein, Robert J., Liya Su, Vahram Haroutunian, Azra Shahidi, and Sheldon T. Brown. 2007. “On the Action of Methotrexate and 6-Mercaptopurine on M. Avium Subspecies Paratuberculosis.” *PLOS ONE* 2 (1): e161. https://doi.org/10.1371/JOURNAL.PONE.0000161.

Grunenwald, Caroline M., Jacob E. Choby, Lillian J. Juttukonda, William N. Beavers, Andy Weiss, Victor J. Torres, and Eric P. Skaar. 2019. “Manganese Detoxification by MntE Is Critical for Resistance to Oxidative Stress and Virulence of *Staphylococcus aureus.*” *MBio* 10 (1). https://doi.org/10.1128/MBIO.02915-18/SUPPL_FILE/MBIO.02915-18-ST002.DOCX.

Guédon, E., P. Serror, S. D. Ehrlich, P. Renault, and C. Delorme. 2001. “Pleiotropic Transcriptional Repressor CodY Senses the Intracellular Pool of Branched-Chain Amino Acids in *Lactococcus Lactis.*” *Molecular Microbiology* 40 (5): 1227–39. https://doi.org/10.1046/J.1365-2958.2001.02470.X.

Gumpenberger, Tanja, Dina Vorkapic, Franz G. Zingl, Katharina Pressler, Stefanie Lackner, Andrea Seper, Joachim Reidl, and Stefan Schild. 2016a. “Nucleoside Uptake in *Vibrio Cholerae* and Its Role in the Transition Fitness from Host to Environment.” *Molecular Microbiology* 99 (3): 470–83. https://doi.org/10.1111/MMI.13143.

Gupta, Radha, Minny Bhatty, Edwin Swiatlo, and Bindu Nanduri. 2013. “Role of an Iron-Dependent Transcriptional Regulator in the Pathogenesis and Host Response to Infection with *Streptococcus pneumoniae*.” *PLoS ONE*. https://doi.org/10.1371/journal.pone.0055157.

Handke, Luke D., Alexey V. Gribenko, Yekaterina Timofeyeva, Ingrid L. Scully, and Annaliesa S. Anderson. 2018. " MntC-Dependent Manganese Transport Is Essential for *Staphylococcus aureus* Oxidative Stress Resistance and Virulence ." *MSphere* 3 (4). https://doi.org/10.1128/MSPHERE.00336-18/SUPPL_FILE/SPH004182591S1.DOCX.

Handke, Luke D., Julio C. Hawkins, Alita A. Miller, Kathrin U. Jansen, and Annaliesa S. Anderson. 2013. "Regulation of *Staphylococcus aureus* MntC Expression and Its Role in Response to Oxidative Stress." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0077874.

Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss. 1991. "Catabolite Repression of Alpha-Amylase Gene Expression in Bacillus Subtilis Involves a Trans-Acting Gene Product Homologous to the *Escherichia coli* Lacl and GalR Repressors." *Molecular Microbiology* 5 (3): 575–84. https://doi.org/10.1111/J.1365-2958.1991.TB00728.X.

Herbig, Andrew F., and John D. Helmann. 2001. "Roles of Metal Ions and Hydrogen Peroxide in Modulating the Interaction of the *Bacillus subtilis* PerR Peroxide Regulon Repressor with Operator DNA." *Molecular Microbiology* 41 (4): 849–59. https://doi.org/10.1046/J.1365-2958.2001.02543.X.

Herman, S., N. Zurgil, and M. Deutsch. 2005. "Low Dose Methotrexate Induces Apoptosis with Reactive Oxygen Species Involvement in T Lymphocytic Cell Lines to a Greater Extent than in Monocytic Lines." *Inflammation Research* 54 (7): 273–80. https://doi.org/10.1007/s00011-005-1355-8.

Hood, M Indriati, and Eric P Skaar. n.d. "Nutritional Immunity: Transition Metals at the Pathogen-Host Interface NIH Public Access." https://doi.org/10.1038/nrmicro2836.

Horsburgh, M. J., M. O. Clements, H. Crossley, E. Ingham, and S. J. Foster. 2001. "PerR Controls Oxidative Stress Resistance and Iron Storage Proteins and Is Required for Virulence in *Staphylococcus aureus*." *Infection and Immunity* 69 (6): 3744–54. https://doi.org/10.1128/IAI.69.6.3744-3754.2001.

Horsburgh, M. J., E. Ingham, and S. J. Foster. 2001. "In *Staphylococcus aureus*, Fur Is an Interactive Regulator with PerR, Contributes to Virulence, and Is Necessary for Oxidative Stress Resistance through Positive Regulation of Catalase and Iron Homeostasis." *Journal of Bacteriology* 183 (2): 468–75. https://doi.org/10.1128/JB.183.2.468-475.2001.

Horsburgh, Malcolm J., Stephen J. Wharton, Alan G. Cox, Eileen Ingham, Sharon Peacock, and Simon J. Foster. 2002a. “MntR Modulates Expression of the PerR Regulon and Superoxide Resistance in *Staphylococcus aureus* through Control of Manganese Uptake.” *Molecular Microbiology*. https://doi.org/10.1046/j.1365-2958.2002.02944.x.

Hurley, L. S., C. L. Keen, and D. L. Baly. 1984. “Manganese Deficiency and Toxicity: Effects on Carbohydrate Metabolism in the Rat.” *Neurotoxicology* 5 (1): 97–104. https://europepmc.org/article/med/6371607.

Inoshima, Ichiro, Naoko Inoshima, Georgia A. Wilke, Michael E. Powers, Karen M. Frank, Yang Wang, and Juliane Bubeck Wardenburg. 2011. “A *Staphylococcus aureus* Pore-Forming Toxin Subverts the Activity of ADAM10 to Cause Lethal Infection in Mice.” *Nature Medicine* 17 (10): 1310–14. https://doi.org/10.1038/NM.2451.

Inoshima, Naoko, Yang Wang, and Juliane Bubeck Wardenburg. 2012. “Genetic Requirement for ADAM10 in Severe *Staphylococcus aureus* Skin Infection.” *Journal of Investigative Dermatology* 132 (5): 1513–16. https://doi.org/10.1038/JID.2011.462/ATTACHMENT/1F74E1B8-DA22-4467-AAF1-E0E708280D53/MMC1.PDF.

Jakubovics, N. S., and H. F. Jenkinson. 2001. “Out of the Iron Age: New Insights into the Critical Role of Manganese Homeostasis in Bacteria.” *Microbiology* 147 (7): 1709–18. https://doi.org/10.1099/00221287-147-7-1709/CITE/REFWORKS.

Jarry, Todd M., Guido Memmi, and Ambrose L. Cheung. 2008. “The Expression of Alpha-Haemolysin Is Required for *Staphylococcus aureus* Phagosomal Escape after Internalization in CFT-1 Cells.” *Cellular Microbiology* 10 (9): 1801–14. https://doi.org/10.1111/J.1462-5822.2008.01166.X.

Jendresen, Christian Bille, Jan Martinussen, and Mogens Kilstrup. 2012. “The PurR Regulon in *Lactococcus Lactis* - Transcriptional Regulation of the Purine Nucleotide Metabolism and Translational Machinery.” *Microbiology (United Kingdom)* 158 (8): 2026–38. https://doi.org/10.1099/MIC.0.059576-0/CITE/REFWORKS.

Kaiser, Julienne C., Alyssa N. King, Jason C. Grigg, Jessica R. Sheldon, David R. Edgell, Michael E.P. Murphy, Shaun R. Brinsmade, and David E. Heinrichs. 2018. “Repression of Branched-Chain Amino Acid Synthesis in *Staphylococcus aureus* Is Mediated by Isoleucine via CodY, and by a Leucine-Rich Attenuator Peptide.” *PLoS Genetics*. https://doi.org/10.1371/journal.pgen.1007159.

Kaiser, Julienne C., Suranjana Sen, Anshul Sinha, Brian J. Wilkinson, and David E. Heinrichs. 2016. “The Role of Two Branched-Chain Amino Acid Transporters in *Staphylococcus aureus* Growth, Membrane Fatty Acid Composition and Virulence.” *Molecular Microbiology*. https://doi.org/10.1111/mmi.13495.

Karavolos, Michail H., Malcom Horsburgh, Eileen Ingham, and Simon J. Foster. 2003a. “Role and Regulation of the Superoxide Dismutases of *Staphylococcus aureus*.” *Microbiology*. https://doi.org/10.1099/mic.0.26353-0.

Kehl-Fie, Thomas E., Seth Chitayat, M. Indriati Hood, Steven Damo, Nicole Restrepo, Carlos Garcia, Kim A. Munro, Walter J. Chazin, and Eric P. Skaar. 2011. “Nutrient Metal Sequestration by Calprotectin Inhibits Bacterial Superoxide Defense, Enhancing Neutrophil Killing of *Staphylococcus aureus*.” *Cell Host & Microbe* 10 (2): 158–64. https://doi.org/10.1016/J.CHOM.2011.07.004.

Kehl-Fie, Thomas E., Yaofang Zhang, Jessica L. Moore, Allison J. Farrand, M. Indriati Hood, Subodh Rathi, Walter J. Chazin, Richard M. Caprioli, and Eric P. Skaar. 2013. “MntABC and MntH Contribute to Systemic *Staphylococcus aureus* Infection by Competing with Calprotectin for Nutrient Manganese.” *Infection and Immunity*. https://doi.org/10.1128/IAI.00420-13.

Kehres, David G., and Michael E. Maguire. 2003. “Emerging Themes in Manganese Transport, Biochemistry and Pathogenesis in Bacteria.” *FEMS Microbiology Reviews* 27 (2–3): 263–90. https://doi.org/10.1016/S0168-6445(03)00052-4.

Keshav, Satish, Ping Chung, Genevieve Milon, and Siamon Gordon. 1991. “Lysozyme Is an Inducible Marker of Macrophage Activation in Murine Tissues as Demonstrated by in Situ Hybridization.” *Journal of Experimental Medicine* 174 (5): 1049–58. https://doi.org/10.1084/JEM.174.5.1049.

Kilstrup, Mogens, Karin Hammer, Peter Ruhdal Jensen, and Jan Martinussen. 2005. “Nucleotide Metabolism and Its Control in Lactic Acid Bacteria.” *FEMS Microbiology Reviews*. https://doi.org/10.1016/j.femsre.2005.04.006.

Kilstrup, Mogens, Karin Hammer, Peter Ruhdal Jensen, and Jan Martinussen. 2005. “Nucleotide Metabolism and Its Control in Lactic Acid Bacteria.” *FEMS Microbiology Reviews* 29 (3): 555–90. https://doi.org/10.1016/J.FMRRE.2005.04.006.

Klebanoff, S. J., A. J. Kettle, H. Rosen, C. C. Winterbourn, and W. M. Nauseef. 2013. "Myeloperoxidase: A Front-Line Defender against Phagocytosed Microorganisms." *Journal of Leukocyte Biology* 93 (2): 185–98. https://doi.org/10.1189/JLB.0712349.

Kobayashi, Scott D., Natalia Malachowa, and Frank R. DeLeo. 2018. "Neutrophils and Bacterial Immune Evasion." *Journal of Innate Immunity* 10 (5–6): 432–41. https://doi.org/10.1159/000487756.

Kofoed, Eric M., Donghong Yan, Anand K. Katakam, Mike Reichelt, Baiwei Lin, Janice Kim, Summer Park, et al. 2016a. "De Novo Guanine Biosynthesis but Not the Riboswitch-Regulated Purine Salvage Pathway Is Required for *Staphylococcus aureus* Infection in Vivo." *Journal of Bacteriology*. https://doi.org/10.1128/JB.00051-16.

Kofoed, Eric M, Donghong Yan, Anand K Katakam, Mike Reichelt, Baiwei Lin, Janice Kim, Summer Park, et al. 2016b. *"De Novo* Guanine Biosynthesis but Not the Riboswitch-Regulated Purine Salvage Pathway Is Required for *Staphylococcus aureus* Infection In Vivo." https://doi.org/10.1128/JB.00051-16.

Kretschmer, Dorothee, Anne Kathrin Gleske, Maren Rautenberg, Rong Wang, Martin Köberle, Erwin Bohn, Torsten Schöneberg, et al. 2010. "Human Formyl Peptide Receptor 2 Senses Highly Pathogenic *Staphylococcus aureus.*" *Cell Host & Microbe* 7 (6): 463–73. https://doi.org/10.1016/J.CHOM.2010.05.012.

Kriegeskorte, Andre, Desiree Block, Mike Drescher, Nadine Windmüller, Alexander Mellmann, Cathrin Baum, Claudia Neumann, et al. 2014. "Inactivation of ThyA in *Staphylococcus aureus* Attenuates Virulence and Has a Strong Impact on Metabolism and Virulence Gene Expression." *MBio* 5 (4): 1–15. https://doi.org/10.1128/MBIO.01447-14/SUPPL_FILE/MBO004141926ST4.DOCX.

Kubica, Malgorzata, Krzysztof Guzik, Joanna Koziel, Miroslaw Zarebski, Walter Richter, Barbara Gajkowska, Anna Golda, et al. 2008. "A Potential New Pathway for *Staphylococcus aureus* Dissemination: The Silent Survival of S. Aureus Phagocytosed by Human Monocyte-Derived Macrophages." *PLOS ONE* 3 (1): e1409. https://doi.org/10.1371/JOURNAL.PONE.0001409.

Lakhundi, Sahreena, and Kunyan Zhang. 2018. "Methicillin-Resistant *Staphylococcus aureus*: Molecular Characterization, Evolution, and Epidemiology." *Clinical Microbiology Reviews*. https://doi.org/10.1128/CMR.00020-18.

Lâm, Thiên Trí, Bernd Giese, Deepak Chikkaballi, Anika Kühn, Wanja Wolber, Jan Pané-Farré, Daniel Schäfer, Susanne Engelmann, Martin Fraunholz, and Bhanu Sinha. 2010. "Phagolysosomal Integrity Is Generally Maintained after *Staphylococcus aureus* Invasion of Nonprofessional Phagocytes but Is Modulated by Strain 6850." *Infection and Immunity* 78 (8): 3392–93. https://doi.org/10.1128/IAI.00012-10/ASSET/D8710130-A0E4-48D0-BF9C-C578FE3A4161/ASSETS/GRAPHIC/ZII9990987310006.JPEG.

Lambris, John D., Daniel Ricklin, and Brian V. Geisbrecht. 2008. "Complement Evasion by Human Pathogens." *Nature Reviews Microbiology 2008 6:2* 6 (2): 132–42. https://doi.org/10.1038/nrmicro1824.

Li, Liang, Wessam Abdelhady, Niles P. Donegan, Kati Seidl, Ambrose Cheung, Yu Feng Zhou, Michael R. Yeaman, Arnold S. Bayer, and Yan Q. Xiong. 2018. "Role of Purine Biosynthesis in Persistent Methicillin-Resistant *Staphylococcus aureus* Infection." *Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jiy340.

Li, Liang, Arnold S. Bayer, Ambrose Cheung, Lou Lu, Wessam Abdelhady, Niles P. Donegan, Jong In Hong, Michael R. Yeaman, and Yan Q. Xiong. 2020. "The Stringent Response Contributes to Persistent Methicillin-Resistant *Staphylococcus aureus* Endovascular Infection Through the Purine Biosynthetic Pathway." *The Journal of Infectious Diseases*. https://doi.org/10.1093/infdis/jiaa202.

Liu, George Y. 2009. "Molecular Pathogenesis of *Staphylococcus aureus* Infection." *Pediatric Research*. https://doi.org/10.1203/PDR.0b013e31819dc44d.

Loir, Yves Le, Florence Baron, and Michel Gautier. 2003. *"Staphylococcus aureus* and Food Poisoning." In *Genetics and Molecular Research*.

Lowy, Franklin D. 1998. " *Staphylococcus aureus Infections* ." *New England Journal of Medicine*. https://doi.org/10.1056/nejm199808203390806.

Ma, Zhen, Melinda J. Faulkner, and John D. Helmann. 2012. "Origins of Specificity and Cross-Talk in Metal Ion Sensing by *Bacillus subtilis* Fur." *Molecular Microbiology* 86 (5): 1144–55. https://doi.org/10.1111/MMI.12049.

Martinussen, Jan, Claus Sørensen, Christian Bille Jendresen, and Mogens Kilstrup. 2010. "Two Nucleoside Transporters in *Lactococcus lactis* with Different Substrate Specificities." *Microbiology*. https://doi.org/10.1099/mic.0.039818-0.

Mazmanian, Sarkis K., Gwen Liu, Eric R. Jensen, Eileen Lenoy, and Olaf Schneewind. 2000. "*Staphylococcus aureus* Sortase Mutants Defective in the Display of Surface Proteins and in the Pathogenesis of Animal Infections." *Proceedings of the National Academy of Sciences of the United States of America* 97 (10): 5510–15. https://doi.org/10.1073/PNAS.080520697/ASSET/4C7570DC-4711-429A-A8F0-D21383535976/ASSETS/GRAPHIC/PQ0805206006.JPEG.

McClure, Jo Ann, and Kunyan Zhang. 2017. "Complete Genome Sequence of a Community-Associated Methicillin-Resistant *Staphylococcus aureus* Hypervirulent Strain, USA300-C2406, Isolated from a Patient with a Lethal Case of Necrotizing Pneumonia." *Genome Announcements* 5 (22). https://doi.org/10.1128/GENOMEA.00461-17.

Mei, Ji Min, Fahimeh Nourbakhsh, Charles W. Ford, and David W. Holden. 1997. "Identification of *Staphylococcus aureus* Virulence Genes in a Murine Model of Bacteraemia Using Signature-Tagged Mutagenesis." *Molecular Microbiology* 26 (2): 399–407. https://doi.org/10.1046/J.1365-2958.1997.5911966.X.

Melter, O., and B. Radojevič. 2010. "Small Colony Variants of *Staphylococcus aureus* - Review." *Folia Microbiologica*. https://doi.org/10.1007/s12223-010-0089-3.

Miller, Erica F., Soumya Vaish, and Robert J. Maier. 2012. "Efficiency of Purine Utilization by *Helicobacter pylori*: Roles for Adenosine Deaminase and a NupC Homolog." *PLOS ONE* 7 (6): e38727. https://doi.org/10.1371/JOURNAL.PONE.0038727.

Mills, Stephen A., and Michael A. Marletta. 2005. "Metal Binding Characteristics and Role of Iron Oxidation in the Ferric Uptake Regulator from *Escherichia coli*." *Biochemistry* 44 (41): 13553–59. https://doi.org/10.1021/BI0507579.

Mishra, Nagendra N., Soo Jin Yang, Ayumi Sawa, Aileen Rubio, Cynthia C. Nast, Michael R. Yeaman, and Arnold S. Bayer. 2009. "Analysis of Cell Membrane Characteristics of in Vitro-Selected Daptomycin-Resistant Strains of Methicillin-Resistant *Staphylococcus aureus*." *Antimicrobial Agents and Chemotherapy* 53 (6): 2312–18. https://doi.org/10.1128/AAC.01682-08.

Moisan, Hélène, Eric Brouillette, Christian Lebeau Jacob, Philippe Langlois-Bégin, Sophie Michaud, and François Malouin. 2006. "Transcription of Virulence Factors in *Staphylococcus aureus* Small-Colony Variants Isolated from Cystic Fibrosis Patients Is Influenced by SigB." *Journal of Bacteriology* 188 (1): 64–76.

Mongodin, Emmanuel, Jon Finan, Michael W. Climo, Adriana Rosato, Steven Gill, and Gordon L. Archer. 2003. “Microarray Transcription Analysis of Clinical *Staphylococcus aureus* Isolates Resistant to Vancomycin.” *Journal of Bacteriology*. https://doi.org/10.1128/JB.185.15.4638-4643.2003.

Morrissey, Julie A., Alan Cockayne, Kirsty Brummell, and Paul Williams. 2004. “The Staphylococcal Ferritins Are Differentially Regulated in Response to Iron and Manganese and via PerR and Fur.” *Infection and Immunity* 72 (2): 972–79. https://doi.org/10.1128/IAI.72.2.972-979.2004.

Murray, Christopher JL, Kevin Shunji Ikuta, Fablina Sharara, Lucien Swetschinski, Gisela Robles Aguilar, Authia Gray, Chieh Han, et al. 2022. “Global Burden of Bacterial Antimicrobial Resistance in 2019: A Systematic Analysis.” https://doi.org/10.1016/S0140-6736(21)02724-0.

“NupC - Nucleoside Permease NupC - *Staphylococcus aureus* (Strain COL) - NupC Gene & Protein.” n.d. Accessed April 24, 2022. https://www.uniprot.org/uniprot/A0A0H2WYX5.

Otto, Michael. 2014. “*Staphylococcus aureus* Toxins.” *Current Opinion in Microbiology*. https://doi.org/10.1016/j.mib.2013.11.004.

Patching, Simon G., Stephen A. Baldwin, Alexander D. Baldwin, James D. Young, Maurice P. Gallagher, Peter J.F. Henderson, and Richard B. Herbert. 2005. “The Nucleoside Transport Proteins, NupC and NupG, from Escherichia Coli: Specific Structural Motifs Necessary for the Binding of Ligands.” *Organic and Biomolecular Chemistry*. https://doi.org/10.1039/b414739a.

Patti, J. M., B. L. Allen, M. J. McGavin, and M. Hook. 2003. “MSCRAMM-mediated adherence of microorganisms to host tissues.” *Https://Doi.Org/10.1146/Annurev.Mi.48.100194.003101* 48 (November): 585–617. https://doi.org/10.1146/ANNUREV.MI.48.100194.003101.

Peacock, Sharon J., Catrin E. Moore, Anita Justice, Maria Kantzanou, Lisa Story, Kathryn Mackie, Gael O’Neill, and Nicholas P.J. Day. 2002. “Virulent Combinations of Adhesin and Toxin Genes in Natural Populations of *Staphylococcus aureus*.” *Infection and Immunity* 70 (9): 4987–96. https://doi.org/10.1128/IAI.70.9.4987-4996.2002/ASSET/46A949F6-BD89-4096-AE80-16E3F97670D6/ASSETS/GRAPHIC/II0920413003.JPEG.

Perego, Marta, Philippe Glaser, Antonia Minutello, Mark A. Strauch, Klaus Leopold, and Werner Fischer. 1995. “Incorporation of D-Alanine into Lipoteichoic Acid and Wall Teichoic Acid in *Bacillus subtilis*: Identification of Genes and Regulations.” *Journal of Biological Chemistry* 270 (26): 15598–606. https://doi.org/10.1074/JBC.270.26.15598.

Periasamy, Saravanan, Som S. Chatterjee, Gordon Y. C. Cheung, and Michael Otto. 2012. “Phenol-Soluble Modulins in Staphylococci: What Are They Originally For?” *Communicative & Integrative Biology* 5 (3): 275. https://doi.org/10.4161/CIB.19420.

Phillips, Darren C., Kevin J. Woollard, and Helen R. Griffiths. 2003. “The Anti-Inflammatory Actions of Methotrexate Are Critically Dependent upon the Production of Reactive Oxygen Species.” *British Journal of Pharmacology* 138 (3): 501–11. https://doi.org/10.1038/SJ.BJP.0705054.

Pohl, Konstanze, Patrice Francois, Ludwig Stenz, Frank Schlink, Tobias Geiger, Silvia Herbert, Christiane Goerke, Jacques Schrenzel, and Christiane Wolz. 2009. “CodY in Staphylococcus Aureus: A Regulatory Link between Metabolism and Virulence Gene Expression.” *Journal of Bacteriology*. https://doi.org/10.1128/JB.01492-08.

Popov, Lauren M., Caleb D. Marceau, Philipp M. Starkl, Jennifer H. Lumb, Jimit Shah, Diego Guerrera, Rachel L. Cooper, et al. 2015. “The Adherens Junctions Control Susceptibility to *Staphylococcus aureus* α-Toxin.” *Proceedings of the National Academy of Sciences of the United States of America* 112 (46): 14337–42. https://doi.org/10.1073/PNAS.1510265112/SUPPL_FILE/PNAS.1510265112.SM02.MOV.

Porcheron, Gaëlle, Amélie Garénaux, Julie Proulx, Mourad Sabri, and Charles M. Dozois. 2013. “Iron, Copper, Zinc, and Manganese Transport and Regulation in Pathogenic Enterobacteria: Correlations between Strains, Site of Infection and the Relative Importance of the Different Metal Transport Systems for Virulence.” *Frontiers in Cellular and Infection Microbiology* 3 (DEC): 90. https://doi.org/10.3389/FCIMB.2013.00090/ABSTRACT.

Price, Erin E., and Jeffrey M. Boyd. 2020a. “Genetic Regulation of Metal Ion Homeostasis in *Staphylococcus aureus*.” *Trends in Microbiology*. https://doi.org/10.1016/j.tim.2020.04.004.

Proctor, Richard A., André Kriegeskorte, Barbara C. Kahl, Karsten Becker, Bettina Löffler, and Georg Peters. 2014. “*Staphylococcus aureus* Small Colony Variants (SCVs): A

Road Map for the Metabolic Pathways Involved in Persistent Infections." *Frontiers in Cellular and Infection Microbiology* 4 (JUL). https://doi.org/10.3389/FCIMB.2014.00099.

Prüfer, Jasmin, Mirjam Schuchardt, Markus Tölle, Nicole Prüfer, Matthias Höhne, Walter Zidek, and Markus Van Der Giet. 2014. "Harmful Effects of the Azathioprine Metabolite 6-Mercaptopurine in Vascular Cells: Induction of Mineralization." *PLOS ONE* 9 (7): e101709. https://doi.org/10.1371/JOURNAL.PONE.0101709.

Rauch, Sabine, Portia Gough, Hwan Keun Kim, Olaf Schneewind, and Dominique Missiakas. 2014. "Vaccine Protection of Leukopenic Mice against *Staphylococcus aureus* Bloodstream Infection." *Infection and Immunity*. https://doi.org/10.1128/IAI.02328-14.

Rian, B, G F Eagan, J Ames, R Ochon, Ichard N F Edorak, E J An, I Rvine, et al. 2009. "Methotrexate for the Treatment of Crohn's Disease." *Http://Dx.Doi.Org/10.1056/NEJM199502023320503* 332 (5): 292–97. https://doi.org/10.1056/NEJM199502023320503.

Rice, Kelly C. 2016. "Creation of Staphylococcal Mutant Libraries Using Transposon Tn917." *Methods in Molecular Biology* 1353: 97–102. https://doi.org/10.1007/7651_2014_188.

Richardson, Anthony R., Stephen J. Libby, and Ferric C. Fang. 2008. "A Nitric Oxide-Inducible Lactate Dehydrogenase Enables *Staphylococcus aureus* to Resist Innate Immunity." *Science (New York, N.Y.)* 319 (5870): 1672–76. https://doi.org/10.1126/SCIENCE.1155207.

Rowe, Sarah E., Nikki J. Wagner, Lupeng Li, Jenna E. Beam, Alec D. Wilkinson, Lauren C. Radlinski, Qing Zhang, Edward A. Miao, and Brian P. Conlon. 2020. "Reactive Oxygen Species Induce Antibiotic Tolerance during Systemic *Staphylococcus aureus* Infection." *Nature Microbiology*. https://doi.org/10.1038/s41564-019-0627-y.

Roy, F. Van, and G. Berx. 2008. "The Cell-Cell Adhesion Molecule E-Cadherin." *Cellular and Molecular Life Sciences 2008 65:23* 65 (23): 3756–88. https://doi.org/10.1007/S00018-008-8281-1.

Ruffing, Ulla, Ruslan Akulenko, Markus Bischoff, Volkhard Helms, Mathias Herrmann, and Lutz von Müller. 2012. "Matched-Cohort DNA Microarray Diversity Analysis of Methicillin Sensitive and Methicillin Resistant *Staphylococcus aureus* Isolates from

Hospital Admission Patients." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0052487.

Sabri, Maurad, Simon Léveillé, and Charles M. Dozois. 2006. "A SitABCD Homologue from an Avian Pathogenic *Escherichia coli* Strain Mediates Transport of Iron and Manganese and Resistance to Hydrogen Peroxide." *Microbiology (Reading, England)* 152 (Pt 3): 745–58. https://doi.org/10.1099/MIC.0.28682-0.

Salazar, Natália, Mónica Marcela Castiblanco-Valencia, Ludmila Bezerra Da Silva, Íris Arantes De Castro, Denize Monaris, Hana Paula Masuda, Angela Silva Barbosa, and Ana Paula Mattos Arêas. 2014. "*Staphylococcus aureus* Manganese Transport Protein C (MntC) Is an Extracellular Matrix-and Plasminogen-Binding Protein." *PLoS ONE*. https://doi.org/10.1371/journal.pone.0112730.

Sause, William E., Divya Balasubramanian, Irnov Irnov, Richard Copin, Mitchell J. Sullivan, Alexis Sommerfield, Rita Chan, et al. 2019. "The Purine Biosynthesis Regulator PurR Moonlights as a Virulence Regulator in *Staphylococcus aureus*." *Proceedings of the National Academy of Sciences of the United States of America*. https://doi.org/10.1073/pnas.1904280116.

Saxena, Sneha, Naeem Khan, Ruchika Dehinwal, Ajay Kumar, and Devinder Sehgal. 2015. "Conserved Surface Accessible Nucleoside ABC Transporter Component SP0845 Is Essential for Pneumococcal Virulence and Confers Protection In Vivo." *PLOS ONE* 10 (2): e0118154. https://doi.org/10.1371/JOURNAL.PONE.0118154.

Schnaith, Annabelle, Hamid Kashkar, Sonja A. Leggio, Klaus Addicks, Martin Krönke, and Oleg Krut. 2007. "*Staphylococcus aureus* Subvert Autophagy for Induction of Caspase-Independent Host Cell Death *." *Journal of Biological Chemistry* 282 (4): 2695–2706. https://doi.org/10.1074/JBC.M609784200.

Seeger, W., R. G. Birkemeyer, L. Ermert, N. Suttorp, S. Bhakdi, and H. R. Duncker. 1990. "Staphylococcal Alpha-Toxin-Induced Vascular Leakage in Isolated Perfused Rabbit Lungs." *Laboratory Investigation; a Journal of Technical Methods and Pathology* 63 (3): 341–49. https://europepmc.org/article/med/2395329.

Sendi, Parham, and Richard A. Proctor. 2009. "Staphylococcus Aureus as an Intracellular Pathogen: The Role of Small Colony Variants." *Trends in Microbiology* 17 (2): 54–58. https://doi.org/10.1016/J.TIM.2008.11.004.

Serganov, Alexander, and Evgeny Nudler. 2013. “A Decade of Riboswitches.” *Cell* 152 (1): 17–24. https://doi.org/10.1016/J.CELL.2012.12.024.

Sinha, Bhanu, Patrice P. François, Oliver Nüße, Michelangelo Foti, Orla M. Hartford, Pierre Vaudaux, Timothy J. Foster, Daniel P. Lew, Mathias Herrmann, and Karl Heinz Krause. 1999. “Fibronectin-Binding Protein Acts as *Staphylococcus aureus* Invasin via Fibronectin Bridging to Integrin Alpha5beta1.” *Cellular Microbiology* 1 (2): 101–17. https://doi.org/10.1046/J.1462-5822.1999.00011.X.

Skurnik, David, Colette Cywes-Bentley, and Gerald B. Pier. 2016. “The Exceptionally Broad-Based Potential of Active and Passive Vaccination Targeting the Conserved Microbial Surface Polysaccharide PNAG.” *Expert Review of Vaccines* 15 (8): 1041–53. https://doi.org/10.1586/14760584.2016.1159135.

Soehnlein, Oliver, Ylva Kai-Larsen, Robert Frithiof, Ole E. Sorensen, Ellinor Kenne, Karin Scharffetter-Kochanek, Einar E. Eriksson, Heiko Herwald, Birgitta Agerberth, and Lennart Lindbom. 2008. “Neutrophil Primary Granule Proteins HBP and HNP1-3 Boost Bacterial Phagocytosis by Human and Murine Macrophages.” *The Journal of Clinical Investigation* 118 (10): 3491–3502. https://doi.org/10.1172/JCI35740.

Somerville, Greg A., and Richard A. Proctor. 2009a. “At the Crossroads of Bacterial Metabolism and Virulence Factor Synthesis in Staphylococci.” *Microbiology and Molecular Biology Reviews : MMBR* 73 (2): 233–48. https://doi.org/10.1128/MMBR.00005-09.

Stocker, B. A., S. K. Hoiseth, and B. P. Smith. 1983. “Aromatic-Dependent ‘Salmonella Sp.’ as Live Vaccine in Mice and Calves.” *Developments in Biological Standardization* 53 (January): 47–54. https://europepmc.org/article/med/6307785.

Stoll, Hartmut, Jörn Dengjel, Christiane Nerz, and Friedrich Götz. 2005. “*Staphylococcus aureus* Deficient in Lipidation of Prelipoproteins Is Attenuated in Growth and Immune Activation.” *Infection and Immunity*. https://doi.org/10.1128/IAI.73.4.2411-2423.2005.

Stryjewski, Martin E., and Henry F. Chambers. 2008. “Skin and Soft-Tissue Infections Caused by Community-Acquired Methicillin-Resistant *Staphylococcus aureus*.” *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America* 46 Suppl 5 (SUPPL. 5). https://doi.org/10.1086/533593.

Surewaard, Bas G.J., Justin F. Deniset, Franz J. Zemp, Matthias Amrein, Michael Otto, John Conly, Abdelwahab Omri, Robin M. Yates, and Paul Kubes. 2016. “Identification and

Treatment of the *Staphylococcus aureus* Reservoir in Vivo." *Journal of Experimental Medicine* 213 (7): 1141–51. https://doi.org/10.1084/JEM.20160334/VIDEO-10.

Takeichi, Masatoshi. 1977. "Functional Correlation between Cell Adhesive Properties and Some Cell Surface Proteins." *Journal of Cell Biology* 75 (2): 464–74. https://doi.org/10.1083/JCB.75.2.464.

Taylor, Anna L., Christopher J.E. Watson, and J. Andrew Bradley. 2005. "Immunosuppressive Agents in Solid Organ Transplantation: Mechanisms of Action and Therapeutic Efficacy." *Critical Reviews in Oncology/Hematology* 56 (1): 23–46. https://doi.org/10.1016/J.CRITREVONC.2005.03.012.

Terakawa, Ayako, Ayane Natsume, Atsushi Okada, Shogo Nishihata, Junko Kuse, Kosei Tanaka, Shinji Takenaka, Shu Ishikawa, and Ken ichi Yoshida. 2016. "*Bacillus subtilis* 5′-Nucleotidases with Various Functions and Substrate Specificities." *BMC Microbiology* 16 (1): 1–13. https://doi.org/10.1186/S12866-016-0866-5/TABLES/4.

Thammavongsa, Vilasack, Justin W. Kern, Dominique M. Missiakas, and Olaf Schneewind. 2009. "*Staphylococcus aureus* Synthesizes Adenosine to Escape Host Immune Responses." *Journal of Experimental Medicine* 206 (11): 2417–27. https://doi.org/10.1084/JEM.20090097.

Thammavongsa, Vilasack, Hwan Keun Kim, Dominique Missiakas, and Olaf Schneewind. 2015. "Staphylococcal Manipulation of Host Immune Responses." *Nature Reviews Microbiology 2015 13:9* 13 (9): 529–43. https://doi.org/10.1038/nrmicro3521.

Thammavongsa, Vilasack, Dominique M. Missiakas, and Olaf Schneewind. 2013. "*Staphylococcus aureus* Degrades Neutrophil Extracellular Traps to Promote Immune Cell Death." *Science* 342 (6160): 863–66. https://doi.org/10.1126/SCIENCE.1242255/SUPPL_FILE/THAMMAVONGSA.SM.PDF.

Treffon, Janina, Diego Chaves-Moreno, Silke Niemann, Dietmar Helmut Pieper, Thomas Vogl, Johannes Roth, and Barbara C. Kahl. 2020. "Importance of Superoxide Dismutases A and M for Protection of *Staphylococcus aureus* in the Oxidative Stressful Environment of Cystic Fibrosis Airways." *Cellular Microbiology* 22 (5): e13158. https://doi.org/10.1111/CMI.13158.

Tsompanidou, Eleni, Emma L. Denham, Dörte Becher, Anne de Jong, Girbe Buist, Marleen van Oosten, Willem L. Manson, Jaap Willem Back, Jan Maarten van Dijl, and Annette

Dreisbach. 2013. “Distinct Roles of Phenol-Soluble Modulins in Spreading of *Staphylococcus aureus* on Wet Surfaces.” *Applied and Environmental Microbiology* 79 (3): 886. https://doi.org/10.1128/AEM.03157-12.

Turner, Nicholas A., Batu K. Sharma-Kuinkel, Stacey A. Maskarinec, Emily M. Eichenberger, Pratik P. Shah, Manuela Carugati, Thomas L. Holland, and Vance G. Fowler. 2019. “Methicillin-Resistant *Staphylococcus aureus*: An Overview of Basic and Clinical Research.” *Nature Reviews. Microbiology* 17 (4): 203–18. https://doi.org/10.1038/S41579-018-0147-4.

Wang, Rong, Kevin R. Braughton, Dorothee Kretschmer, Thanh Huy L. Bach, Shu Y. Queck, Min Li, Adam D. Kennedy, et al. 2007. “Identification of Novel Cytolytic Peptides as Key Virulence Determinants for Community-Associated MRSA.” *Nature Medicine* 13 (12): 1510–14. https://doi.org/10.1038/NM1656.

Wertheim, Heiman F.L., Damian C. Melles, Margreet C. Vos, Willem Van Leeuwen, Alex Van Belkum, Henri A. Verbrugh, and Jan L. Nouwen. 2005. “The Role of Nasal Carriage in Staphylococcus aureus Infections.” *Lancet Infectious Diseases*. https://doi.org/10.1016/S1473-3099(05)70295-4.

Westh Hansen, Svend Erik, Nina Jensen, and Agnete Munch-Petersen. 1987. “Studies on the Sequence and Structure of the *Escherichia Coli* K-12 NupG Gene, Encoding a Nucleoside-transport System.” *European Journal of Biochemistry*. https://doi.org/10.1111/j.1432-1033.1987.tb13431.x.

Wilke, Georgia A., and Juliane Bubeck Wardenburg. 2010. “Role of a Disintegrin and Metalloprotease 10 in *Staphylococcus aureus* α-Hemolysin - Mediated Cellular Injury.” *Proceedings of the National Academy of Sciences of the United States of America* 107 (30): 13473–78. https://doi.org/10.1073/PNAS.1001815107/SUPPL_FILE/PNAS.201001815SI.PDF.

Wise, Robert I. 1964. “*Staphylococcus aureus* Infections.” *Annals of Internal Medicine*. https://doi.org/10.7326/0003-4819-61-4-808_1.

Xie, Hao, Simon G. Patching, Maurice P. Gallagher, Gary J. Litherland, Adrian R. Brough, Henrietta Venter, Sylvia Y.M. Yao, et al. 2004. “Purification and Properties of the *Escherichia coli* Nucleoside Transporter NupG, a Paradigm for a Major Facilitator Transporter Sub-Family.” *Molecular Membrane Biology* 21 (5): 323–36. https://doi.org/10.1080/09687860400003941.

Yu, Wei, Di Yao, Simiao Yu, Xintong Wang, Xiaoting Li, Mengyao Wang, Shuo Liu, et al. 2018. “Protective Humoral and CD4+ T Cellular Immune Responses of *Staphylococcus aureus* Vaccine MntC in a Murine Peritonitis Model.” *Scientific Reports*. https://doi.org/10.1038/s41598-018-22044-y.

Zakataeva, Natalia P. 2021. “Microbial 5′-Nucleotidases: Their Characteristics, Roles in Cellular Metabolism, and Possible Practical Applications.” *Applied Microbiology and Biotechnology 2021 105:20* 105 (20): 7661–81. https://doi.org/10.1007/S00253-021-11547-W.

Zecconi, Alfonso, and Federico Scali. 2013. “*Staphylococcus aureus* Virulence Factors in Evasion from Innate Immune Defenses in Human and Animal Diseases.” *Immunology Letters* 150 (1–2): 12–22. https://doi.org/10.1016/J.IMLET.2013.01.004.

Curriculum Vitae

Name: Tothong (Mai) Sonpaveerawong

Post-secondary education and degrees:

Western University

London, Ontario, Canada

2015-2020 HBSc. with 1-year internship

2020-2022 MSc.

Scholarships

- Global Opportunity Awards, $2,000, 2018
- International Learning Awards, $1000, 2018
- Dr. FW Luney Graduate Entrance Scholarship, $2,000, 2020

Honours and Awards

Dean's Honour List (2015-2020)

Related Work experience:

- Teaching Assistant
  Western University, London, ON, Canada
  Fall semester2021
- Research technologist (Khan Lab)
  Agriculture and Agri-Food Canada, Ottawa, ON, Canada
  2018-2019