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Master of Science

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ABSTRACT

Staphylococcus aureus Metal Acquisition in Milk and Mammary Gland Tissue

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Mastitis resulting from mammary gland infection is a common and painful disease associated with lactation. In addition to the impact on human and animal health, mastitis causes substantial economic losses in the dairy industry. *Staphylococcus aureus* is a frequent cause of mastitis worldwide. Despite significant progress in understanding *S. aureus* pathogenesis in general, much remains to be learned regarding virulence factors relevant in the context of mastitis. In mammary gland infections, it is not fully understood which metal acquisition systems are required for *S. aureus* survival.

To help understand molecular mechanisms by which *S. aureus* might acquire essential metals, such as iron, within lactating mammary glands, *S. aureus* mutants were tested for growth defects *in vitro.* A low-iron media (TMM) was created and supplemented with differing iron sources relevant to mastitis infection such as host iron-binding proteins lactoferrin and transferrin. Mutants were grown in the various iron sources to determine which genes were involved with iron acquisition for each specific media tested. Results show that a double knock-out (Δ*htsA/sirA::ba*) involved with two iron siderophore receptors and the ATPase, *fhuC::ba,* which powers those receptors are essential for growth in media supplemented with human lactoferrin, while mutants involved with the iron siderophore Staphyloferrin B (*sbnE::ba*) and its specific receptor (*sirA::ba*) proved important for growth in bovine lactoferrin. Additionally, *S. aureus* mutants were grown in bovine and human milk. Significant growth defects in human milk were found for mutants involved with zinc (*znuBC::ba*) and manganese (*psaA::ba*) acquisition. Iron limitations leading to growth defects were also found in Δ*htsA/sirA::ba* and *fhuC::ba* grown in human milk. Growth defects in bovine milk were seen for *psaA::ba* but not zinc genes. Growth of the *fhuC* mutant was shown to be significant, but not the double knock-out, indicating that iron acquisition in bovine milk does not involve the SirABC or HtsABC siderophore receptors. A mutant involved in purine synthesis, *purH::ba,* was also shown to have a significant growth defect in bovine milk.

The importance of *S. aureus* metal acquisition has been well established, but there is a significant need to research these multifaceted processes further. Increased understanding of how metal acquisition facilitates bacterial survival in the lactating mammary gland can provide therapeutic targets for more effective mastitis prevention and treatment.

Keywords: mastitis, iron acquisition, metal acquisition, nutritional immunity, *Staphylococcus aureus*

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1.1 Mastitis

Mastitis, or inflammation of the mammary gland, is one of the most prevalent and persistent diseases among dairy cattle [1]. Several factors can lead to inflammation of the mammary gland including an obstructed or plugged milk duct, chemical irritations, physical trauma or as a result of microorganisms, usually bacteria, which can cause intramammary infections [1,2]. In dairy herds, intramammary infections can develop and spread quickly from cow to cow through improperly cleaned milking equipment, milk handlers, and the environment.

One or more mastitis cases have been reported across essentially every dairy operation in the United States, with a clinical manifestation reportedly affecting 1 in 4 dairy cows nationwide [3]. Mastitis is a problem throughout the world, leading to substantial economic losses in the dairy industry. The reported incidence of bovine mastitis can vary greatly due to differences in diagnostic techniques and clinical definitions of the disease, as well as, due to breed, season, year, and region (Table 1).

Table 1: Incidence of mastitis around the world

Location	% Incidence of clinical mastitis	% Incidence of sub-clinical mastitis	Reference
Belgium	17.1 (quarter)	NR	[4]
Brazil	NR	46.4 (cow)	[5]
Canada	23 (cow)	NR	[6]
	20.9 (cow)	NR	[7]
China	8.7 (cow) 3.7 (quarter)	48.8 (cow) 19 (quarter)	[8]
Ecuador	12 (cow)	60 (cow)	[9]
England and Wales	47-65 (cow)	NR	[10]
Ethiopia	12.5 (cow) 10.7 (quarter)	51.8 (cow) 46.4 (quarter)	[11]
India	25.63-97.61 (cow)	NR	[12]*
	NR	46.35 (cow) 23.24 (quarter)	[13]
Netherlands	32.2 (cow)	71.2 (cow)	[14]

Sweden	10 (cow)	NR	[15]
United States	24.8 (cow)	NR	[3]

Recent reports highlighting the average incidence rate of mastitis from around the world. Clinical and subclinical rates are reported on a quarter or cow basis as indicated. It is important to note that differences in diagnostic techniques and clinical definitions of mastitis may impact reported cases of the disease.

NR – Not reported in the indicated study

*The indicated reference is a review

Clinical mastitis leads to severe pain, inflammation, and hardening of the mammary tissue and can be accompanied by milk abnormalities (flakey, watery and/or purulent milk), fever, fatigue, and changes in animal behavior [16-18]. It also leads to an increase of somatic cells, or white blood cells, in the infected mammary gland [18,19]. Somatic cell counts (SCC) are commonly used within the dairy industry as an indication of intramammary infection. Uninfected cows generally have an individual milk SCC ≤70,000-100,000 cells/mL [1,19,20]. This number does fluctuate depending on age and days of lactation, therefore, an increased SCC level of ≥200,000 cells/mL is commonly accepted as a marker for mastitis [1,19]. This cell count threshold does not indicate a clear line between healthy and infected mammary glands, but rather offers a practical operational value for milk handlers to estimate the likelihood that a cow is infected. This somewhat arbitrary marker fails to identify numerous cows with low SCC but with active infections as identified by culture [21,22]. The term subclinical mastitis is used to describe mammary gland infections that are culture positive yet lack any visible symptoms or obvious changes in the milk. Practical limitations make identifying every case of subclinical mastitis very difficult and these cases may spontaneously resolve, progress and develop into clinical mastitis or persist chronically at subclinical levels. Additionally, untreated subclinical infections serve as a reservoir of infection for other members of the herd.

Mastitis infections lead to increased treatment and labor costs, lowered milk quality, and overall lowered milk production rates [18,23,24]. Antibiotic treatment of mastitic cows is often the highest out-of-pocket expenditure due to mastitis, however, the loss of revenue as a result of

decreased milk production has been shown to have the largest economic impact to the dairy industry [23,25]. For example, Hand *et al* showed that for every 24 hours SCC exceeded 200,000 cells/mL an estimated 0.35 to 1.09 kg of milk production was lost from that cow; as SCC increased so did the loss of milk production with the greatest losses effecting the highest milk-producing cows and those with higher parity number [24]. Milk loss increased as the average overall SCC for the lactation period increased, however, even having low SCC (between 100,000-200,000 cells/mL) for 5 or more consecutive days was a significant risk factor for milk loss [24].

Mastitis is also a concern for nursing women within all countries and socioeconomic backgrounds. An estimated 10-20% of mothers will develop mastitis during some point of lactation [26-32]. Along with the pain and swelling of the infection, mastitis can result in abscess formation which may require antibiotic treatment and surgical drainage of the infected site [33-36]. Although it is usually a self-limiting disease, mastitis can lead to chronic or recurrent infections and in rare cases can cause severe clinical disease [37-42].

1.2 *Staphylococcus aureus*

S. aureus is a serious pathogen causing a variety of severe diseases ranging from skin to heart infections [43]. Additionally, it's an important causative agent of both human and bovine mastitis [3,6,12,15,36,44,45]. The efficiency with which *S. aureus* is able to invade and colonize many different tissue types can be attributed to its wide array of virulence factors which promote nutrient uptake from the host, as well as, tissue adherence, invasion, and destruction [2,43,46]. The ability of *S. aureus* to colonize and cause disease within the mammary gland is well established, yet much remains to be learned regarding virulence factors that promote mastitis.

Due to the clear economic impact of mastitis on the dairy industry and the public health concerns associated with mastitis in nursing mothers, there is a significant need to understand the

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fundamental processes which promote *S. aureus* colonization and pathogenicity in the mammary gland. The purpose of this introduction is to highlight advances and gaps in our current understanding of pathogenic factors that promote *S. aureus* mastitis. Countless components contribute to the success of these bacteria, all of which cannot be addressed here. Rather, special focus will be given to those factors influencing initial establishment of infection and nutrient acquisition required for bacterial survival.

1.3 Pathogenesis of *S. aureus* mastitis

1.3.1 Colonization

S. aureus strains can be isolated from bovine teat, nasal, and vaginal flora, milk handlers, milking equipment, and the dairy farm environment [47]. The association between colonization of these sites and intramammary infections remains a topic of discussion. *S. aureus* colonization of the teat skin has been linked as a potential reservoir for the bacterium in mammary infections [48-51]. However, conflicting reports indicate a significant difference between genotypes isolated from mastitic milk and those found on the skin, concluding that although skin-strains can act as opportunistic pathogens to cause intramammary infection, generally milk-strains are the main cause of *S. aureus* mastitis [52-54]. Milk-strains can be isolated from the teat skin of infected cows after milking and from the milking equipment used on that cow providing a relevant source of *S. aureus* contamination to spread within the herd [53].

The teat itself is comprised of a thick epidermis layer, with keratin to give the teat both the flexibility and rigidity it needs to endure suckling, as well as a secondary internal dermis layer containing the nerves and blood vessels. Unlike normal skin, the teat does not contain any oil or sweat glands to keep it moist, so it is prone to drying out and cracking which can increase susceptibility to infection [17]. On the inside, the rosette of Fürstenberg is formed by folds in the

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mucosa of the teat canal and designates the junction between the teat cistern and teat canal. The folds allow for the uninhibited flow of milk down, while providing a barrier against upward travel of debris that might have entered the teat canal during milking. The teat canal is relatively small (averaging 10.3 mm in length and 1.45 mm in width) and surrounded by a circular sphincter muscle to keep the teat tightly closed between milkings [55]. Milking loosens these muscles and elongates the teat canal length. Complete recovery of the teat after milking can take more than 8 hours, and frequent milking (often multiple times in a day) leaves the teat vulnerable to potential bacterial contamination during those long recovery times [56,57]. Despite efforts to maintain cleanliness before and after milking, the teat canal remains the main point of entry for bacteria into a mammary gland.

Although 20-30% of the human population is colonized by *S. aureus* within the nasal passages, no association between nasal colonization in mothers and the development of mastitis has been found [26,58,59]. However, women who experience nipple damage while nursing, or have *S. aureus* isolates on the breast tissue and nipple, or in expressed milk, do have an increased risk of mastitis [26,59]. Interestingly, a significantly higher rate of infant nasal colonization of *S. aureus* was observed for mothers with mastitis [26,59]. As maternal to infant transmission is one of the main routes of *S. aureus* colonization during the early postnatal period, nursing infants can potentially act as a source for transfer of *S. aureus* back to the mother and into the mammary gland [60-62].

1.3.2 Adherence

As the bacteria spread from the teat canal, they gain access to the proximal parts of the mammary gland where they can adhere to the ductular and alveolar epithelium including the extracellular matrix (ECM). Adherence of *S. aureus* to bovine mammary gland cells has been

documented in numerous *in vivo* and *in vitro* studies (reviewed by [2]). *S. aureus* possesses over 20 different cell-wall-anchored proteins with an array of functions to help in adhesion, biofilm production, invasion, and protection from host immune response (reviewed by [63,64]). Many of these proteins are classified as <u>m</u>icrobial <u>s</u>urface <u>c</u>omponents <u>r</u>ecognizing <u>a</u>dhesive <u>m</u>atrix <u>m</u>olecules (MSCRAMMs), which aid in the attachment of *S. aureus* to the ECM. Fibronectin-binding proteins A and B (FnBPs) bind both fibronectin and fibrinogen, and bone sialoprotein-binding protein also binds fibrinogen [65-69]. Collagen-rich tissues are abundant along larger milk ducts in mammary glands and to a small degree at the distal branch points. Collagen binding protein (Cna) may be important at these locations, and carriage of the *cna* gene is prevalent among bovine mastitis *S. aureus* strains [70,71].

The ability of *S. aureus* to adhere has been shown to be strain-dependent [2,72,73]. Differences in the production of adhesions, growth phase, cell type, and growth media might account for these variations, as well as the presence or absence of a capsule [2,72-74]. Strains producing an exopolysaccharide capsule have difficulty adhering to epithelial cells and collagen, while the effects of α-toxin (Hla) and β-toxin (Hlb) on epithelial cells increased the ability of *S. aureus* to bind the cells [72,74,75].

1.3.3 Invasion

Adhesion is an important first step for invasion of epithelial cells. When FnBPs bind fibronectin they form a bridge between the bacterial FnBP and the host fibronectin receptor integrin $\alpha_5\beta_1$ [76,77]. FnBPs are enough to trigger successful invasion into bovine mammary epithelial cells, while the lack of these adhesins prevents the ability of *S. aureus* to invade [76,78,79]. Attachment onto the host cell activates a signaling cascade to alter the actin cytoskeleton near the plasma membrane. This leads to the formation of an actin cytoskeleton

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pocket which engulfs the bacterium in a process resembling professional phagocytosis and is thought to occur without any active bacterial processes [76,80-83]. The ability to invade epithelial and endothelial cells, fibroblasts, keratinocytes, and osteoblasts is highly conserved among *S. aureus* strains, highlighting its success as a facultative intracellular pathogen [84-87].

Upon cell invasion, the phagosome is broken down by β-toxin (Hlb) and δ-toxin (Hld), releasing *S. aureus* into the cytoplasm [88,89]. Recently, *S. aureus* infection was found to induce autophagy within bovine mammary epithelial cells, however, proper fusion of the autophagosome and lysosome is prevented thereby avoiding *S. aureus* lysosomal degradation and allowing the bacteria to continue replicating within the autophagosome [90]. Autophagy can be induced as a response to intracellular infection, but more commonly as a catabolic mechanism in response to cell stress, nutrient starvation, and as a means of recycling organelles [91]. *S. aureus* invasion alters cell metabolic levels, lowering glucose and essential amino acids which may induce autophagy as an additional means of nutrient scavenging and energy acquisition within the cell to aid in intracellular survival for the bacteria [92].

The majority of bovine mammary epithelial cells infected with *S. aureus* undergo apoptosis within 18 hours of infection [89]. In fact, some cells showed signs of apoptosis within as little as 2 hours; however, not all strains of *S. aureus* are capable of inducing apoptosis [85,89,93]. Conversely, *S. aureus* may actively prevent apoptosis when infecting macrophages, helping the infected cell live longer and allowing bacteria to disseminate into deeper tissues [20]. Living *S. aureus* cells have been isolated from bovine alveolar cells and macrophages obtained from chronically infected cows, highlighting the likelihood of intracellular persistence during infection [94].

1.3.4 Biofilms and persistence

S. aureus may also form aggregates or microcolonies at the surface of epithelial cells. Aggregates are recalcitrant to phagocytosis and can lead to the formation of biofilms which offer the bacteria even greater protection [2,95]. Biofilm formation has increasingly been recognized as an important factor in chronic mastitis [95,96]. Mature biofilms produced by *S. aureus* can be held together by polysaccharide, which requires the *icaADBC* locus, in a biofilm matrix known as polysaccharide intercellular adhesin/poly-N-acetylglucosamine (PIA/PNAG) [97]. The *ica* locus genes are highly prevalent among *S. aureus* clinical bovine isolates, however the presence of these genes does not guarantee biofilm will be produced *in vitro* [98,99].

Alternatively, PIA/PNAG-independent biofilms may form where protein and DNA primarily comprise the extracellular matrix [95]. A surface protein, so far found only in bovine mastitis strains and designated as Bap (biofilm-associated protein), induces biofilm formation independent of the *ica* cluster *in vitro* [95,100]. Strains expressing Bap often result in lower SCC, allowing the infection to persist without detection, causing the host to become a reservoir for the spread of the disease throughout the herd [95]. The genes encoding for Bap are located on a pathogenicity island (SaPIbov2) in which toxin genes (those encoding for toxic shock syndrome toxin, among others) have been replaced with a transposon containing Bap. The inactivation of toxin production may account for the less severe reaction and lowered SCC production, however, the prevalence of Bap among dairy isolates remains very low, making it difficult to draw definitive conclusions regarding the pathogenesis of Bap-positive strains [95,99,101-103].

1.3.5 Toxins

Bovine mastitis strains of *S. aureus* have been shown to express various combinations of α-toxin (Hla/Hly), β-toxin (Hlb), λ-toxin (HlgAB, HlgCB), δ-toxin (Hld), leukocidins (LukMF', LukSF-PV, LukED, LukAB), enterotoxins (SEA, SEB, SEC, SED, SEG, SEI, SEJ, SEM, SEN,

SEO, SEU), and toxic shock syndrome toxin (TSST-1) [2,71,104-107]. Testing mastitis isolates for toxins revealed a high percentage of genes for hemolysins present in the samples: Hla (97.4%), Hlg (88.8%), Hld (87.9%), Hlb (76.7%), and 64.6% of samples were positive for all [107]. However, actual expression of these genes may vary. For instance, Hlb was seen highly expressed while Hla expression was lowered in bovine mastitis strains in comparison to human nasal and septicemia isolates [108].

Mastitis strains harboring multiple enterotoxins and leukocidins are common, as well as the TSST-1 gene [107,109,110]. LukMF' is highly secreted during mastitis infections, especially clinical infections, and has been identified as the main killing agent of bovine neutrophils [105,111,112]. LukSF-PV (Panton-Valentine Leukocidin) induces rapid expression of neutrophil extracellular traps (NETs) [113,114]. Unfortunately for the host, *S. aureus* also produces a nuclease to degrade those NETs and the degradation products of this interaction can then be converted to deoxyadenosine by adenosine synthase (AdsA) which induces apoptosis in macrophages [115,116].

Hemolysins and leukocidins also aid in bacterial growth by lysing host cells and thus gaining greater access to essential nutrients such as iron. The cell damage and necrosis induced by these toxins can lead to greater inflammation and an influx of polymorphonuclear neutrophils (PMNs) which can lead to even more cell damage [2,75,106,117,118]. Continued toxin secretion can lead to scar tissue formation in the mammary gland and permanently reduce milk production [119].

1.3.6 Nutrient acquisition

Iron, zinc, manganese, copper, cobalt and nickel play critical roles in cellular functions necessary for bacterial survival. Host mechanisms that limit the availability of metals, or

alternatively, expose bacteria to toxic levels, are an important feature of innate immunity, collectively known as nutritional immunity. In response, bacteria have evolved elaborate mechanisms to overcome or bypass the nutritional immune defenses of the host, and many of the genes involved in metal homeostasis are critical virulence factors during infection.

Despite the clinical and economic importance of *S. aureus* induced mastitis, we know surprisingly little about how this bacterium acquires essential metals in the lactating mammary gland. Significant progress has been made in our understanding of *S. aureus* metal acquisition in other infections which may also be relevant during mastitis (reviewed by [120-122]). However, given the unique physiology and host sequestration proteins present in lactating mammary glands, the metal scavenging mechanisms employed by *S. aureus* in milk and mammary glands are likely different from other tissues. Potential mechanisms of metal acquisition in the mammary gland will be discussed herein. For more information on bacterial strategies to overcome metal toxicity please refer to previous reviews [121,122].

1.3.6.1 The role of host iron sequestration in nutritional immunity

Systemic iron levels are strictly regulated within the host. The majority of iron is stored intracellularly bound to ferritin or heme in erythrocytes. Additionally, extracellular iron-binding proteins such as transferrin and lactoferrin bind residual free iron in serum and secretory fluids respectively (reviewed by [46,123]). A significant portion of iron in milk is bound by lactoferrin and iron limitation caused by lactoferrin has bacteriostatic effects on *S. aureus in vitro* [124]. Human lactoferrin is reported to bind Fe^{3+} atoms with a K_D ~10^{-20} M [125,126]. Lactoferrin is comprised of two domains or lobes, each capable of binding one iron ion [127]. Bovine lactoferrin has approximately 66% and 73% sequence homology to human lactoferrin within the N-lobe and C-lobe iron-binding regions respectively [127]. The amount of lactoferrin present in

milk varies drastically between species. Human milk contains some of the highest known concentrations of lactoferrin (1.0-5.0 mg/mL), while cow milk contains between 0.01–0.1 mg/mL [127]. At the beginning of bovine lactation (excluding colostrum), lactoferrin levels are low but increase gradually throughout the lactation period, reaching highest levels during involution (dry-off) [128-131]. Surprisingly, this is in direct contrast to reported human lactoferrin levels in breastmilk, which are highest during early lactation [132,133]. Lactoferrin concentrations in bovine and human milk rapidly increase as a result of mammary gland infection [128,134-137]. This infection-induced lactoferrin response can be attributed to increased production from mammary epithelial cells and an influx of neutrophils during inflammation [127,134]. Lactoferrin is a major constituent of secondary neutrophil granules released at sites of infection [138,139].

Lactoferrin also has direct antibacterial activity independent and complementary to its metal sequestration effects (reviewed by [140]). These additional properties have been associated primarily with the apo- (iron-free) state of lactoferrin [141,142]. Proteolytic cleavage of apolactoferrin results in formation of the antibacterial peptide lactoferricin which is largely responsible for the antibacterial properties of lactoferrin and exhibits stronger activity against a wide range of bacteria, including *S. aureus,* than its parental apolactoferrin [143-146]. Bovine lactoferricin has greater bactericidal activity against *S. aureus* than human lactoferricin tested *in vitro* [147]. Strain variations and stage of growth phase may also impact sensitivity of *S. aureus* to lactoferrin killing [148]. Expression of IsdA helps to protect the bacteria by binding lactoferrin at its surface [149].

In addition to lactoferrin, a significant portion of iron in milk is bound to citrate. Citrate is abundant during early lactation and decreases significantly during mastitis [150-152]. Citrate competes with lactoferrin for iron in the milk to form a ferric dicitrate complex. Ferric citrate

dissociation constants vary by the formation of different compounds, but the most biologically relevant form is reported to have a $K_D \sim 10^{-33}$ M at a pH of 7.4 [153].

1.3.6.2 Bacterial iron scavenging

Bacteria have developed sophisticated mechanisms to overcome iron limitation imposed by the host during infection. For example, bacteria may import heme, lactoferrin or transferrin directly. Additionally, they may produce chelators such as siderophores that compete with host proteins for iron or import ferric citrate. Very little is known about bacterial iron scavenging during mammary gland infections compared with other tissue sites.

Recently, the ability to utilize ferric citrate as an iron source was established as an important fitness factor for mammary pathogenic *Escherichia coli* (MPEC) strains [154,155]. Ferric dicitrate can be utilized by *E. coli* encoding a specific receptor (FecA). Mutations in the *fecABCDE* operon result in bacteria which grow poorly in milk and are severely attenuated in a mouse mammary gland model of mastitis [154]. In this study, control experiments using non-lactating mouse mammary glands demonstrated that the growth defect seen in these bacteria was lactation dependent [154]. Later experiments confirmed the importance of FecA in bovine mammary gland infections [155]. *S. aureus* does not have known genes homologous to the *fecABCDE* operon, and specific mechanisms by which *S. aureus* may utilize citrate bound iron have not been elucidated. However, given the abundance of ferric citrate in milk, it would not be surprising to find that successful mastitis-associated *S. aureus* lineages have also acquired mechanisms to exploit this readily available iron resource.

S. aureus produces two high affinity iron-scavenging siderophores designated as staphyloferrin A (SA) and staphyloferrin B (SB) (reviewed by [46]). Production of SA and SB contributes to virulence in murine kidney and intravenous infections, and are produced *in vivo*

within heart, liver and kidney abscesses [156-159]. Notably, citrate is a critical structural component of both SA and SB siderophores. In fact, staphyloferrin A is comprised of two molecules of citrate linked with D-ornithine via amide bonds [160]. Staphyloferrin B is comprised of L-2,3-diaminopropionic acid (Dap), 1,2-diaminoethane (Dae), citric acid, and α-ketoglutaric acid [161,162]. Staphyloferrin A is produced by the gene products encoded by the *sfa* operon, while staphyloferrin B is encoded on the *sbn* operon [156,163]. These operons, as well as their siderophore receptors, are tightly regulated by the ferric uptake regulatory protein (Fur) which serves as a repressor when iron is readily available [46,156,163-165]. In low iron conditions, Fur repression is relieved and both SA and SB siderophores may be produced. Interestingly, the production of SB is also influenced by a heme-responsive regulator encoded within the *sbn* operon [166]. Production of SA relies upon citrate produced by the TCA cycle, which paradoxically is down regulated in low iron conditions [167]. The functions of SA and SB may overlap to some extent, but the regulatory differences existing between them indicate they are likely not redundant. Non-uniform production and distribution of SA and SB have been reported in different tissue abscesses emphasizing differences in the potential regulation of the two siderophores [159]. It remains to be determined which metabolic pathways predominate and which, if any, siderophores are produced during *S. aureus* growth in milk.

Iron-loaded siderophore binding induces a conformational change in the membrane ABC transporters for the uptake of the iron-bound siderophore into the cell [168,169]. The transporter SirABC is highly specific for the uptake of Fe^{3+}- SB, while HtsABC can sustain the transport of both Fe^{3+}- SA and heme (Figure 1) [157,163,168,169]. Stimulation of growth by siderophore-mediated iron acquisition has been linked with virulence of *S. aureus* [121,156,158]. However, the role of SA, SB and their receptors in the context of mammary gland infections remains

unknown. Perhaps the structural similarity of SA and ferric citrate may also allow ferric dicitrate transport via HtsABC (Figure 1). The Hts transporter, initially named for its role as a heme transport system, is important in the uptake of heme via the HtsBC permeases, and disruption of either of those genes leads to attenuated virulence in intestinal infections of *C. elegans*, and IV infections of mice [157]. Expression of SirABC has been linked with increased resistance to oxidative stress and macrophage killing [170].

Figure 1: Known and potential iron uptake mechanisms of *S. aureus*

Solid arrows indicate the path of iron source and its corresponding uptake mechanism, dashed arrows indicate potential uptake mechanisms. Figure modifed from [46].
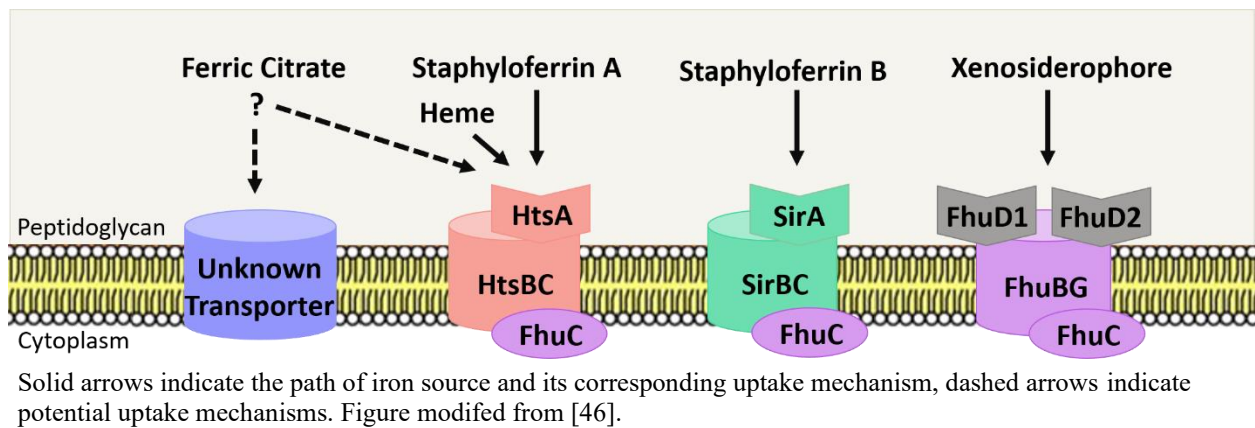
S. aureus siderophores are essential for the use of holo- (iron saturated) transferrin in human serum and mouse intravenous infections [158,171,172]. Transferrin levels in bovine milk average 0.03 mg/mL throughout the milking cycle with a slight increase noted during infection [128]. This is the equivalent of the average lactoferrin concentration in bovine milk at the start of lactation; however, lactoferrin increases 5-6 times that amount by late lactation or during infections [128]. It is unclear if the transferrin found in milk during lactation is iron saturated but could be a potential source of iron.

When available, *S. aureus* can also utilize xenosiderophores produced in mixed bacterial populations. These include ferrichrome, aerobactin, ferrioxamine B, and coprogen which can be

taken up via the *S. aureus* surface lipoproteins FhuD1 and FhuD2, and their ABC transporter complex FhuCBG (Figure 1) [46,173-175].

The iron-responsive surface determinant (Isd) system (Figure 2) also plays an important role in overcoming iron limitation during infection. Encoded within five operons, this system binds heme, hemoglobin and even hemoglobin-haptoglobin complexes when available, with an ancillary role for the direct uptake of holo-transferrin in the blood (via the IsdA protein, which has also been shown to bind lactoferrin) [46,171,176-179]. Briefly, the IsdA, IsdB, and IsdH surface hemoproteins mediate the transfer of heme to IsdC and/or the IsdE heme-receptor lipoprotein. Transport through the cell membrane via IsdDF permeases allows heme degradation by IsdG and IsdI enzymes for the release of iron into the cell [46,177].

Figure 2: The Isd system

The movement of heme through the Isd system into the cytoplasm, where it is broken down into iron and Staphylobilin. *Transferrin (Tn) and lactoferrin (Ln) binding can occur via IsdA. Figure modified from [46].

Heme sources in milk may include lactoperoxidase, an iron containing hemoprotein. Lactoperoxidase produces oxyacids such as hypothiocyanite which can be damaging to bacteria [180,181]. While *S. aureus* has defenses against these antimicrobial reactive oxygen species, it is unknown if the bacteria can utilize the iron-heme component within lactoperoxidase. Heme found within epithelial cells or leukocytes may also be accessible to *S.*

aureus strains that produce lytic toxins [2,118,182]. Levels of haptoglobin, the host binding protein of hemoglobin, rise in bovine milk after *S. aureus* mastitis, supporting the idea that hemoglobin released from damaged cells during the course of infection represents an iron source available to these bacteria [113,183].

1.3.6.3 Additional metals

Other first row transition metals such as zinc, manganese, copper, nickel and cobalt are required for a multitude of cellular processes. However, just as with iron, these important transition metals are concealed from bacteria by various components of host nutritional immunity. For example, metal uptake by host cell transporters in response to infection and intracellular binding by metallothioneins can limit their availability (reviewed by [184,185]).

In extracellular fluids, the S100 family of EF-hand calcium-binding proteins are critical factors of nutritional immunity. In addition to binding calcium, these homo- or heterodimer proteins have a high affinity for transition metals. Psoriasin (S100A7) is produced by keratinocytes and is highly expressed in psoriasis patches and some breast cancers; it is also found abundantly in mammary epithelia, normal goat milk and the bovine streak canal (located at the end of the teat canal) [186-190]. Treatment with even low levels of psoriasin (10 µg/mL) can markedly inhibit *E. coli* growth by sequestering zinc; however, a reduction in *S. aureus* growth occurs only at much higher concentrations (250 µg/mL) [191-193]. It is unclear if physiological levels of psoriasin in the bovine streak canal are high enough to inhibit *S. aureus* growth *in vivo* [189]. Calgranulin C (S100A12) sequesters zinc and copper and is predominately secreted by neutrophils [194,195]. Calgranulin C gene expression is significantly upregulated in mammary gland cells when challenged with *S. aureus,* lipopolysaccharide (LPS) or peptidoglycan (PGN), and during subclinical or acute mastitis [113,196-198]. Calprotectin (S100A8/A9) is also

abundant within neutrophil granules; in fact, about half of the soluble protein within the cytosol of neutrophils is calprotectin [199-201]. This protein tightly binds iron, zinc, nickel and manganese, and its importance in binding to transition metals during infection has been well documented [202-208]. Calprotectin and calgranulin C also have cytokine-like properties, signaling through RAGE and/or TLR4 receptors, resulting in proinflammatory gene expression [209,210]. Calprotectin is present at high levels in normal human breast milk immediately after birth [208]. Vaginal rather than cesarean delivery is correlated with higher calprotectin concentrations in the mother's breastmilk and corresponding limitation of *S. aureus* growth, which has been specifically attributed to manganese starvation [208]. Neutrophils deliver large amounts of calprotectin to sites of infection, and this is reflected in milk samples during *S. aureus* mastitis [113]. Within the context of tissue abscesses, calprotectin limits *S. aureus* growth by sequestering manganese which *S. aureus* uses as a cofactor for superoxide dismutase to combat neutrophil oxidative killing [205]. An influx of neutrophils after *S. aureus* colonization of mammary glands may be expected to further increase calprotectin levels in milk. The cumulative effects of S100 family proteins in normal cow milk, as well as their individual roles in limiting *S. aureus* mastitis, have not been adequately investigated.

In addition to the iron-binding properties of lactoferrin discussed earlier, lactoferrin also binds a variety of other metals. Most of the manganese found in human milk is bound to lactoferrin; however, in bovine milk the majority of manganese is bound to other ligands [211]. Zinc in human milk is bound in large quantities to high molecular weight protein complexes, which contain high amounts of lactoferrin, while casein binding of zinc is predominate in bovine milk [212]. Human lactoferrin has also been shown capable of binding copper and cobalt [213].

Many pathogens, including *S. aureus,* utilize high-affinity membrane transporters to

compete with host restriction of specific metals (Table 2). These include the manganese

transporters MntABC and MntH, the zinc transporter AdcABC (also annotated as ZnuABC in

Table 2: Host sequestration and *S. aureus* metal acquisition mechanisms

Metal	Extracellular Host Sequestration	Bacterial Acquisition Transporter / Siderophore (if applicable)
Cobalt (Co)	Lactoferrin[a,b] [127,128,132,213]	CntABCDF / Staphylopine [214,215]
Copper (Cu)	Calgranulin C (S100A12)[b] [113,194,197,198] Lactoferrin[a,b] [127,128,132,213]	CntABCDF / Staphylopine [214,215]
Iron (Fe)	Heme & Hemoglobin [179] Lactoferrin[a,b] [127,128,132] Transferrin [128] Calprotectin (S100A8/A9)[a,b] [113,206,207] Citrate[a,c] [150-152]	HtsABC / SA[d] [157,163] SirABC / SB[d] [156,168] Isd system[d] [177,178] CntABCDF / Staphylopine [214,215]
Manganese (Mn)	Calprotectin (S100A8/A9)[a,b] [113,204,206,208] Lactoferrin[a,b] [127,128,132,211]	MntABC[d] [216] MntH[d] [216]
Nickel (Ni)	Calprotectin (S100A8/A9)[a,b] [113,202,206]	NikBCDE[d] [217] NikA[d] [217] NixA [217] CntABCDF / Staphylopine [214,215]
Zinc (Zn)	Psoriasin (S100A7) [186,189,193] Calgranulin C (S100A12)[b] [113,195,197,198] Calprotectin (S100A8/A9)[a,b] [113,206,208] Lactoferrin[a,b] [127,128,132,212] Casein[a] [212]	AdcABC (ZnuABC)[e] [214] CntABCDF / Staphylopine [214,215]

Components of host nutritional immunity limiting essential transition metals and the corresponding bacterial mechanisms to uptake these metals. Additional references can be found within the main text.

[a] Naturally abundant in milk (Note: concentrations may depend on stage of lactation and species)

[b] Upregulated during mammary gland infection

[c] Downregulated during mammary gland infection

[d] Known virulence factor during *in vivo* murine infections

[e] Unpublished data from our lab indicates this may be important during growth in milk

some bacteria), and nickel transporters NikBCDE, NikA and NixA [214,216,217]. Manganese has been demonstrated to be a limiting factor for growth of *S. aureus* in human breast milk, and depletion of calprotectin clearly increases bacterial growth [208]. MntABC and MntH are necessary to overcome manganese sequestration in the context of murine systemic infections and abscess models [204,216,218,219].

Another transporter (CntABCDF) was originally named for its ability to import cobalt and nickel [220]. However, more recent work revealed that *S. aureus* secretes a novel metallophore called staphylopine (StP), which can bind zinc, cobalt, nickel, copper, and iron, and these StP-metal complexes are imported through the CntABCDF complex (Table 2) [214]. *In vivo*, the Cnt transporter appears to be important in binding zinc, which allows *S. aureus* to overcome zinc limitation induced by calprotectin in mouse bloodstream infections [214].

1.4 Host and strain specificity

S. aureus is seen to have a high degree of host specificity, with mobile genetic elements (MGEs) and core variable (CV) genes giving the most diversity between species-specific strains [221,222]. There are ten common human lineages of *S. aureus* which contain variations of hundreds of genes, especially in regards to MSCRAMMs [223]. Different surface profiles allow each strain to potentially interact with the host in a unique way. A comparative genomic study found bovine mastitis isolates were predominately within the ST151, ST771 and ST97 lineages (livestock-associated lineages), although some isolates from bovine mastitis cases were from lineages previously associated with humans, including one isolate from the MRSA CC1 lineage [221]. Another study also showed that isolates from bovine mastitis cases tended to be closely related by pulsotype and grouped together, while human *S. aureus* isolates showed more diversity and were spread among different groups [224]. Both human and bovine isolates had many

virulence genes in common, including *hla, hld, fnbA, clfA,* and *icaA,* however, a significantly higher proportion of bovine isolates also had *hlb* and *cna* [224]. Additionally, siderophore production was significantly higher in mastitic strains (regardless of host origin) when compared with controls [224].

The idea that the majority of mastitis cases are the result of only a few clones around the world is a long standing hypothesis, however, specific genotypic patterns and types can vary by region making it difficult to pinpoint specific, consistent differences among *S. aureus* isolates of bovine mastitis [71,225,226]. Additionally, there is a lack of conformity among *S. aureus* researchers regarding the method of categorization. Different studies refer to *S. aureus* pulsotype, sequence type, cluster type, phage type, spa type, exotoxin genotype and more, making it difficult to easily draw reliable conclusions across multiple studies.

1.5 Treatment and antibiotic resistance

Treatment and prevention plans to combat mastitis are responsible for the majority of antibiotics routinely administered to dairy cattle even though the effectiveness of antibiotic treatment is highly variable depending upon the infectious pathogen, stage of lactation, breed of cattle and additional factors [1,227,228]. The most common treatment for mild to moderate mastitis cases are intramammary antibiotic tubes, but these have limited applications for treating different causes of mastitis. Commercially, there are only two antimicrobial classes available for bovine mastitis treatment: β-lactams (amoxicillin, ceftiofur, cephapirin, cloxicillin, hetacillin, and penicillin) and a lincosamide (pirlimycin). Blanket therapy of a herd with intramammary antimicrobial drugs at dry off is a common practice to prevent mastitis, and 80% of all dairy operation in the US reportedly treat every cow regardless of mastitis status or history [3].

It has been shown that culturing milk samples as part of the mastitis treatment plan reduces the amount of antibiotics used by 50% [229]. However, a relatively small proportion of dairy farms in the United States report frequently culturing milk samples for cows with clinical mastitis or high somatic cell counts (18% and 17% respectively) [230]. A study conducted in Wisconsin discovered that the majority (62.1%) of intramammary treatments were given erroneously to cases where the causative agent of mastitis was *E. coli* (drug ineffective against gram-negative organisms) or in cases where no bacterial growth was seen in the sample [231].

Most women suffering with mastitis are also prescribed antibiotics, often without cultures being performed [28]. Common treatments include cephalexin, amoxicillin, ampicillin, augmentin, dicloxacillin and flucloxacillin, or in the case of MRSA, vancomycin and trimethoprim/sulfamethoxazole [28,232]. If no improvement occurs within 48 hours of antibiotic administration culture and MIC testing is recommended [232]. Many doctors will also encourage the use of hot compresses, or changes in breastfeeding techniques, position and frequency to help alleviate symptoms.

MRSA and multidrug resistant (MDR) *S. aureus* is a growing concern. In 2017, one study reported 31% of community-associated *S. aureus* human isolates (nasal, skin tissue) were MDR and most of those fell within the ST8 (USA300) lineage, a common community-associated MRSA strain [233]. Around 42% of the USA300 strains isolated were MDR [233]. During lactation, MRSA has been shown to pass through breastmilk to preterm infants and sometimes lead to serious, even fatal, clinical diseases [37,38]. A study spanning 7 years found that 81% (17 out of 21 cases) of MRSA strains which they isolated from women with mastitis during that time occurred during the last year of the study, the authors concluded that this may demonstrate increasing occurrence [34]. Others have also reported increasing rates of MRSA [234-236].

Incidence of reported MRSA bovine mastitis cases varies greatly. A study encompassing 50 Minnesota dairy farms had only 1.3% of isolates from bulk milk analyses confirmed as MRSA, while another in China reported 47.6% of *S. aureus* dairy isolates to be *mecA*-positive (indicating methicillin resistance) yet clinically many of these strains were still susceptible to oxacillin [237,238]. Methicillin and multidrug resistant strains continue to be found worldwide in milk and there is a growing concern of zoonotic transfer between infected cattle and milk handlers [239,240]. In 2007, the first human-bovine transmission of MRSA was reported, although it is unclear if the cow or the human was the initial source of MRSA [241].

1.6 Conclusion

The complexity and diversity of factors involved in *S. aureus* mastitis infections are impressive. Recent discoveries have added to the general understanding of *S. aureus* pathogenicity in bovine mastitis, but much more work remains to be done. In mammary gland infections, it is not fully understood which metal acquisition systems are essential for *S. aureus* survival. Individual strains may differ in their sensitivities to shortages of specific metals and significant differences between human and bovine milk composition adds additional factors to consider when conducting mastitis studies. Furthermore, it is unclear which components of milk are utilized as a source of iron or other metals by the bacteria and if these sources remain the same between species. Much of what we know regarding host nutritional immunity is human specific, and bovine homologs remain either unknown or understudied.

Determining which specific bacterial systems are responsible for metal acquisition is complicated as most metals are bound and transported by redundant systems, indicating multiple mutations may be necessary to establish their roles *in vivo*. This redundancy of function highlights the importance of these metals in *S. aureus* survival and underscores the need to understand these

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multifaceted processes. Determining available sources of iron within milk and the bacterial genes required to utilize these iron sources is of key importance to the field of mastitis and the focus of this thesis. Understanding the unique factors that promote *S. aureus* iron and metal acquisition in mammary glands can help lead to the production of novel vaccines, and additional treatment methods for this difficult and costly disease.

SECTION 2: EXPERIMENTAL PLAN

Based on previous work demonstrating the importance of iron binding proteins and nutritional immunity in other tissues, I hypothesized that lactoferrin and ferric citrate would bind the majority of iron in milk and the lactating mammary gland. Accordingly, I hypothesized that siderophores and siderophore receptors would be needed for *S. aureus* to effectively acquire iron in these environments. In order to determine mechanisms of *S. aureus* iron acquisition during mastitis, bacteria containing transposon insertions in genes suspected of iron acquisition involvement were tested in two different ways.

First, these transposon insertion mutants were tested for growth defects in a low-iron minimal media (TMM) supplemented with a single iron source hypothesized to be relevant during mastitis infections. I hypothesized that when essential genes for iron acquisition were inactivated, or knocked-out, there would be a significant growth defect when these strains were grown in TMM with a single relevant iron source. Second, all strains were also tested for growth defects in bovine and human milk. Milk provides a biologically relevant growth medium for mastitis infections.

Mutants were selected based on initial research and my hypothesis as to which genes may play an important role in iron acquisition. Additional mutants, including mutants involved in zinc and manganese metal acquisition, and other cellular processes, were selected based on Tn-seq experiments conducted in milk by Dr. Wilson's lab (data not shown). All transposon insertion mutants for this research were obtained from the Nebraska Transposon Mutant Library (NTML) (Table 3). All NTML mutant strains were validated to have transposon insertions in the appropriate location through PCR, as described in Materials and Methods.

Table 3: NTML mutants

NTML ID #	Gene Name	Known/Predicted Function
SAUSA300_0117	*sirA*	Iron ABC transporter, siderophore-binding protein for SB
SAUSA300_0122	*sbnE* *	Staphyloferrin B biosynthesis protein
SAUSA300_0618	*psaA*	Manganese ABC transporter, substrate-binding protein
SAUSA300_0619	*znuB*	Zinc ABC transporter, membrane permease
SAUSA300_0620	*znuC*	Zinc ABC transporter, ATPase
SAUSA300_0633	*fhuC* *	Iron ABC transporter, ATPase
SAUSA300_0798	*nlpA*	(MetQ) Methionine binding lipoprotein
SAUSA300_0975	*purH*	Purine biosynthesis protein
SAUSA300_1015	*ctaA*	Cytochrome oxidase assembly protein
SAUSA300_1032	*isdE* *	Heme ABC transporter, heme-binding protein
SAUSA300_1274	*dppD*	Dipeptide/Nickel ABC transporter, ATPase
SAUSA300_2136	*htsA* *	Iron ABC transporter, siderophore-binding protein for SA
SAUSA300_2140	*sfaD* *	Staphyloferrin A biosynthesis protein
SAUSA300_2413	*cntL* *	Staphylopine biosynthesis protein

Transposon mutants selected for testing from the Nebraska Transposon Mutant Library.
*Designates a gene which also had a clean deletion knock-out created for testing.

Clean deletion knock-out mutants, or mutants in which the target gene was completely removed rather than disrupted, were also generated for this project. Specific genes deleted are listed above (indicated in Table 3 by asterisk). To differentiate between the method of gene inactivation, hereafter the transposon insertion mutants will be designated as *gene*::*ba* (*bursa aurealis* transposon) and the clean deletion knock-out mutants as Δ*gene*.

As a control, each transposon mutant was grown in rich media (TSB), to screen for potential intrinsic growth defects. A double knock-out, created using the NTML *sirA::ba* transposon strain with a clean deletion of the *htsA* gene, was designed to target two separate receptors involved in siderophore mediated iron acquisition and was also tested. As shown in Figure 3, all bacteria grew at rates comparable to wild type (USA300) when grown in TSB.

Figure 3: Growth in TSB

Mutant and wild type growth in TSB. No significant differences were found in the ability of mutants to grow in this media compared to the wild-type strain. Data represents three biological replicates and results are shown as the log transformation of CFU/mL at 0, 5 and 10hrs. Error bars display 95% confidence intervals.
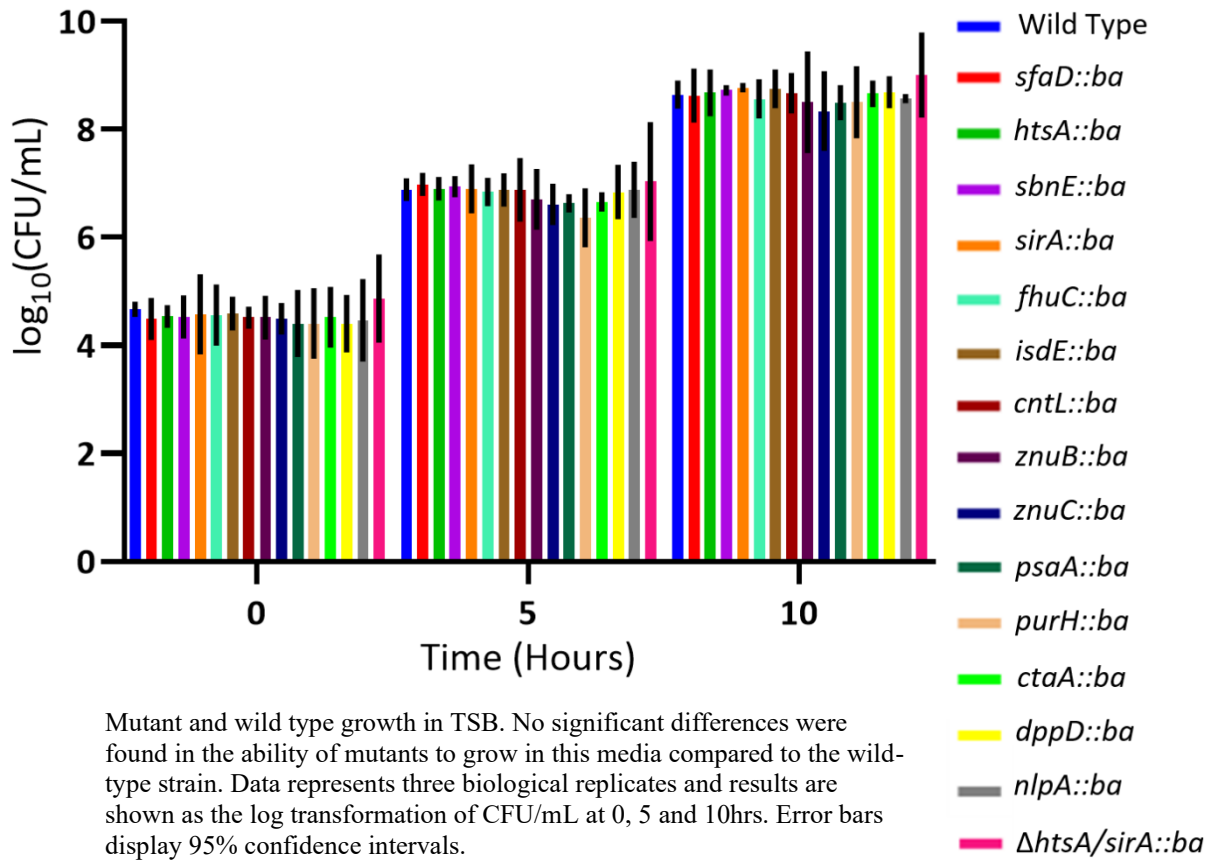
SECTION 3: RESULTS AND DISCUSSION

Due to the high number of mutants and media tested, the results and discussion are broken up into two distinct groups. Group A consists of bacterial strains with transposon insertions targeting genes known to contribute to iron acquisition, while Group B consists of bacterial strains with transposon insertions targeting genes involved with additional metal acquisition (such as zinc and manganese) and other cellular processes. All data will be presented partitioned into these two distinct groupings with the wild-type strain included as a control.

3.1 Group A: Mutants which function in iron acquisition

Table 4: Group A: Mutants which function in iron acquisition

Mutant	Function
sfaD::ba	Staphyloferrin A biosynthesis protein
htsA::ba	Iron ABC transporter, staphyloferrin A binding protein
sbnE::ba	Staphyloferrin B biosynthesis protein
sirA::ba	Iron ABC transporter, staphyloferrin B binding protein
fhuC::ba	ABC transporter ATPase (for SirABC and HtsABC, and more)
isdE::ba	Heme ABC transporter, heme binding protein
cntL::ba	Staphylopine biosynthesis protein (StP binds multiple metals)
Δ*htsA/sirA::ba*	Staphyloferrin A and B binding proteins for iron ABC transporters

Mutant strains and functions selected for testing in Group A. Each mutant has functions involved with iron transport that may be important for iron acquisition during mastitis infections.

3.1.1 Growth in minimal media

Using low-iron Tris minimal media (TMM) 30 hr growth experiments were conducted using an automated OD reader. As a control, bacterial strains were grown in minimal medium (TMM) with and without the addition of free iron ($FeSO_4$). Experimental groups consisted of bacteria grown in minimal media supplemented with specific iron sources hypothesized to be important during mastitis infections. Figures (4-20) display growth curve data organized by media type, while Figures (44-52) display the same data organized by bacterial strain and are included

for reference in the Supplementary Figures section of this thesis.

TMM is a low-iron environment and serves as a baseline indicator of bacterial growth. Notably, the minimal media used in these experiments was not iron-free, but rather iron-depleted. As a result, many strains of the bacteria could grow in the minimal media even without additional iron supplementation (Figure 4). The majority of strains grew slightly below the growth achieved by the wild-type strain. However, *fhuC::ba* and Δ*htsA/sirA::ba* clearly cannot grow in minimal TMM and seem unable to acquire sufficient residual iron present in the medium. The double knock-out Δ*htsA/sirA::ba* prevents the function of two iron siderophore receptors (HtsABC and SirABC). FhuC is the ATPase responsible for powering the movement of iron-loaded siderophores through those ABC transporters. The lack of growth of *fhuC::ba* and Δ*htsA/sirA::ba*

Figure 4: Group A growth in TMM
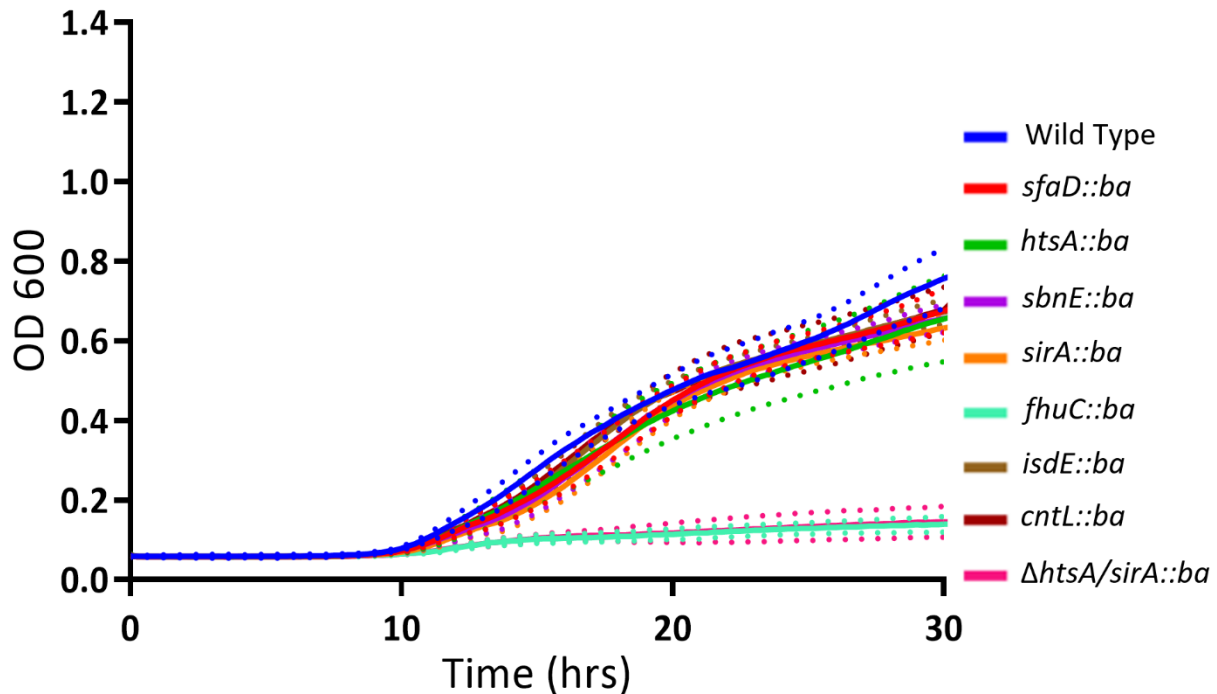
Growth of strains for 30 hrs in TMM. Most mutants grow similar to the wild-type strain, however, *fhuC::ba* and Δ*htsA/sirA::ba* cannot grow in the TMM medium. The lack of growth of *fhuC::ba* and Δ*htsA/sirA::ba* in minimal media, in comparison to the growth of other mutants suggests that in low iron concentrations, iron uptake is dependent on the ATPase activity of FhuC and that siderophore receptors are also involved in this process. Data represents three biological repeats, each consisting of three technical repeats.

in minimal medium, in comparison to the growth of other mutants suggests that in low iron concentrations, iron uptake is dependent on the ATPase activity of FhuC and that siderophore receptors HtsA and SirA are also involved in this process.

As a positive control, all bacterial strains were also grown in TMM media with added ferric sulfate (Figure 5). All strains show growth in ferric sulfate similar to the wild-type control.

Figure 5: Group A growth in ferric sulfate
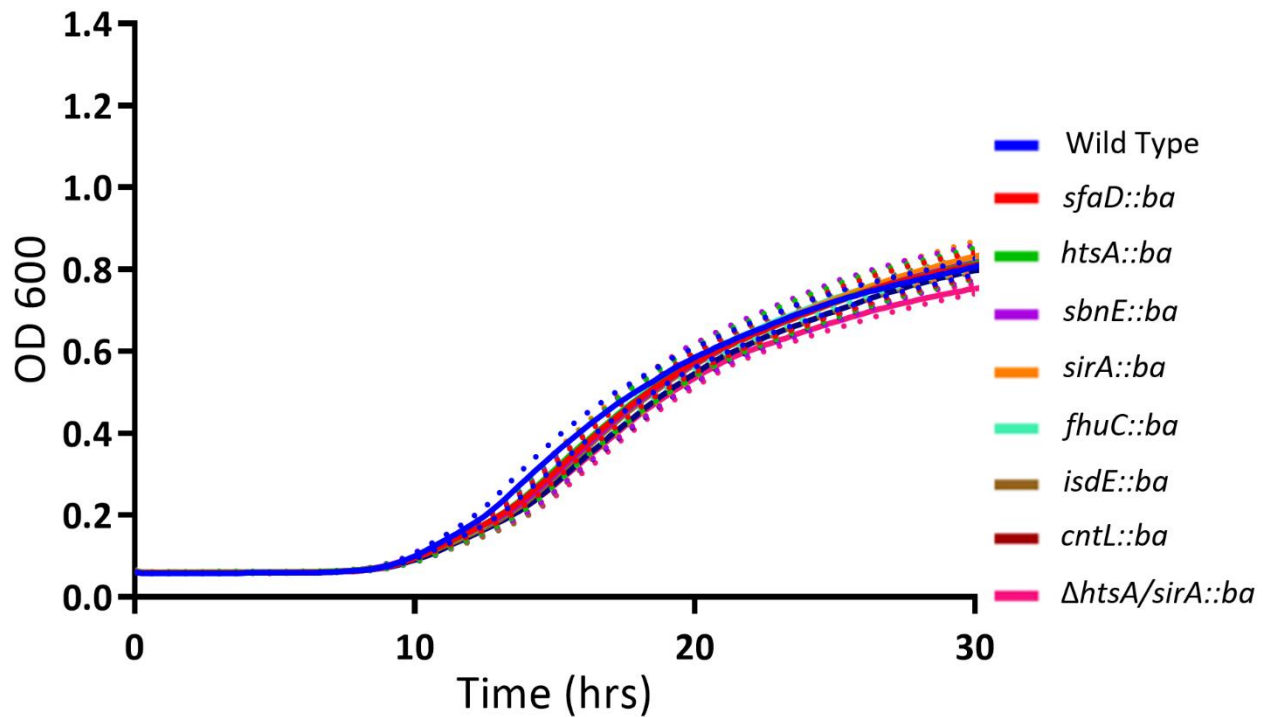
Growth of strains for 30 hrs in TMM supplemented with ferric sulfate. Data represents three biological repeats, each consisting of three technical repeats.

Final growth yield (at 30 hrs) in the ferric sulfate medium was converted to a percent change in growth for each strain in comparison to its growth in TMM (Figure 6). In comparison to the TMM baseline, *sfaD::ba, sbnE::ba, sirA::ba, fhuC::ba, isdE::ba,* and Δ*htsA/sirA::ba* all had increased growth (P-value = <0.0001). *htsA::ba* and *cntL::ba* also had significantly increased growth (P-values 0.0019 and 0.0008 respectively). *fhuC::ba* and Δ*htsA/sirA::ba* show the highest increases in growth. It is important to recognize that this dramatic increase in growth is due to the diminished growth of these strains obtained in TMM. Notably, the wild-type strain does not

exhibit a significant difference in growth between TMM and TMM with ferric sulfate

emphasizing the efficiency with which *S. aureus* can obtain iron even in difficult environments.

Figure 6: Group A percent growth in ferric sulfate
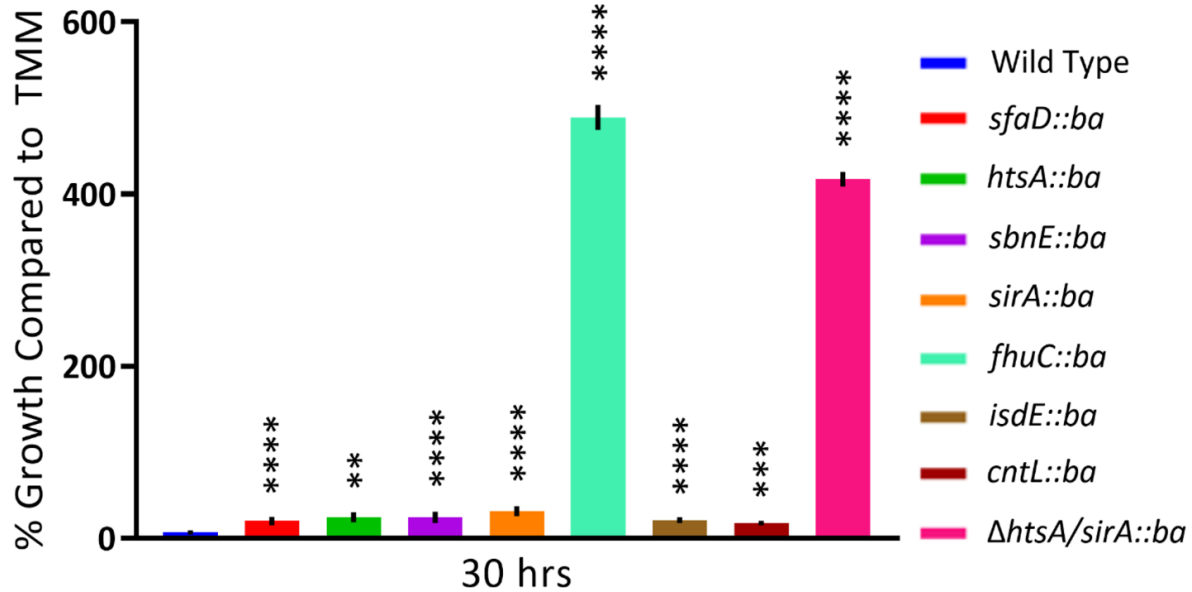
Percent increase of growth for strains in TMM supplemented with ferric sulfate in comparison to growth in TMM at 30 hrs. All mutants are able to utilize ferric sulfate as an iron source and show significant growth increases in comparison to the growth of each strain in minimal TMM. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Taken together, these results demonstrate that the TMM minimal media used in these

experiments contained low enough concentrations of available iron to inhibit the growth of some,

but not all, of the gene-specific insertion mutants. The ability to restore and improve the growth

of all mutants with the addition of free iron demonstrates that the observed growth defect was in

fact a result of iron depletion and not an artifact of media preparation.

As I had hypothesized that potential iron sources in milk included transferrin, lactoferrin

and ferric citrate, experiments were carried out in TMM minimal media supplemented with these

different iron sources. Although similar, there are differences in the amino acid sequence of

human and bovine transferrin as well as lactoferrin. Therefore, both human and bovine versions

of each protein were tested separately.

Growth curves from TMM supplemented with iron saturated bovine transferrin are found in

Figure 7. Percent increase of growth in bovine transferrin as compared to growth in TMM is

**Figure 7: Group A growth in bovine transferrin**
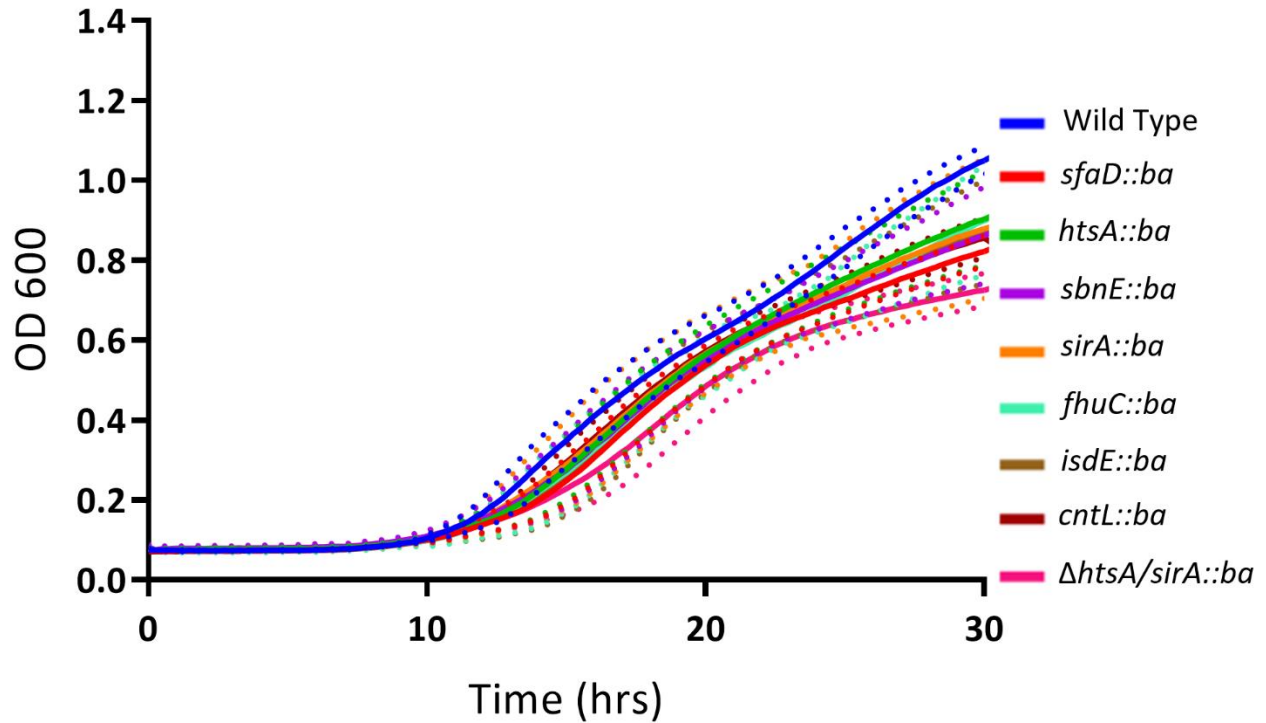
Growth of strains for 30 hrs in TMM supplemented with bovine transferrin. Data represents three biological repeats, each consisting of three technical repeats.

found in Figure 8. The wild-type strain, *sfaD::ba, sirA::ba, fhuC::ba, cntL::ba* and

Δ*htsA/sirA::ba* each had growth increases with P-values = <0.0001. *htsA::ba, sbnE::ba* and

isdE::ba also displayed increased growth (P-values 0.0008, 0.0051, and 0.0021 respectively).

These results suggest that all mutants can grow using iron saturated bovine transferrin as an iron

source. However, these results are very similar to growth in TMM with added ferric sulfate,

which may complicate the interpretation of these results if free iron was also present in the bovine

transferrin supplemented medium. The process of utilizing bovine transferrin may rely on

multiple systems as no single mutant showed a significant defect in growth. Levels of bovine

transferrin during lactation remain relatively low when compared to other host proteins and it is

unclear if transferrin in milk would be iron bound [128], but given the growth of strains in this media, transferrin could serve as an iron source for *S. aureus* during bovine mastitis infections.

Figure 8: Group A percent growth in bovine transferrin

Percent increase of growth for strains in TMM supplemented with bovine transferrin in comparison to growth in TMM at 30 hrs. All strains show significant increases in growth in comparison to minimal TMM media. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ****P-value = <0.0001

Growth in iron saturated human transferrin (Figure 9) and percent of increased growth in comparison to growth in TMM (Figure 10) was very different from growth in bovine transferrin. Many mutant strains, as well as the wild-type strain, did not grow significantly different from growth in the TMM negative control. This was surprising in that growth utilizing iron saturated human transferrin has been documented in *S. aureus* and previous research has showed that SA and SB siderophores play a dominant role in this process [158,171,172]. In my data, no substantial growth defect is found for the siderophore knock-outs (*sfaD::ba* and *sbnE::ba*). However, as I was unable to generate a double knock-out for these two genes, redundancy of function between the siderophores may explain why no growth defect was detected. This

discrepancy could also be strain dependent or due to differences in methods.
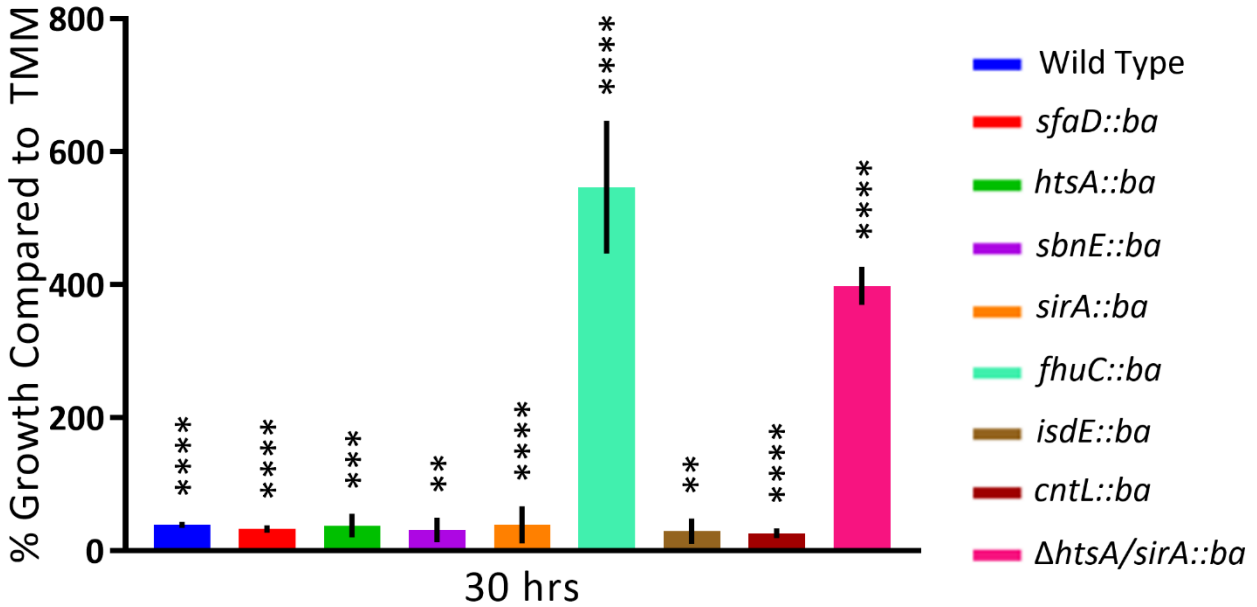
Figure 9: Group A growth in human transferrin
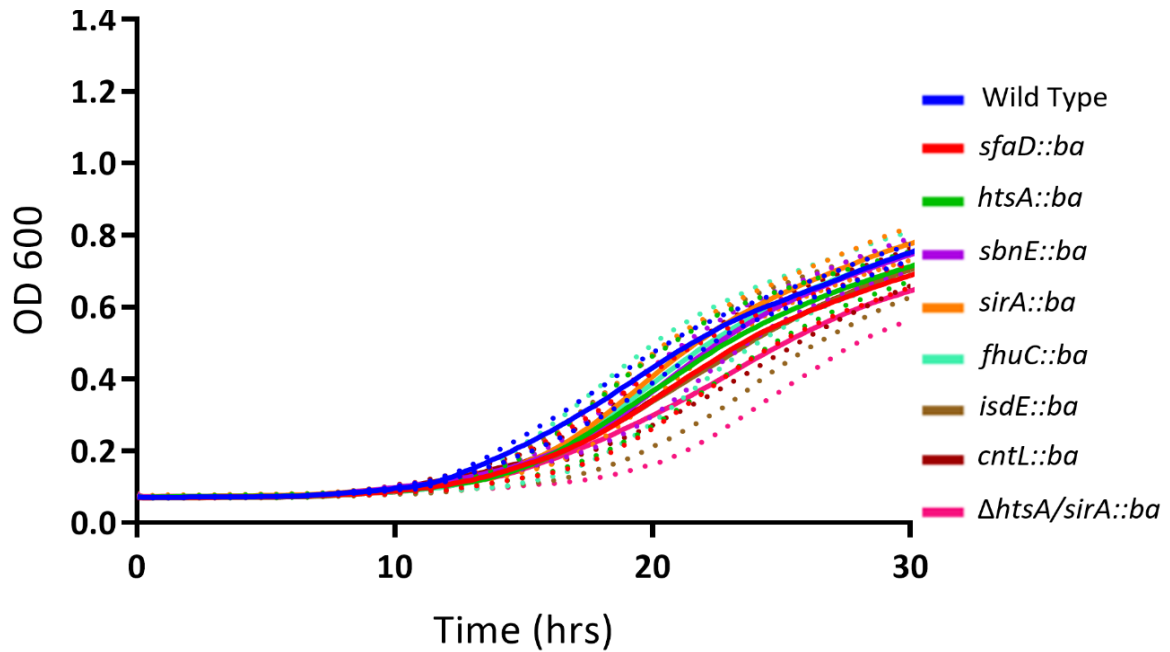
Growth of strains for 30 hrs in TMM supplemented with human transferrin. Data represents three biological repeats, each consisting of three technical repeats.

As most strains, including the wild-type strain, struggled to grow beyond their baseline levels in human transferrin, it seems the important genes to look at may be the ones that do show growth. *sbnE::ba* displays an increased growth with a P-value = 0.0017, while *sirA::ba, fhuC::ba* and Δ*htsA/sirA::ba* all have P-values = <0.0001. Notably, these four mutants all influence genes responsible for staphyloferrin B production (*sbnE*), its receptor (*sirA*), or its ATPase (*fhuC*). This may indicate that SB and its receptor are not essential for uptake of iron from human transferrin. However, since *fhuC::ba* and the double knock-out Δ*htsA/sirA::ba* inhibit both siderophore receptors and still grow well in media supplemented with human transferrin, this may indicate that growth in human transferrin does not involve either receptor tested. Knocking out those genes, could potentially lead to growth increases by reducing the energy expended by the bacterium to express those genes.

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Figure 10: Group A percent growth in human transferrin
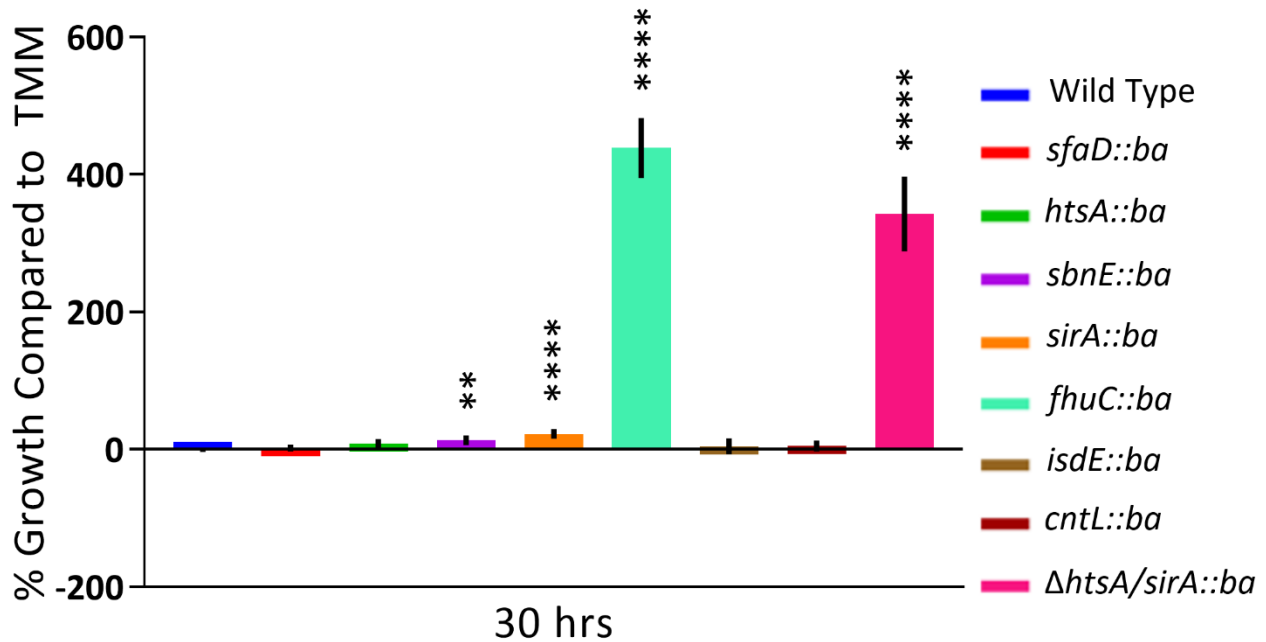
Percent increase of growth for strains in TMM supplemented with human transferrin in comparison to growth in TMM at 30 hrs. Most strains, including the wild type, did not grow beyond the baseline growth achieved in minimal TMM. However, four strains involved with SB and its receptor (*sbnE::ba, sirA::ba, fhuC::ba,* and Δ*htsA/sirA::ba*) do show growth in this media indicating they are nonessential for iron uptake from human transferrin. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ****P-value = <0.0001

Growth in TMM supplemented with bovine lactoferrin isolated from bovine milk and the percent increase of growth in bovine lactoferrin when compared to growth in TMM are shown in Figures 11 and 12 respectively. However, it is important to note that due to the limited availability of this iron source, results of this growth curve were conducted only once in triplicate and have not been repeated. Please also note, *cntL::ba* is not represented in this data set. All tested strains are able to grow well in this media with the wild-type strain, *sfaD::ba, htsA::ba, sirA::ba, cntL::ba* and Δ*htsA/sirA::ba* strains each displaying improved growth in comparison to TMM at 30 hrs (P-values = <0.0001). *sbnE::ba, fhuC::ba,* and *isdE::ba* also show increased growth (P-values 0.0007, 0.0100, and 0.0053 respectively). Unfortunately, the higher level of growth achieved by all strains also means that the mechanism by which *S. aureus* utilizes bovine

Figure 11: Group A growth in bovine lactoferrin isolated from milk
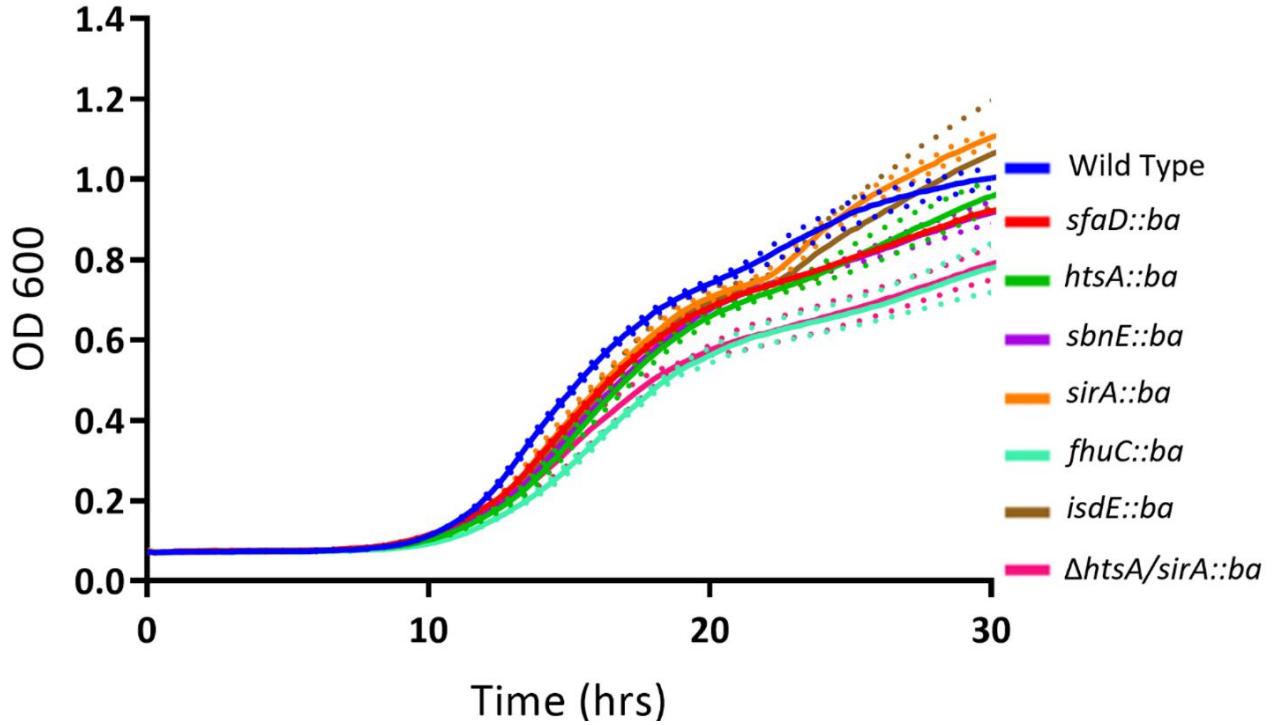
Growth of strains for 30 hrs in TMM supplemented with bovine lactoferrin isolated from milk. Data represents one experiment conducted with three technical repeats.

Figure 12: Group A percent growth in bovine lactoferrin isolated from milk
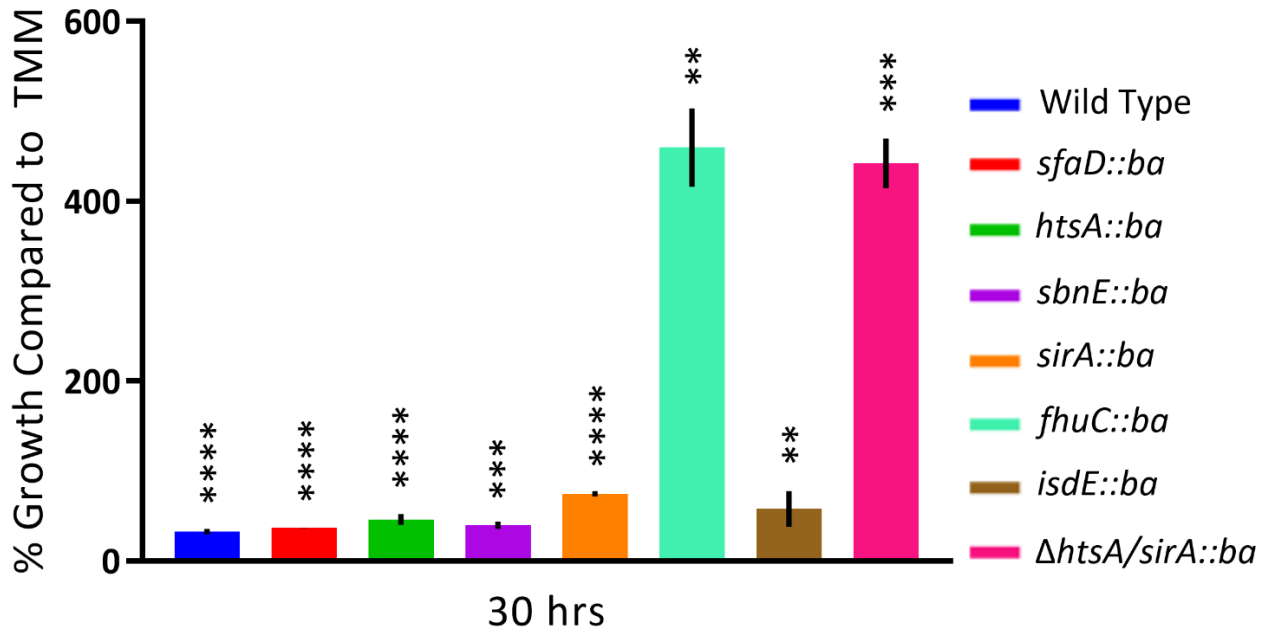
Percent increase of growth for strains in TMM supplemented with bovine lactoferrin isolated from milk in comparison to growth in TMM at 30 hrs. All strains show significantly increased growth in comparison to growth in TMM. Data represents one experiment conducted with three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

lactoferrin is still unclear; being either obscured by overlapping functions, or because *cntL* or other untested genes are responsible for utilization of this iron source. If repeated data support the current findings, bovine lactoferrin isolated from milk would be a viable iron source during mastitis infections.

Due to the long backorder on bovine lactoferrin isolated from milk, bovine lactoferrin isolated from colostrum was used for testing. Interestingly, when TMM was supplemented with bovine lactoferrin isolated from bovine colostrum, all strains struggled to grow (Figure 13). In fact, *sbnE::ba, sirA::ba,* and *fhuC::ba* all show significantly worse growth in bovine lactoferrin isolated from colostrum as a percentage of growth achieved in comparison to growth in TMM at 30 hrs (Figure 14) (P-values = <0.0001). *sfaD::ba* and Δ*htsA/sirA::ba* also showed a decrease in growth (P-values 0.0042 and 0.0008 respectively). Recall that SbnE is essential for production of the siderophore SB, while SirA is the binding portion of the ABC-transport system responsible for

Figure 13: Group A growth in bovine lactoferrin isolated from colostrum

Growth of strains for 30 hrs in TMM supplemented with bovine lactoferrin isolated from colostrum. Data represents three biological repeats, each consisting of three technical repeats.

the uptake of SB, and that FhuC functions as the ATPase for this transporter. The four mutant strains that were completely unable to grow in bovine lactoferrin from colostrum are each connected to SB production or uptake.

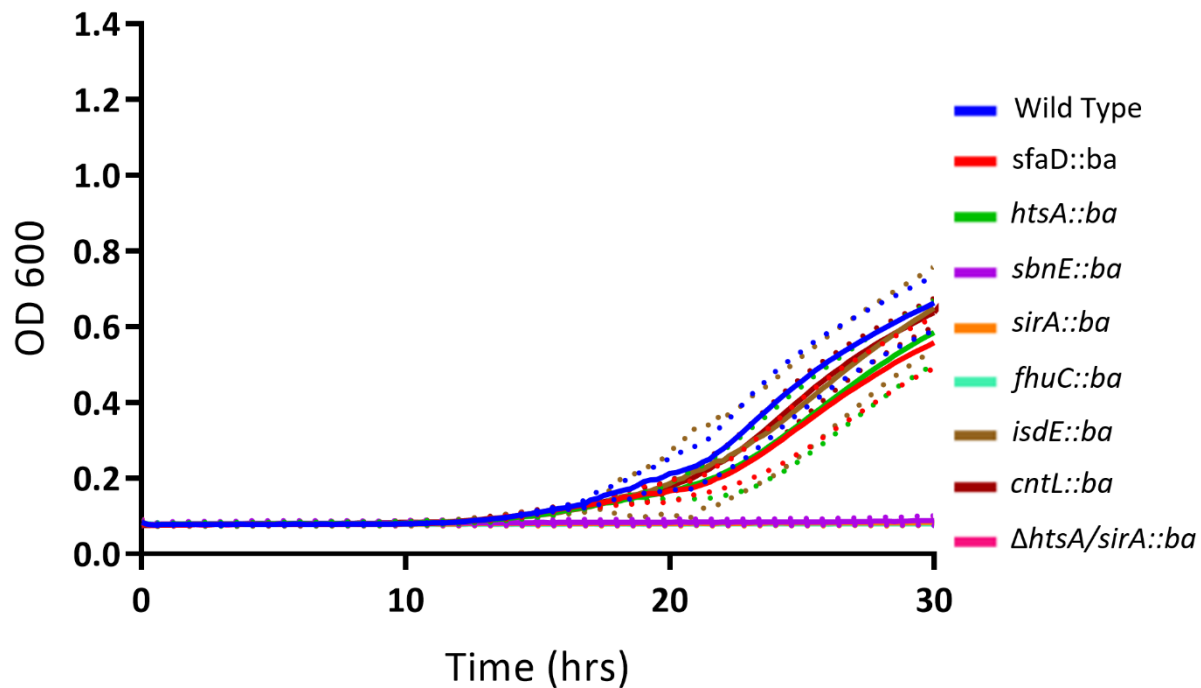**Figure 14: Group A percent growth in bovine lactoferrin isolated from colostrum**
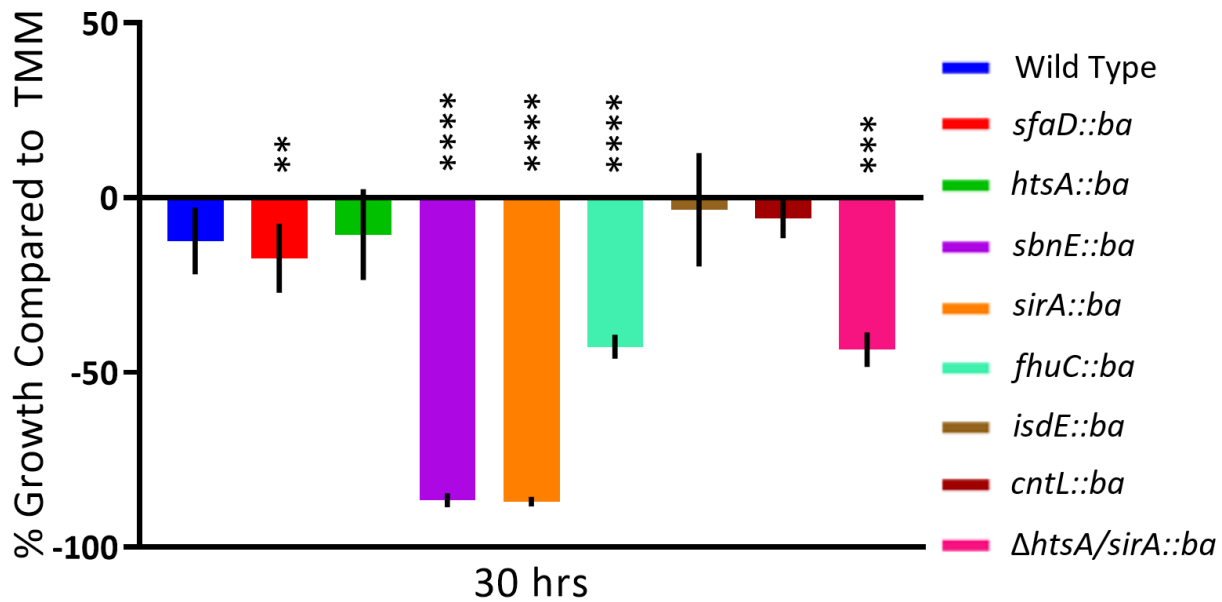
Percent decrease of growth for strains in TMM supplemented with bovine lactoferrin isolated from colostrum in comparison to growth in TMM at 30 hrs. All strains, including the wild type, struggle to grow and exhibit lower growth than what was seen in the negative control (TMM). Four of these strains, the ones completely incapable of growth in bovine lactoferrin from colostrum are connected to SB production or uptake and indicates that this siderophore and its receptor are important for survival in this medium. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

These results suggest that this siderophore and its receptor are essential for survival in this medium. Each iron source was tested to ensure the same amount of iron was added to each media. As a result, the difference in growth between bovine lactoferrin isolated from milk or from colostrum cannot be attributed to differences in iron concentration between the two media. Most likely, a higher amount of apolactoferrin is present in the colostrum and without the function of SB or its receptor these strains are unable to compete effectively with the abundance of lactoferrin for iron. As these defects are not seen in bovine lactoferrin isolated from milk, it seems there are also redundant systems involved in this process, however, when extreme pressure is applied to the

bacteria (by the high abundance of apolactoferrin) the essential nature of SB and its receptor as the dominate mechanisms in up taking iron from bovine lactoferrin becomes apparent (Figure 15).

Figure 15: Proposed model for iron uptake from bovine lactoferrin
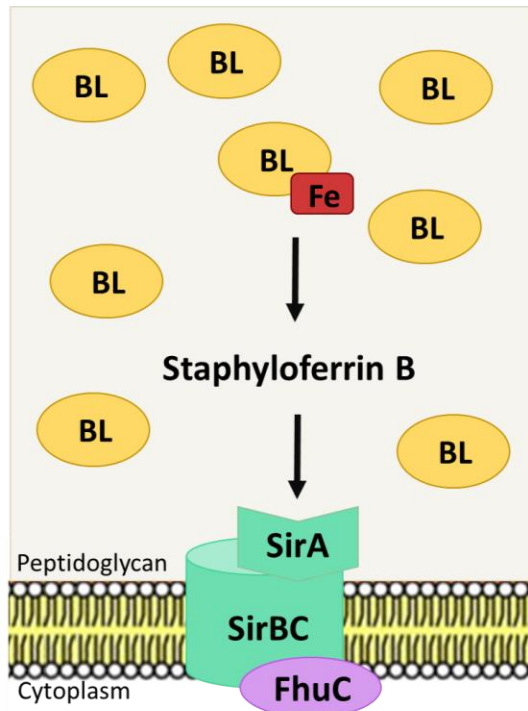
Based on the results discussed above, this figure shows the proposed model for iron uptake from bovine lactoferrin. The inability of mutants relating to SB production and uptake to grow in bovine lactoferrin isolated from colostrum demonstrates the essential nature of these genes. When bovine apolactoferrin (BL) levels are high and competing for iron in the media, staphyloferrin B is essential for iron acquisition from bovine hololactoferrin (BL-Fe). Staphyloferrin B transports the iron through the SirABC receptor powered by the FhuC ATPase and into the bacterial cell.

Growth curves of TMM supplemented with iron saturated human lactoferrin are shown in Figure 16. *fhu::ba* and Δ*htsA/sirA*::ba both display growth deficiencies when compared to the other strains. The percent change in growth for strains in TMM supplemented with human lactoferrin in comparison to growth in TMM at 30 hrs are found in Figure 17. All strains display an increase of growth in comparison to TMM media (wild type, *sfaD::ba, htsA::ba, sbnE::ba, sirA::ba, fhuC::ba,* and Δ*htsA/sirA::ba* have a P-value = <0.0001, while *isdE::ba* and *cntL::ba* have P-values 0.0002 and 0.0022 respectively). However, regardless of the increase in growth relative to TMM, *fhuC::ba* and Δ*htsA/sirA::ba* exhibit diminished growth in human lactoferrin supplemented media. Despite growth in bovine transferrin and human transferrin these mutants exhibit strong growth defects in this media. The data indicates that the genes targeted in *fhuC::ba*

Figure 16: Group A growth in human lactoferrin
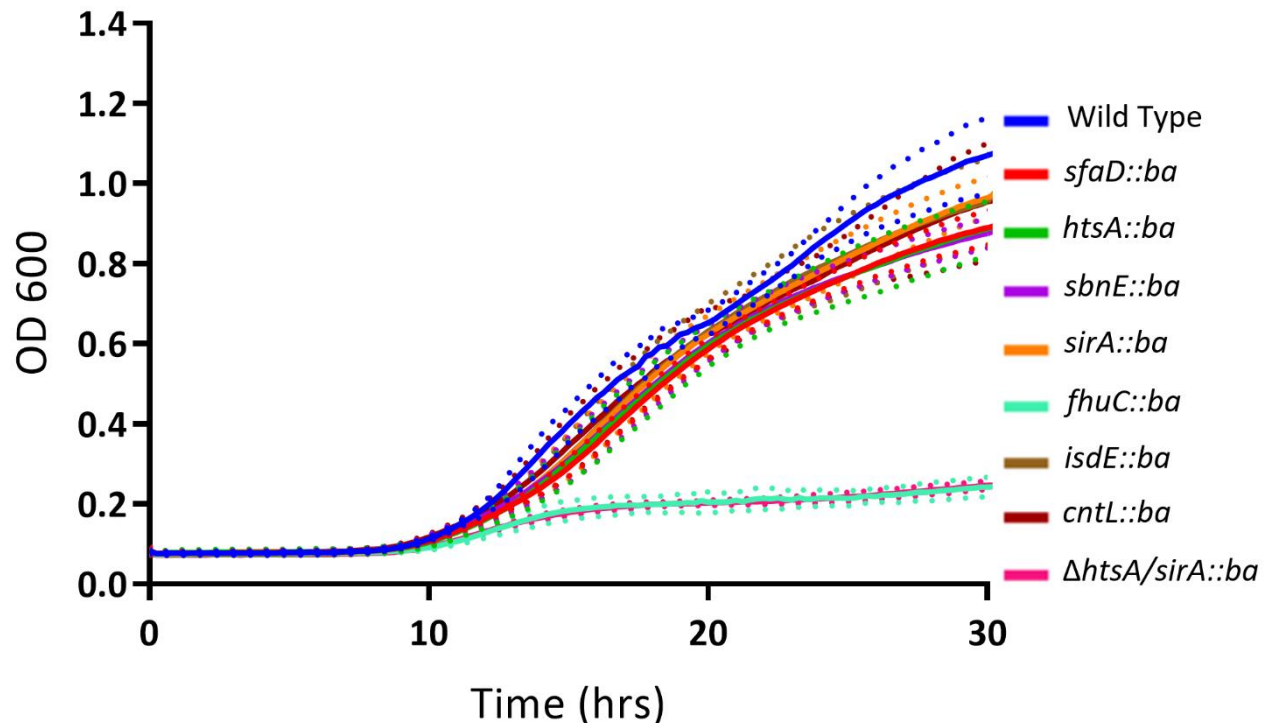
Growth of strains for 30hrs in TMM supplemented with human lactoferrin. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Figure 17: Group A percent growth in human lactoferrin
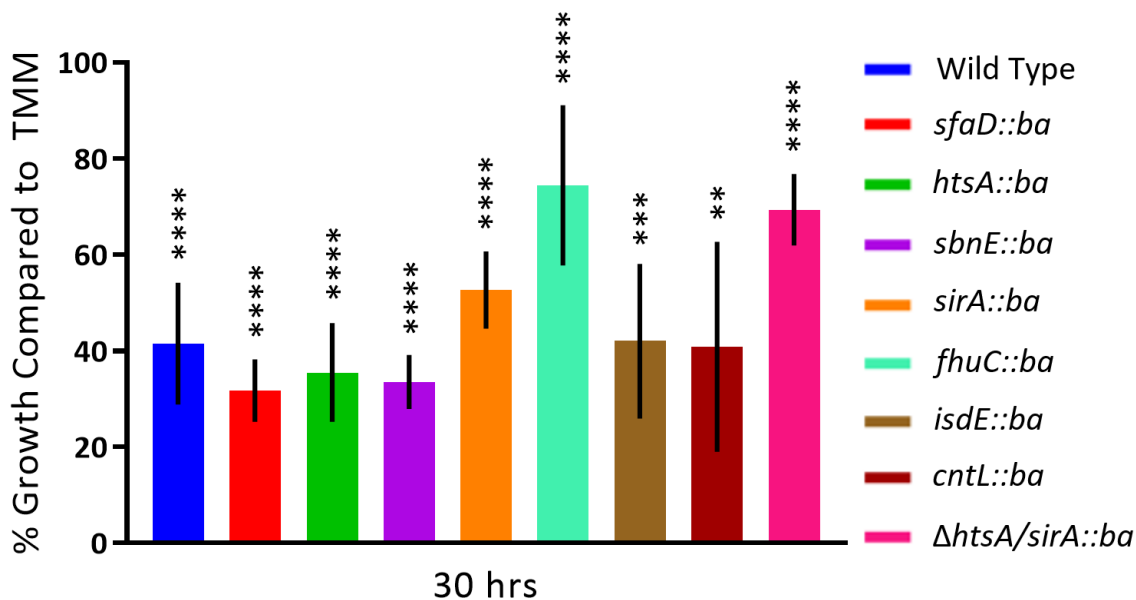
Percent increased growth of strains in TMM supplemented with human lactoferrin in comparison to growth in TMM at 30 hrs. While all strains display a significant increase of growth in comparison to the TMM baseline, *fhuC::ba* and Δ*htsA/sirA::ba* both exhibit growth deficiencies in human lactoferrin and indicates the importance of these genes for growth in this media type. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

and Δ*htsA/sirA::ba* are essential for growth in human lactoferrin, but not in human transferrin. It is also notable that the growth defect is only seen when both siderophore receptors are non-functional (either directly knocked-out or nonfunctional via nonfunctional ATPase). This indicates there are overlapping functions between HtsA and SirA in utilizing iron from human lactoferrin, and if only one gene is knocked-out the other functional gene can compensate allowing the strain to still grow. Construction of a double knock-out for the siderophores staphyloferrin A and staphyloferrin B was also attempted but remains unsuccessful. Based on these results it is unclear if the interaction between human lactoferrin and these two receptors is siderophore mediated. However, as HtsA and SirA are known to bind siderophore-bound iron this interaction is likely mediated by siderophores staphyloferrin A and staphyloferrin B. Figure 18 displays a visual model of these findings.

Figure 18: Proposed model of iron uptake from human lactoferrin
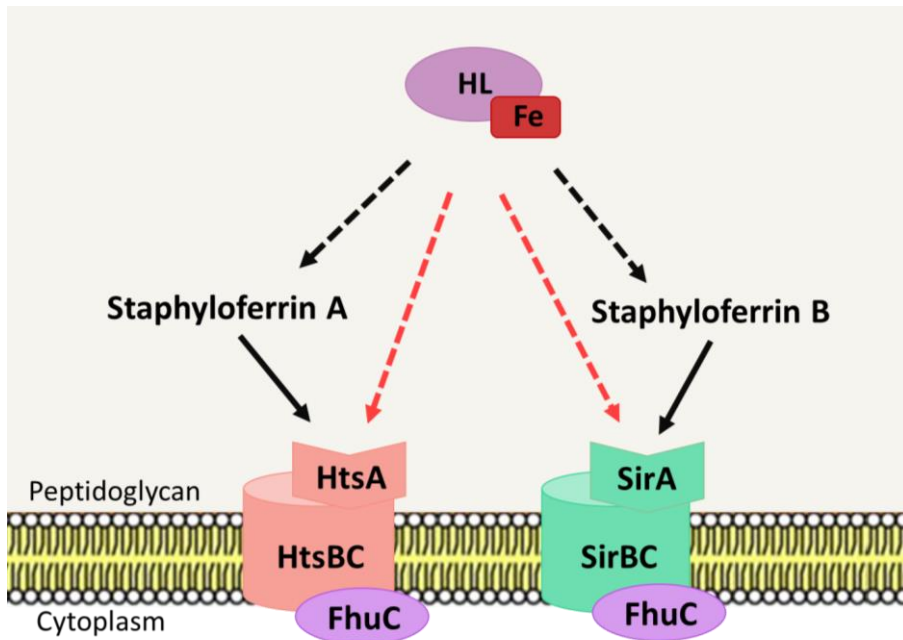
Based on the results discussed above, this figure displays the proposed model for iron uptake from human lactoferrin (HL). When human lactoferrin is bound to iron, *S. aureus* can utilize this iron source either through siderophore mediated interactions (dashed black arrows), or potentially through direct contact with the two essential iron ABC transporters HtsABC and SirABC (dashed red arrows). FhuC functions as an ATPase for HtsABC and SirABC transporters.

Ferric citrate was also tested as an iron source for *S. aureus* (Figure 19). Surprisingly, the growth patterns of all mutants are nearly identical to the growth seen in the positive control with ferric citrate. As a percent growth comparison to the TMM negative control *sfaD::ba, sbnE::ba, sirA::ba, fhuC::ba, isdE::ba,* and Δ*htsA/sirA::ba* all have growth increases with P-values = <0.0001. *htsA::ba* and *cntL::ba* have increased growth with P-values 0.0021 and 0.0001 respectively. Even with the addition of 10X the amount of citrate (to ensure this growth was not due to an abundance of unbound iron) the strains are unaffected. Ferric citrate is a natural component of milk and is likely a major iron contributor in both human and bovine milk. Despite mutations in prominent iron acquisition systems, *S. aureus* can still obtain iron from ferric citrate, and the mechanism by which it uptakes this iron source remains unclear. Although I had speculated that the similarity in structure of Staphyloferrin A and ferric citrate might also allow

Figure 19: Group A growth in ferric citrate with 10X citrate
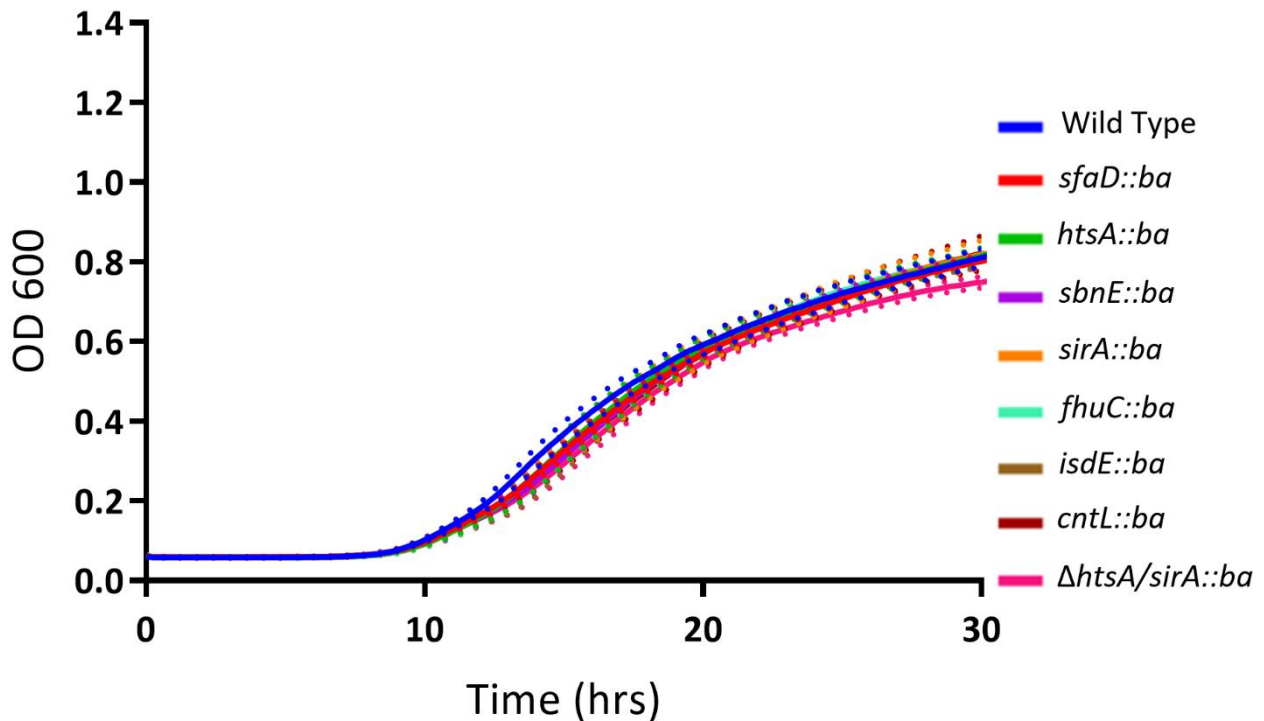
Growth for 30 hrs in TMM supplemented with ferric citrate plus an additional 10X citrate. Data represents three biological repeats, each consisting of three technical repeats.

for ferric dicitrate transport into *S. aureus* via HtsABC, this does not appear to be the case. Likely, ferric citrate uptake is accomplished through a still undiscovered receptor or potentially from redundancy of function among several iron transporter systems.

Figure 20: Group A percent growth in ferric citrate with 10X citrate
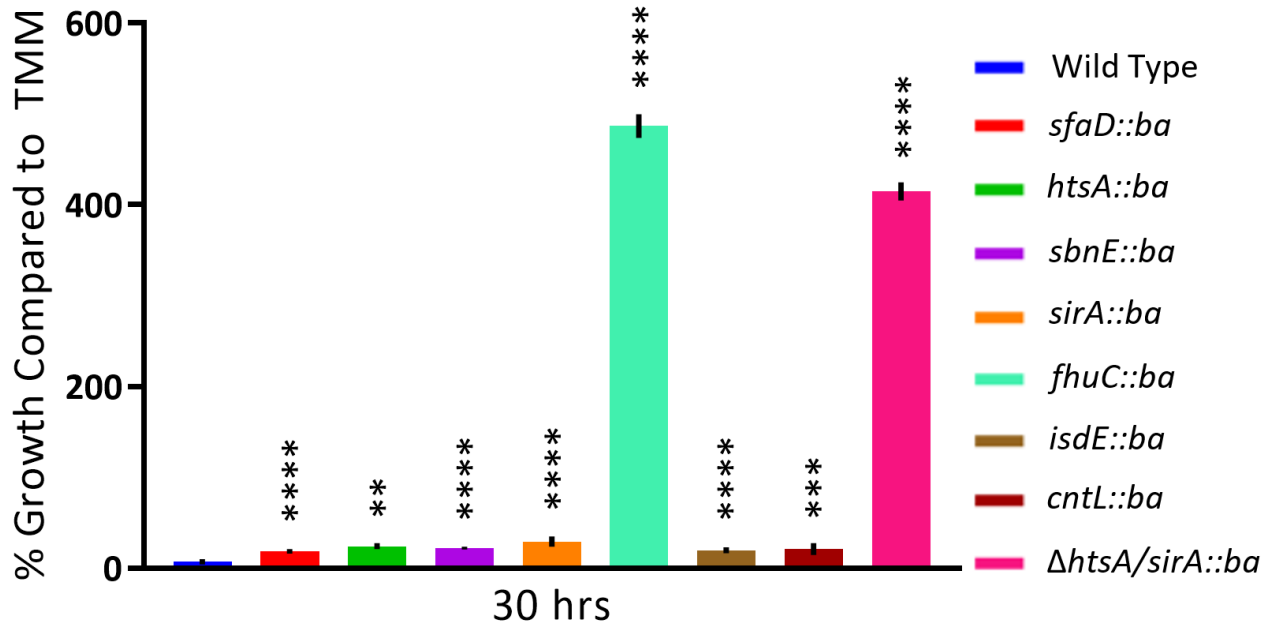
Percent increased growth for strains in TMM supplemented with ferric citrate plus an additional 10X citrate in comparison to TMM at 30 hrs. All strains display significantly increased growth compared to minimal TMM. Data represents three biological repeats, each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

3.1.2 Growth in bovine milk

TMM supplemented with physiological relevant iron sources provided useful data in a very defined environment. However, growth curves in milk were also conducted to provide data in a more biologically relevant growth medium. High levels of *S. aureus* contamination was found naturally in unpasteurized bovine milk. This was problematic for performing colony counts as there was no way to easily distinguish between the existing flora in the milk and the *S. aureus* used to inoculate the experiment. Several methods were tested to remove these bacteria, including centrifuging the milk to remove contamination and working with antibiotic plates. Unfortunately,

these methods did not sufficiently resolve the issue and therefore I decided to proceed with using

whole, pasteurized milk in my growth curves (Figure 21).

Figure 21: Group A growth in bovine pasteurized milk
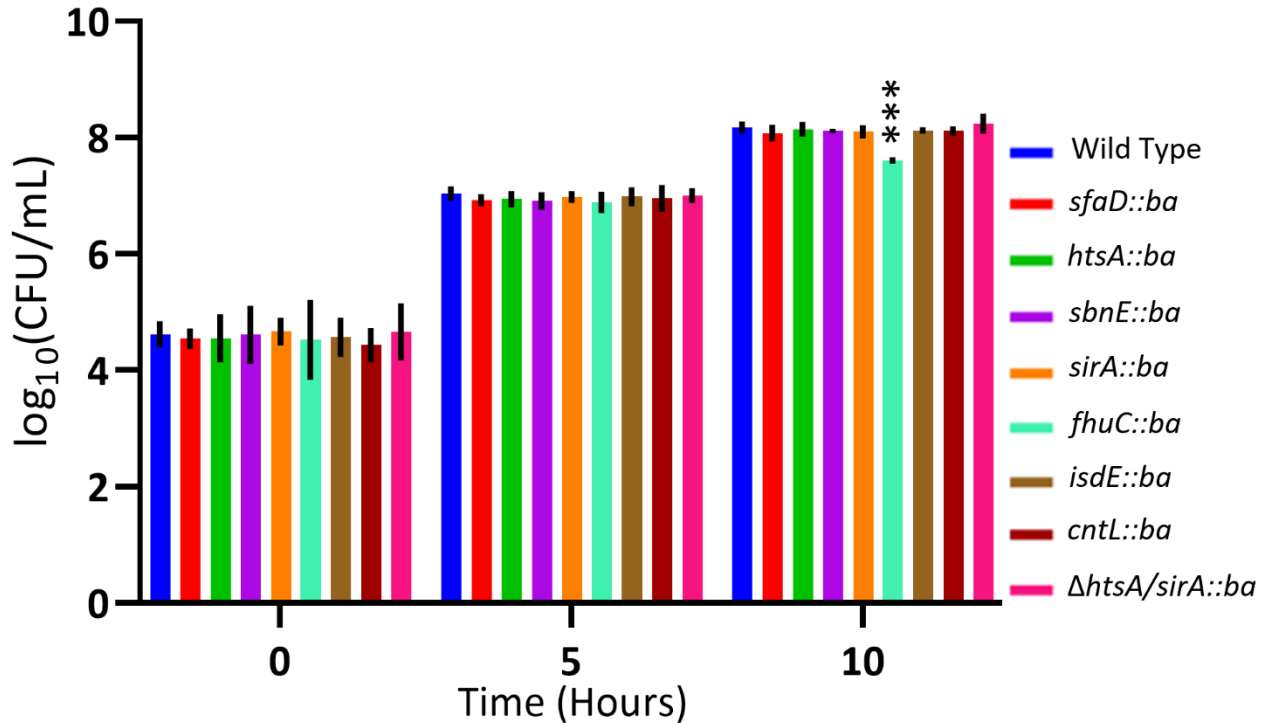
Growth in bovine pasteurized milk reported as the log transformation of CFU/mL at 0, 5, and 10hrs. At 10 hrs a small but significant growth defect is seen in *fhuC:ba*. Data represents three biological repeats. Error bars represent 95% confidence intervals. ***P-value = <0.001

Although no significant difference is found at the 5 hr timepoint, *fhuC:ba* shows a small

yet significant growth defect at the 10 hr timepoint (P-value = 0.0003). FhuC functions as an

ATPase for many different ABC transporters including iron ABC transporters (SirA and HtsA),

but since the double knock-out did not show a significant growth defect, it seems that FhuC is

functioning as an ATPase for genes important in milk growth that were not tested in my panel of

mutants.

Although statistically significant results were obtained, additional growth defects

potentially were masked by partial protein function due to possible transposon insertions late into

the gene. Therefore, clean-deletion knock-out mutants were generated for several mutants initially

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hypothesized to be important in *S. aureus* iron acquisition. These clean-deletion mutants were tested, and the results were compared to the transposon strain growth in bovine pasteurized milk (Figure 22). Comparison of the transposon and clean deletion mutants revealed that no clean deletion knock-out had a greater growth defect than the transposon insertion mutant when grown in pasteurized bovine milk. In fact, several clean deletion mutants grew better than the transposon mutant, likely because the clean deletion knock-out saves energy by not having to replicate the transposon insertion.

Figure 22: Comparison of transposon and clean-deletion mutants
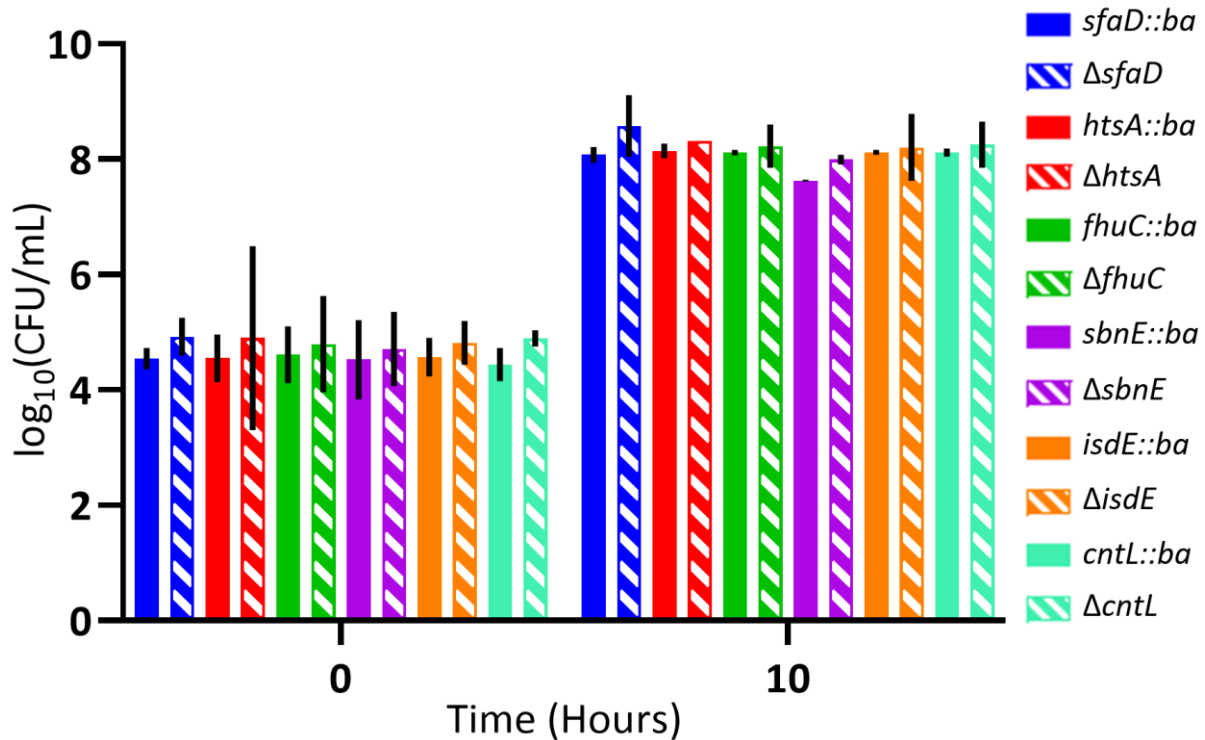
Growth of transposon mutants (solid bars) compared to clean-deletion knock-out mutants (striped bars of the same color) in pasteurized bovine milk. Shown as a log transformation of CFU/mL at 0 and 10hrs. Data represents at least two biological repeats. Error bars represent 95% confidence intervals.

When the testing of clean deletion mutants did not reveal significant defects, the possibility that pasteurization may be masking, or obscuring growth defects was explored. Testing in unpasteurized milk was accomplished by first centrifuging and then filtering the milk to

remove contaminating bacteria. Again, only *fhuC:ba* showed a significant decrease in growth in unpasteurized bovine milk (P-value at 10 hrs = 0.0246) (Figure 23).

Figure 23: Group A growth in unpasteurized bovine milk
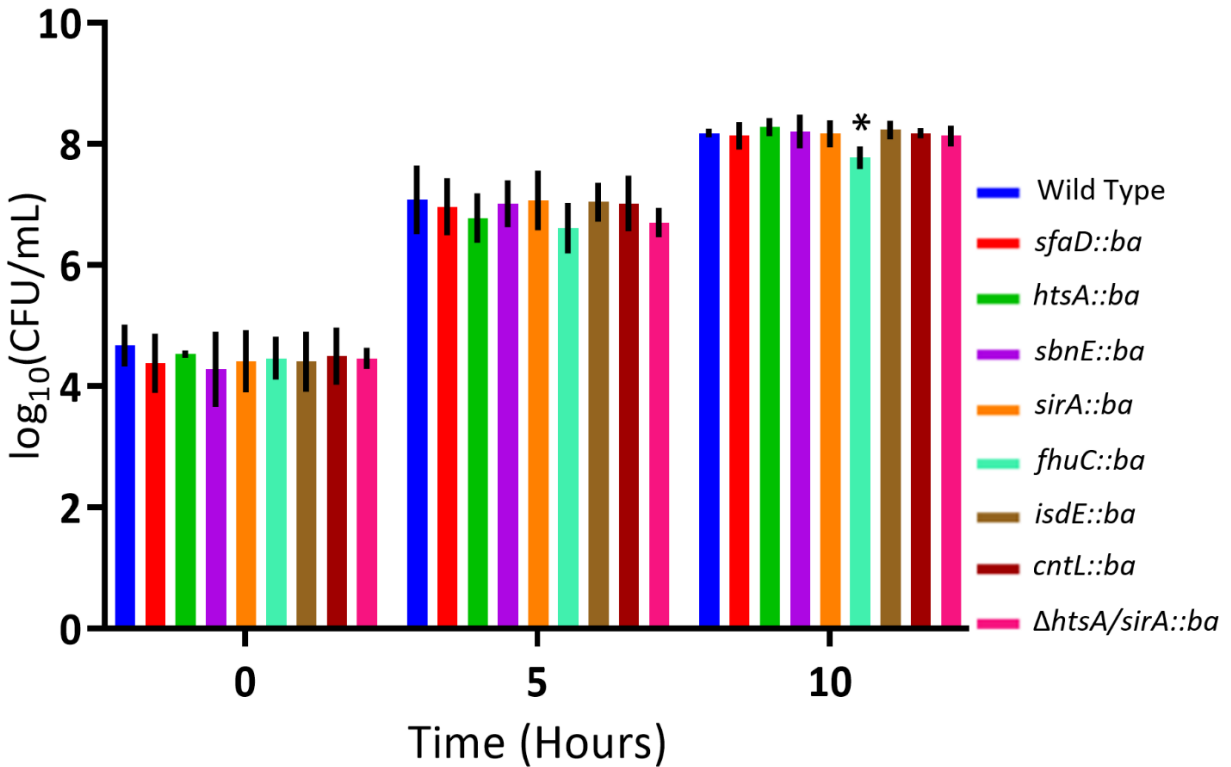
Growth in bovine unpasteurized milk reported as the log transformation of CFU/mL at 0, 5, and 10hrs. At 10 hrs *fhuC::ba* displays a slight but significant growth defect. Data represents three biological repeats. Error bars represent 95% confidence intervals. *P-value = <0.05

These data indicate that pasteurization does not affect the availability of tested strains to grow in bovine milk. Growth of the *fhuC::ba* mutant was altered in bovine milk. However, since the double siderophore receptor knock-out mutant, Δ*htsA/sirA::ba*, did not show a similar defect it would seem that multiple systems utilizing FhuC as an ATPase are involved in iron uptake.

3.1.3 Growth in human milk

Filtered human milk was also used to test the mutants for growth defects (Figure 24). At 10 hr growth in human milk *fhuC::ba* shows a slight growth defect as does Δ*htsA/sirA::ba* (P-values = 0.0183 and 0.0264 respectively). In TMM media with human lactoferrin these two

mutants exhibited a strong growth defect. Likely the human lactoferrin present in breastmilk limits the ability of *fhuC::ba* and Δ*htsA/sirA::ba* to acquire iron, but other iron sources present in the milk help compensate for this loss of function, resulting in a smaller growth difference in human milk compared to TMM supplemented with human lactoferrin. The fact that any defect is seen highlights the importance of these genes for growth in breastmilk and shows that human lactoferrin is a relevant and important iron source for *S. aureus* in milk.

Figure 24: Group A growth in human milk
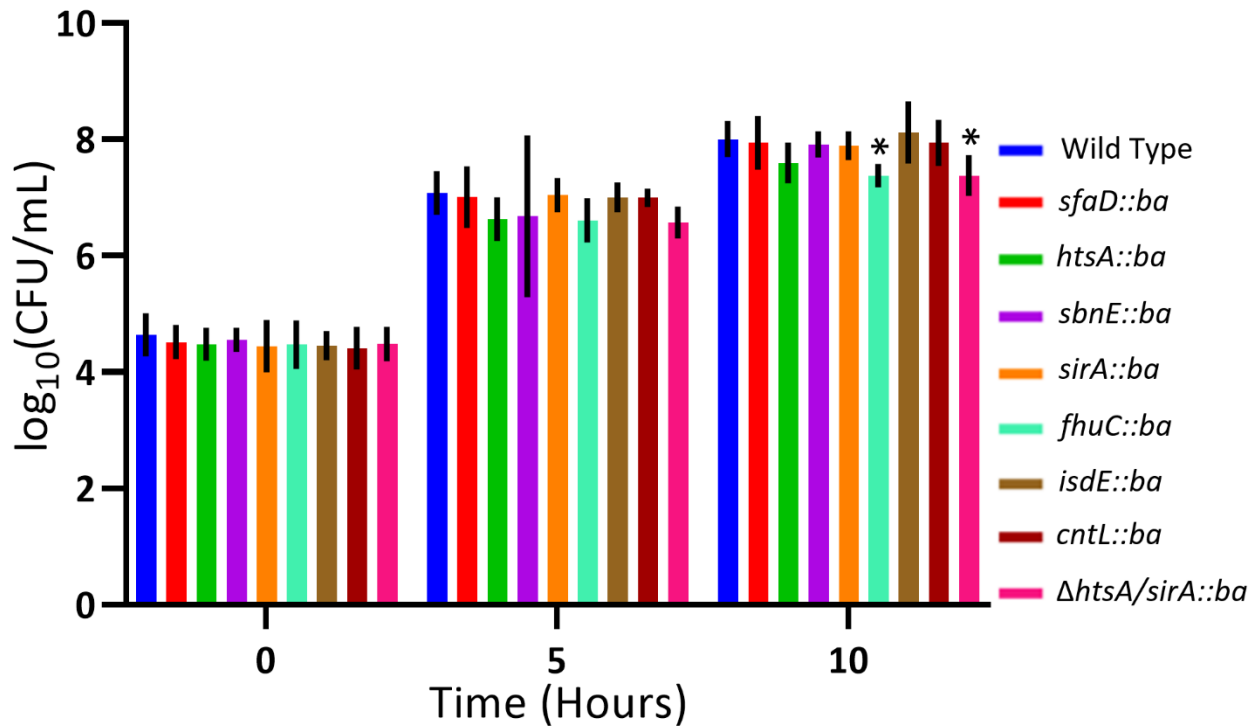
Growth in human milk reported as the log transformation of CFU/mL at 0, 5, and 10hrs. At 10 hrs *fhuC:ba* and Δ*htsA/sirA::ba* have growth defects. Data represents three biological repeats. Error bars represent 95% confidence intervals. *P-value = <0.05

3.2 Group B: Mutants involved with additional metals and cellular processes

Table 5: Group B: Mutants involved with additional metals and cellular processes

Mutant	Function
znuB::ba	Metal ABC transporter, membrane permease
znuC::ba	Metal ABC transporter, ATPase
psaA::ba	Manganese ABC transporter, substrate-binding protein
purH::ba	Purine biosynthesis protein
ctaA::ba	Cytochrome oxidase assembly protein
dppD::ba	Dipeptide/Nickle ABC transporter, ATPase
nlpA::ba	(MetQ) Methionine binding lipoprotein

Mutant strains and functions selected for testing in Group B. Each mutant has functions associated with metal acquisition (other than iron) or other cellular processes. These mutants were identified through Tn-seq data produced in the Wilson Lab.

3.2.1 Growth in minimal media

As had been done previously with Group A mutants, Group B mutants were grown in TMM media with differing iron sources relevant to mastitis. Figures 25-39 show the growth of *S. aureus* Group B mutants in each type of media for comparison against one another. In addition, Figures 53-59 display these data by strain rather than media type and are included for reference in the Supplementary Figures section of this thesis.

Minimal TMM media was used as the baseline negative control for growth. Group B mutants display unique growth patterns in TMM (Figure 25). Three strains (*znuC::ba, znuB::ba,* and *psaA::ba* discussed in greater detail below) show a delayed growth pattern in the minimal media. TMM media was designed to only be deficient in iron. However, without the proper functioning of several genes important in acquisition of other metals, such as zinc and manganese, growth inhibition was observed. The mutant *ctaA::ba*, involved in cytochrome oxidase assembly, has markedly inhibited growth compared to the wild-type strain and *purH::ba*, involved in purine biosynthesis, appears incapable of growing in TMM media.

Figure 25: Group B growth in TMM
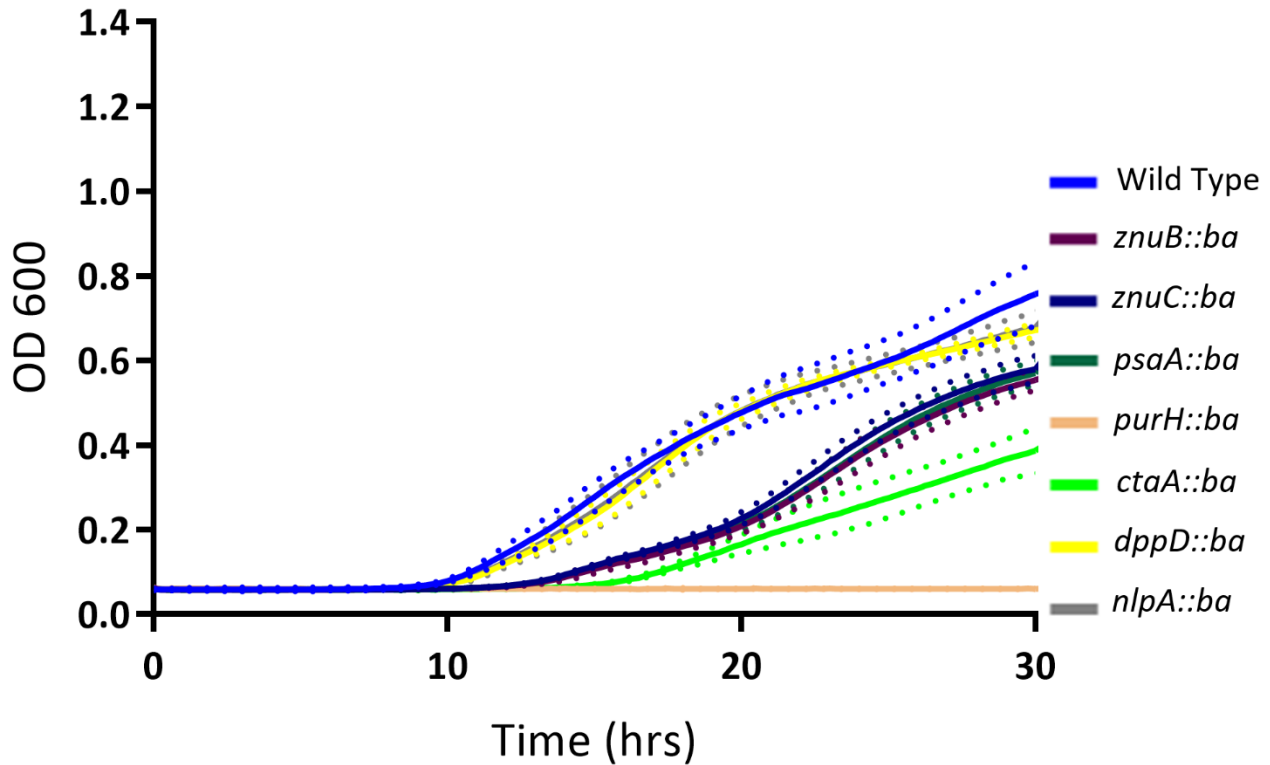
Growth of strains for 30 hrs in TMM. While *dppD::ba* and *nlpA::ba* appear to grow similar to the wild-type strain, *znuB::ba, znuC::ba,* and *psaA::ba* all display a delayed growth phenotype and lowered growth overall in TMM. *ctaA::ba* also has distinctively low growth, and *purH::ba* does not grow at all in the TMM media. Data represents three biological repeats each consisting of three technical repeats.

As a positive control, ferric sulfate was supplemented into the TMM media (Figure 26). *znuB::ba, znuC::ba,* and *psaA::ba* again show a delayed growth suggesting that the observed growth defect was not due to iron limitation. Most striking is that two of these mutants target the same operon (*znuB* and *znuC*). The *znuABC* operon is commonly annotated as *adcABC* in *S. aureus,* however, to be consistent with the NTML database the *znuABC* annotation will be used for the duration of this paper. The operon consists of a substrate binding protein (ZnuA), membrane permease (ZnuB) and ATPase (ZnuC) which function together as a metal ABC-type transporter for zinc and manganese. ZnuA was not tested simply because it did not appear in the Tn-seq screen conducted by Dr. Wilson (data not shown). The other mutant with the same delayed growth, *psaA::ba*, is located adjacent to *znuBC* and is reported to function as a

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Figure 26: Group B growth in ferric sulfate
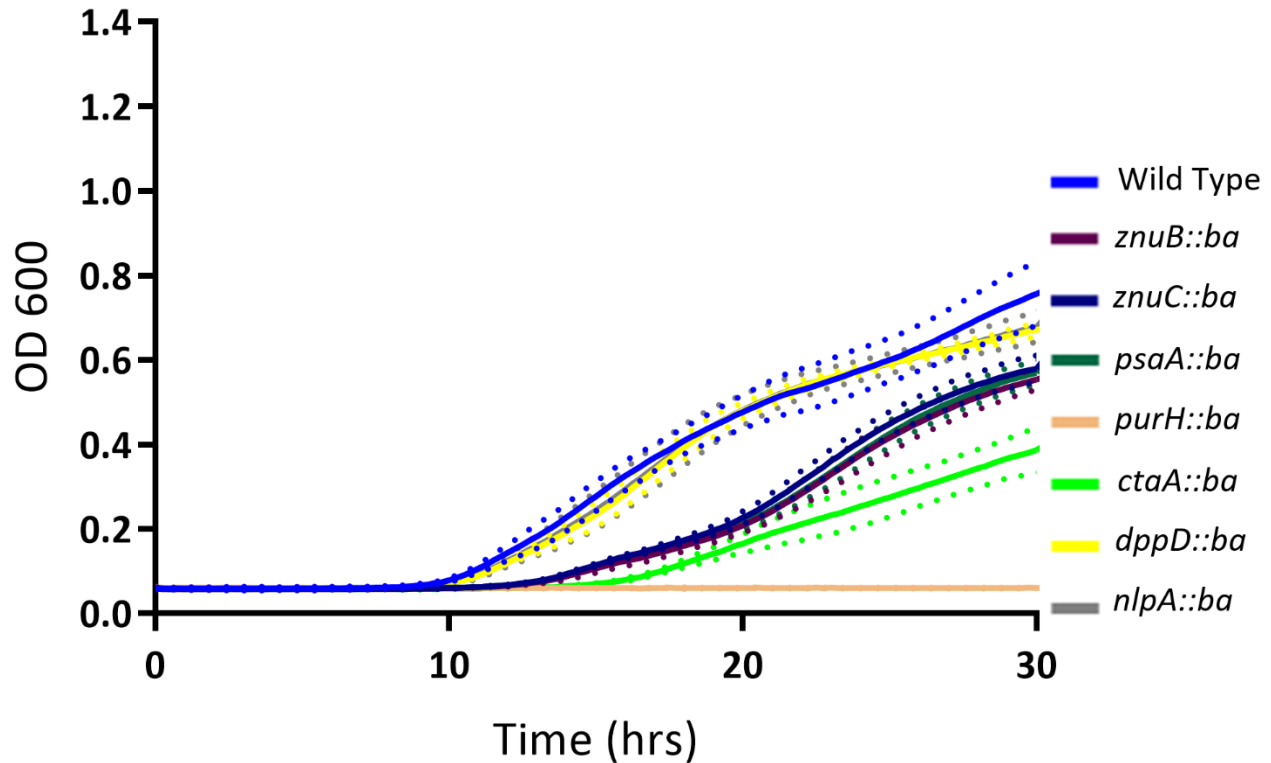
Growth of strains for 30 hrs in TMM supplemented with ferric sulfate. Data represents three biological repeats each consisting of three technical repeats.

manganese binding protein of an ABC-type transporter. This gene also has the common

alternative annotation MntC for manganese transport protein C. This delayed growth pattern is

found in TMM and in the positive control (TMM with ferric sulfate) and therefore should be

considered an inherent growth defect of these mutants in the minimal media, and not as an

indicator of poor growth in other supplemented media types. The same can be said of *ctaA::ba*

which shows greater growth in ferric sulfate when compared to minimal TMM. This growth,

however, remains far below the wild-type standard. Figure 27 displays percent growth increase of

strains in TMM supplemented with ferric sulfate in comparison to minimal TMM. The mutant

purH::ba is unable to grow in ferric sulfate supplemented media, or in any of the other tested

TMM media (Supplementary Figure 56), and thus no reliable inferences can be made regarding

this strain in these growth curves, to avoid potential confusion, this strain has been removed from the remainder of the TMM growth curves.

Figure 27: Group B percent growth in ferric sulfate
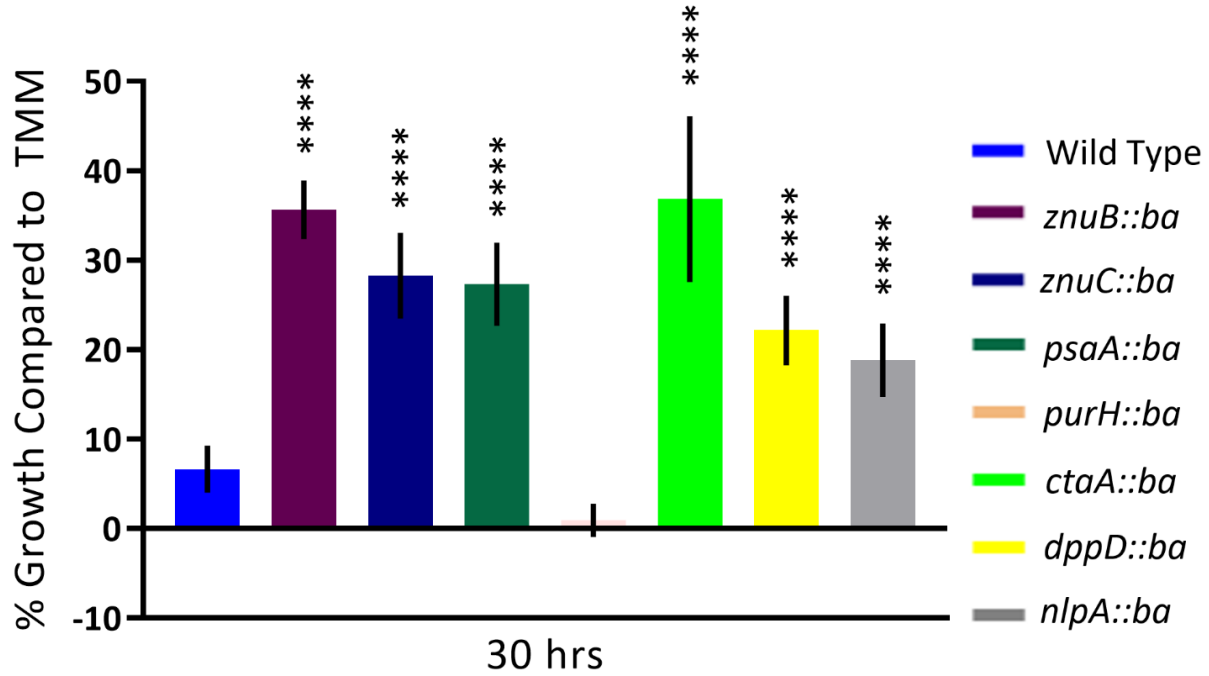
Percent growth increase of strains in TMM supplemented with ferric sulfate compared to TMM at 30 hrs. All strains (except for the wild type and purH::ba) are able to grow in the positive control significantly better than in the negative control. Data represents three biological repeats each consisting of three technical repeats. ****P-value = <0.0001

Although the experimental plan was intended to test for iron acquisition, these mutants which function in other metal acquisition and additional cellular processes also show significantly increased growth at 30 hrs with the addition of iron into the system (every strain, excluding the wild type and *purH::ba* had an increased growth with a P-value = <0.0001). This could be because the minimal media is a stressful environment and the addition of iron results in increased growth of non-iron related mutants because the strains no longer need to waste energy producing siderophores or other iron uptake related proteins. Unpublished research conducted in the Wilson lab by Abraham Quaye showed that ZnuB functioned primarily in manganese transport, however, it could also be involved in iron uptake (data not shown). This data could also help explain why

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iron supplementation improves growth of *znuB::ba* in minimal media (the other strains were not tested).

The addition of iron saturated bovine transferrin also results in increased growth (Figure 28). Figure 29 also displays this data as a percent increase of growth in comparison to TMM. The wild type, *znuB::ba, znuC::ba, psaA::ba, dppD::ba,* and *nlpA::ba* each had increased growth in comparison to their baseline growth in the negative control (P-values = <0.0001), while *ctaA::ba* had a P-value = 0.0328. The wild-type strain grows the best in bovine transferrin, but since all strains showed higher growth in media supplemented with bovine transferrin than in plain TMM, this data supports my previous findings that bovine transferrin is a viable iron source for *S. aureus in vitro*.

**Figure 28: Group B growth in bovine transferrin**
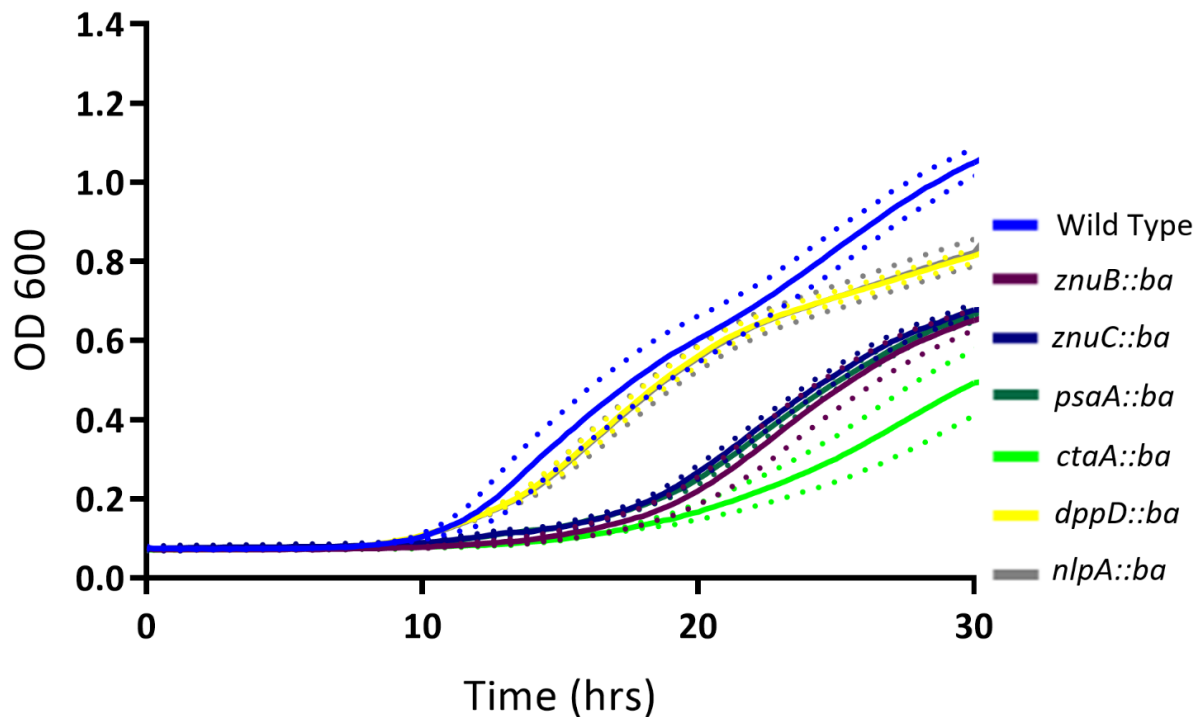
Growth of strains for 30 hrs in TMM supplemented with bovine transferrin. Data represents three biological repeats each consisting of three technical repeats. *P-value = <0.05 ****P-value = <0.0001

Figure 29: Group B percent growth in bovine transferrin
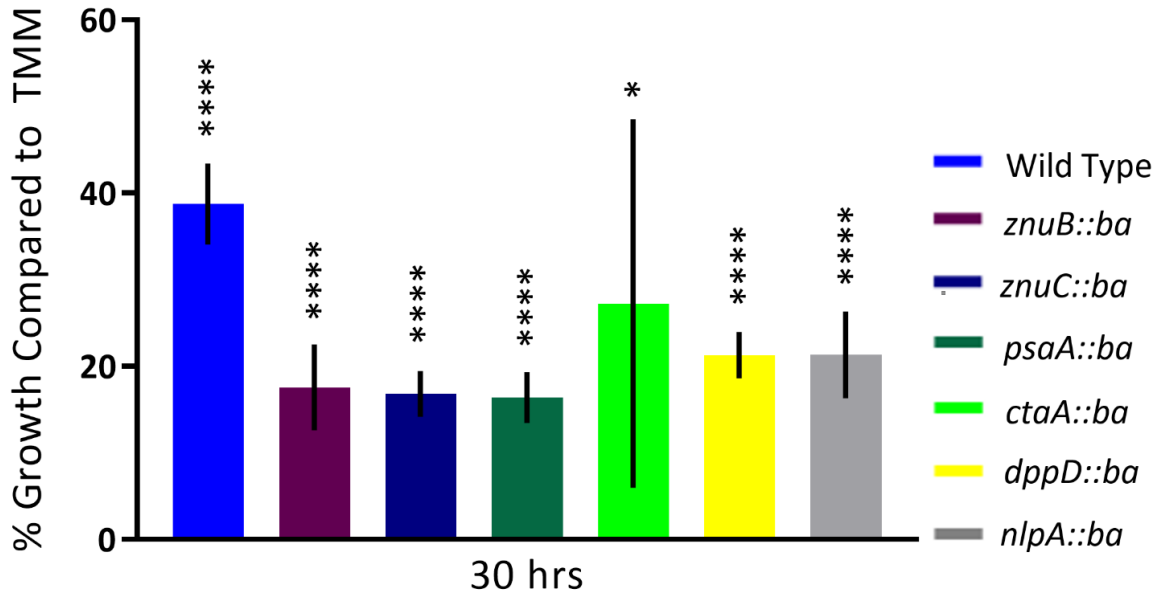
Percent increased growth of strains in TMM supplemented with bovine transferrin in comparison to growth in TMM at 30 hrs. Although each strain tested grows considerably less than the wild type control, all strains do show increased growth in comparison to their baseline growth in minimal TMM (Figure 25). Data represents three biological repeats each consisting of three technical repeats. *P-value = <0.05 ****P-value = <0.0001

Figure 30: Group B growth in human transferrin
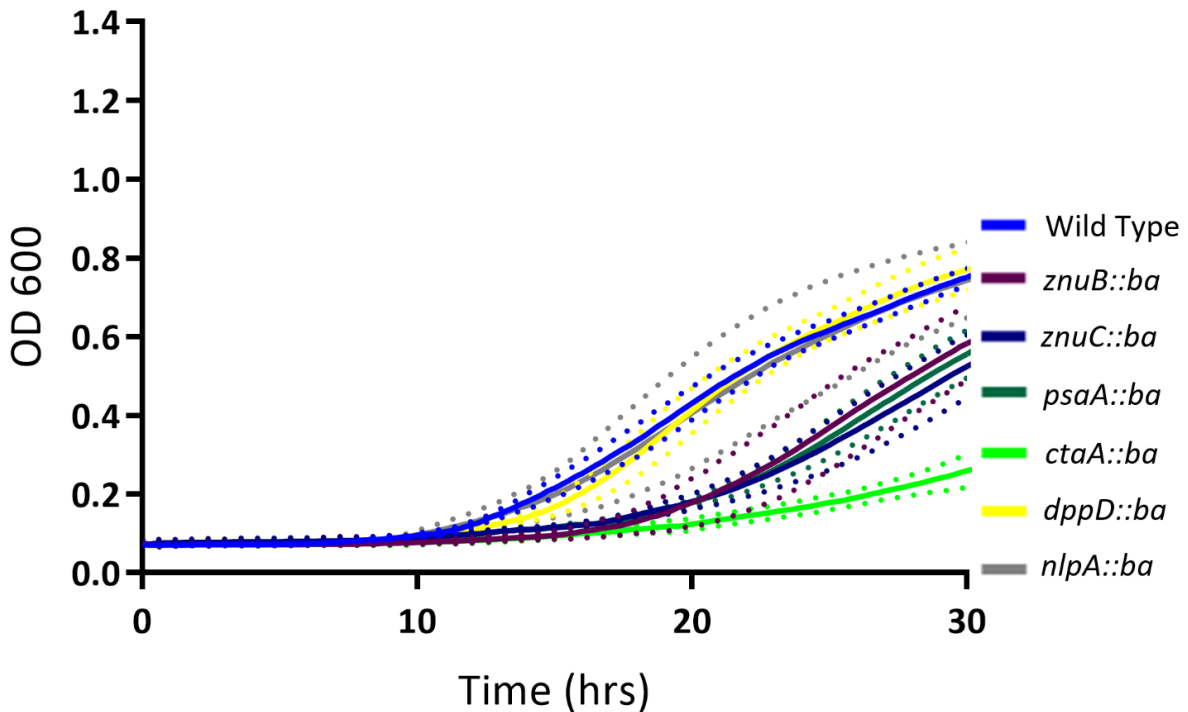
Growth of strains for 30 hrs in TMM supplemented with human transferrin. Data represents three biological repeats each consisting of three technical repeats. **P-value = <0.01

When iron saturated human transferrin was utilized as an iron source in the medium, many strains, including the wild type, grew at levels very similar to growth in plain TMM. (Figure 30). Percent increase of growth in TMM supplemented with human transferrin in comparison to TMM is shown in Figure 31. While *dppD::ba* showed a slight increase in growth (P-value = 0.0017), *ctaA::ba* was the only mutant to show a decrease in growth (P-value = 0.0003) compared to its already poor growth in TMM. Its function in cytochrome oxidase assembly could be important for survival in human transferrin.

Figure 31: Group B percent growth in human transferrin
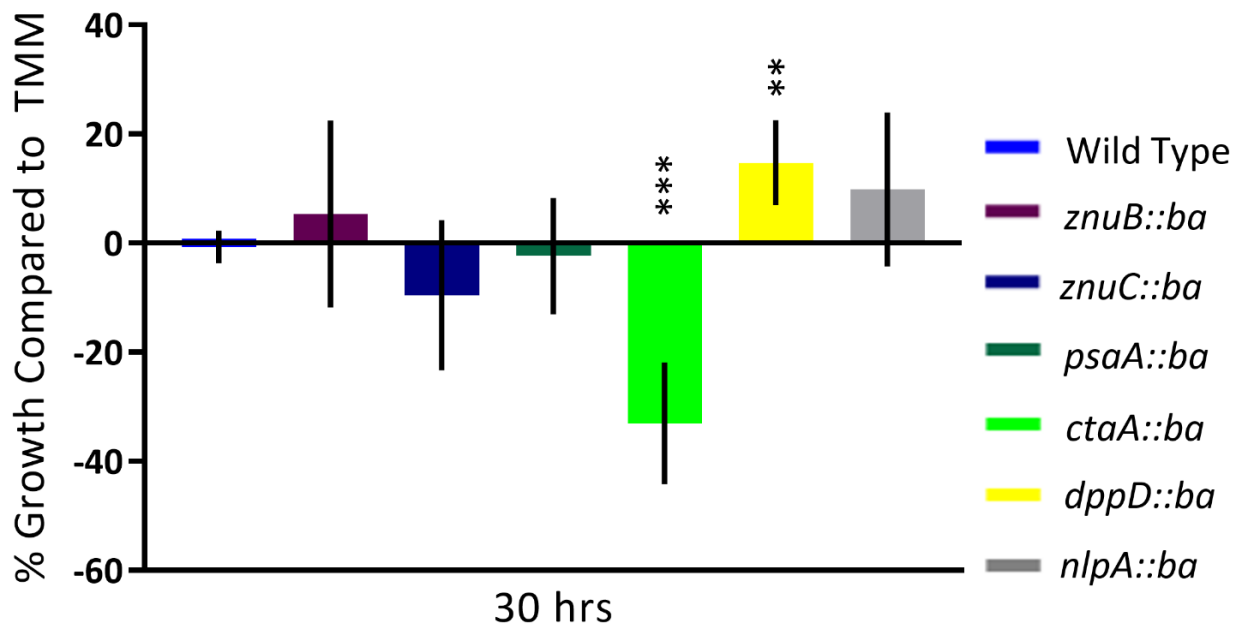
Percent change in growth of strains TMM supplemented with human transferrin compared to growth in TMM at 30 hrs. Overall, the strains exhibit poor growth in human transferrin, similar to the base line growth in TMM. *ctaA::ba* did have a slight decrease of growth, while *dppD::ba* appeared to have a slight increase of growth in this media. Data represents three biological repeats each consisting of three technical repeats. **P-value = <0.01 ***P-value = <0.001

Due to the limited availability of bovine lactoferrin isolated from bovine milk, only *znuB::ba* was selected to be tested in this media (Figure 32). The growth curve was conducted only once in triplicate and has not been repeated. Percent increase in growth of *znuB::ba* in bovine lactoferrin in comparison to TMM (Figure 33) with a P-value of 0.0024. Despite the high

level of growth achieved, this mutant maintains its delayed growth phenotype indicating that the strain takes time adapting to the media in order to grow. Again, this highlights the importance of ZnuB in obtaining other metals in the media.

Figure 32: Group B growth in bovine lactoferrin isolated from milk
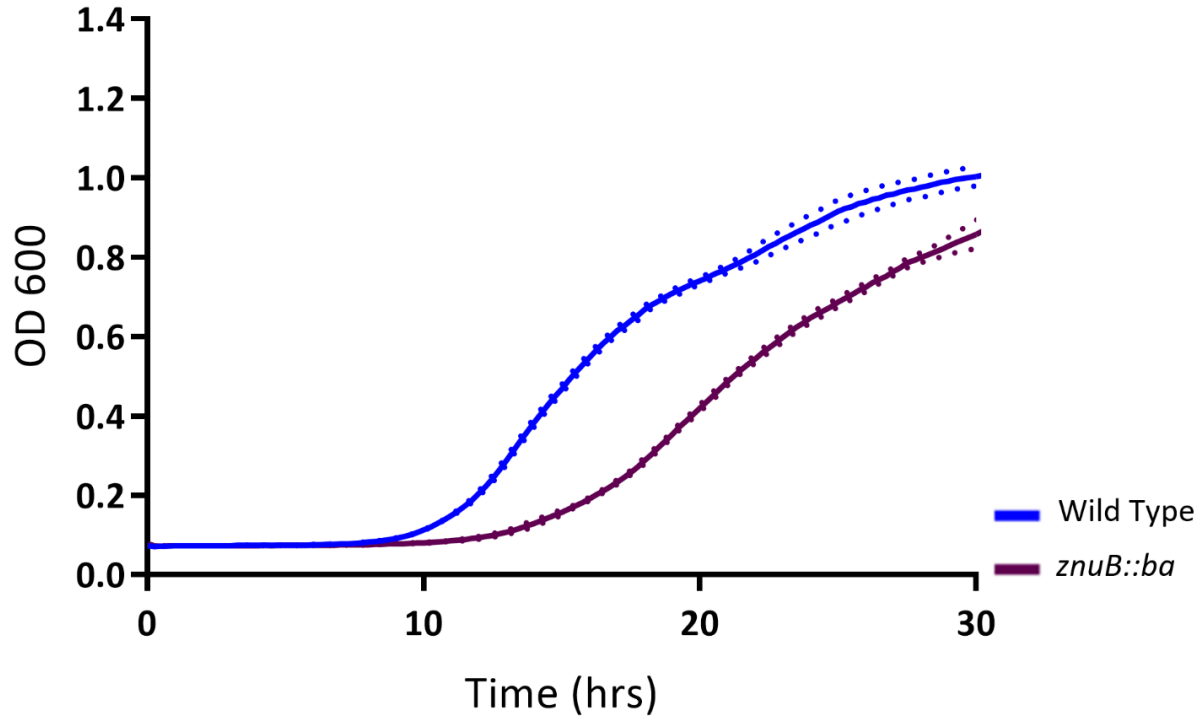
Only the *znuB::ba* strain was tested in TMM media supplemented with bovine lactoferrin isolated from milk. Data represents one experiment consisting of three technical repeats. **P-value = <0.01

Figure 33: Group B percent growth in bovine lactoferrin isolated from milk
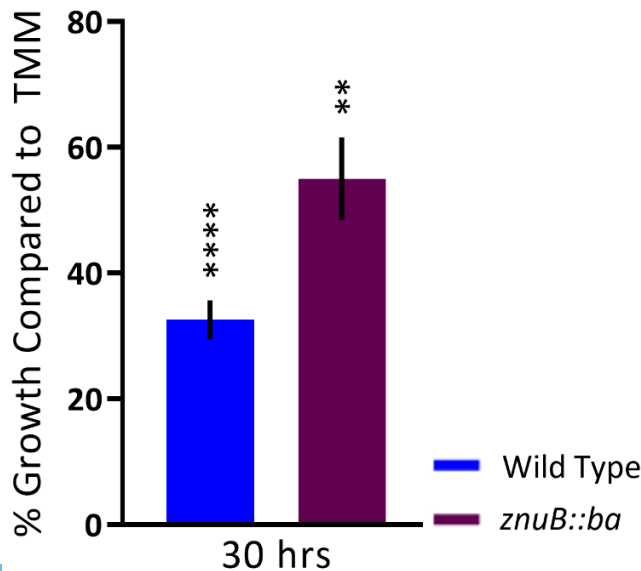
Percent increase of growth for the wild-type and *znuB::ba* strains in TMM supplemented with bovine lactoferrin isolated from milk in comparison to growth in TMM at 30 hrs. *znuB::ba* shows increased growth in comparison to the TMM media, and in comparison to its previous growth in bovine transferrin. Data represents one experiment consisting of three technical repeats. **P-value = <0.01

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Similar to what was seen in Group A mutants, growth in bovine lactoferrin isolated from bovine colostrum was depressed in all strains (Figure 34). While the wild type, *dppD::ba,* and *nlpA::ba* showed no significant percent change in growth in comparison to the negative control, *znuB::ba, znuC::ba, psaA::ba,* and *ctaA::ba* all were unable to grow on this media type (P-values = <0.0001) (Figure 35). As discussed before, I hypothesize that this is due to an increase of apolactoferrin for the amount of iron supplemented into the media. Lactoferrin is capable of binding other metals such as zinc and manganese, therefore a high amount of excess apolactoferrin could also explain why these other metal binding mutants struggled to grow in this media as well. *ctaA::ba* which already displays comparatively low growth across all the different media also could not grow, possibly due to increased stress on the cells due to the added competition of excess lactoferrin.

Figure 34: Group B growth in bovine lactoferrin isolated from colostrum
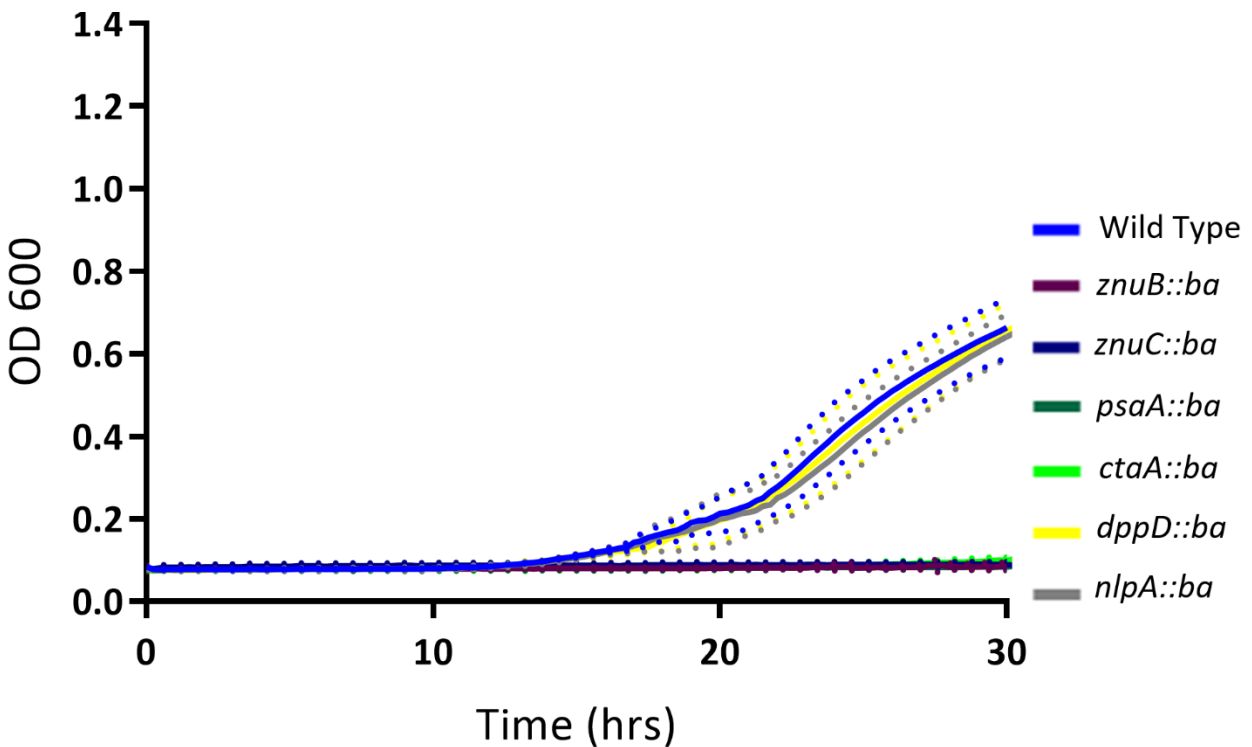
Growth of strains for 30 hrs in TMM supplemented with bovine lactoferrin isolated from colostrum. Data represents three biological repeats each consisting of three technical repeats.
****P-value = <0.0001

Figure 35: Group B percent growth in bovine lactoferrin isolated from colostrum
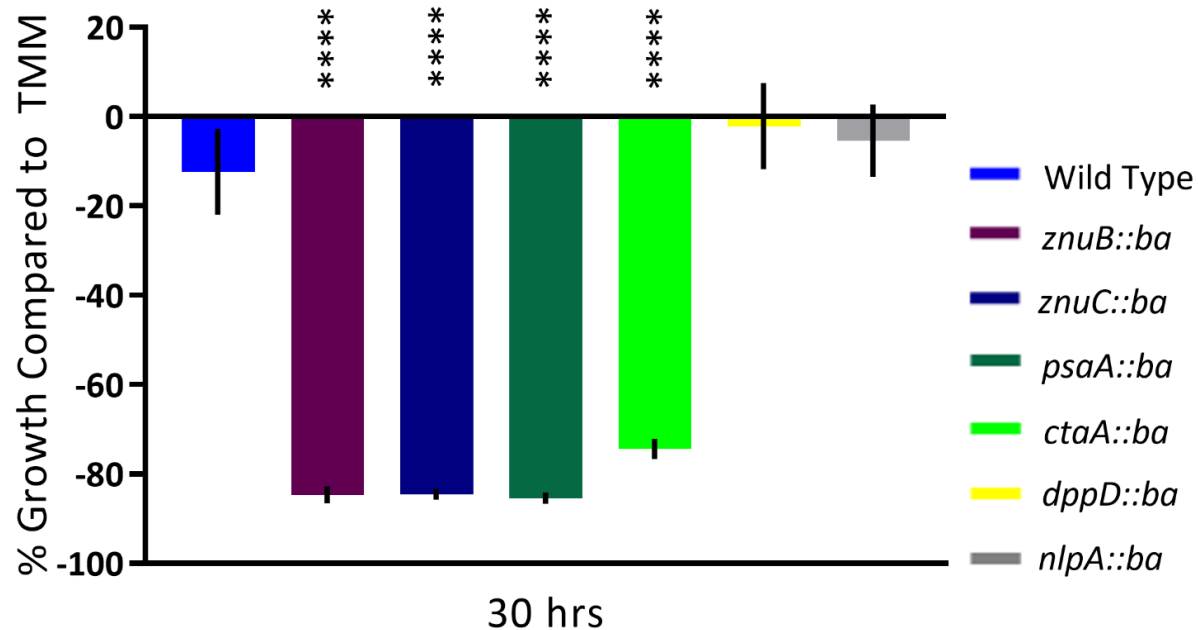
Percent decrease in growth of strains in TMM supplemented with bovine lactoferrin isolated from colostrum as compared to growth in TMM at 30 hrs. All strains struggle to grow, with *znuB::ba, znuC::ba, psaA::ba,* and *ctaA::ba* exhibiting significantly lowered growth in this media. Potentially this could be due to excess apolactoferrin present in the media which adds additional stress and competition in binding up zinc and manganese. Data represents three biological repeats each consisting of three technical repeats. ****P-value = <0.0001

Each mutant shows an increased growth in human lactoferrin compared to the TMM control (Figure 36). Percent increase of growth in this medium compared to TMM reveals all strains significantly improved growth (Figure 37). The wild type, *znuB::ba, znuC::ba, psaA::ba, dppD::ba,* and *nlpA::ba* each have growth increases with a P-value = <0.0001. *ctaA::ba* has an increased growth with a P-value = 0.0015. It is also notable that *znuB::ba, znuC::ba,* and *psaA::ba* do not exhibit as strong of a growth delay as is usually seen. This could indicate that either the human lactoferrin was contaminated with additional metals, or that *S. aureus* has means to utilize other metals, such as zinc and manganese, which are bound to human lactoferrin.

Figure 36: Group B growth in human lactoferrin
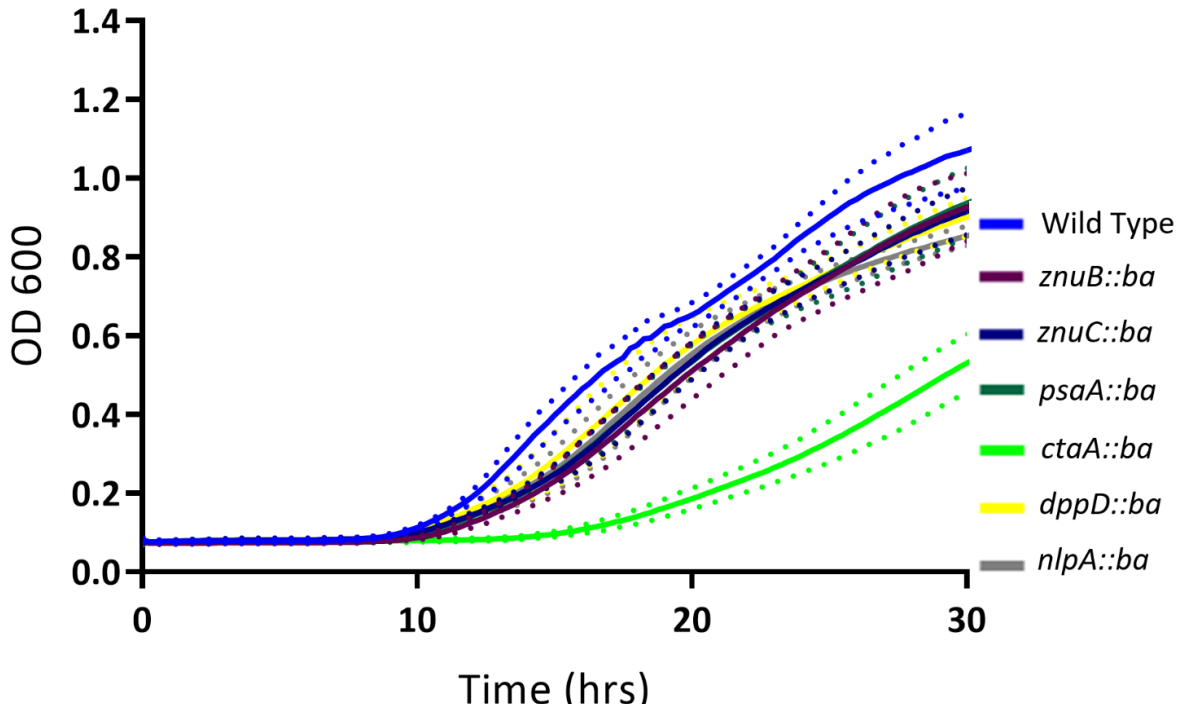
Growth of strains for 30 hrs in TMM supplemented with human lactoferrin. Data represents three biological repeats each consisting of three technical repeats.

Figure 37: Group B percent growth in human lactoferrin
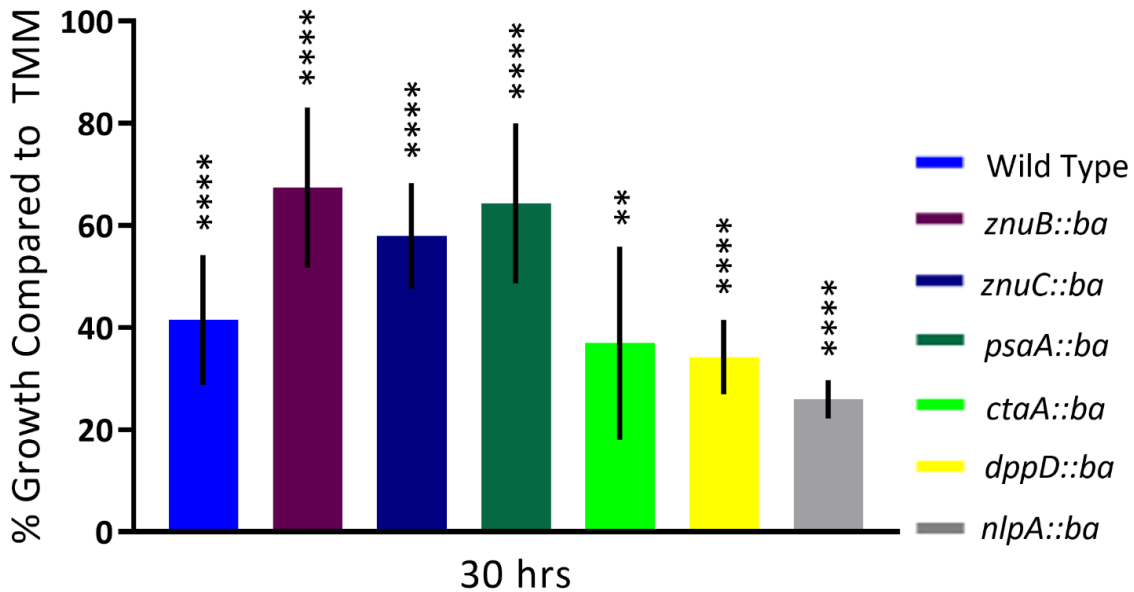
Percent increased growth of strains for 30 hrs in TMM supplemented with human lactoferrin. All strains show significantly increased growth in the media in comparison to the TMM negative control. Data represents three biological repeats each consisting of three technical repeats. **P-value = <0.01 ****P-value = <0.0001

Ferric citrate was also tested with the Group B mutants (Figure 38) and percent change in growth determined as a comparison to growth in TMM (Figure 39). Growth patterns of all mutants are nearly identical to the growth seen in the positive control with ferric citrate with every strain, except the wild-type strain, showing increased growth with a P-value= <0.0001. An additional 10 times the amount of citrate was used to help bind up any residual iron that may be present in the ferric citrate. It is unclear how the ferric citrate is being taken up by the bacteria.

Figure 38: Group B growth in ferric citrate with 10X citrate
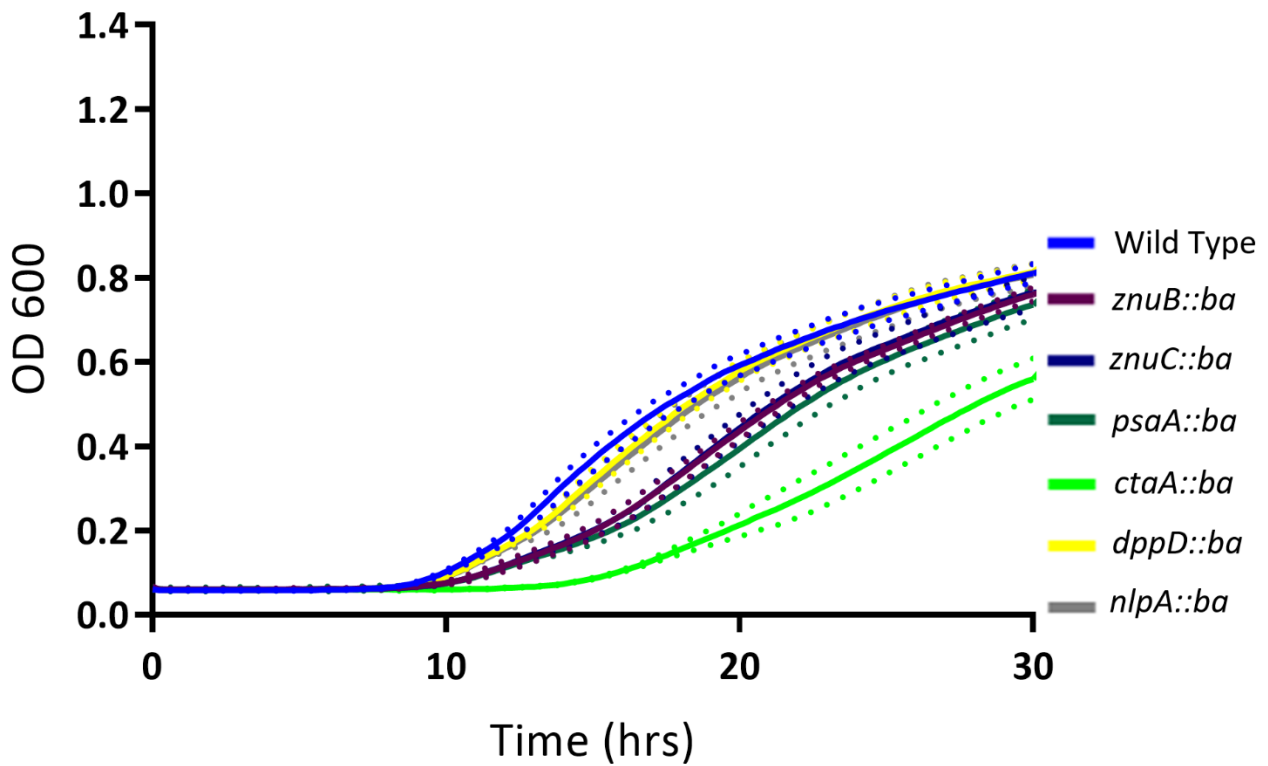
Growth of strains for 30 hrs in TMM supplemented with ferric citrate with the addition of 10X citrate. Data represents three biological repeats each consisting of three technical repeats.

Figure 39: Group B percent growth in ferric citrate with 10X citrate
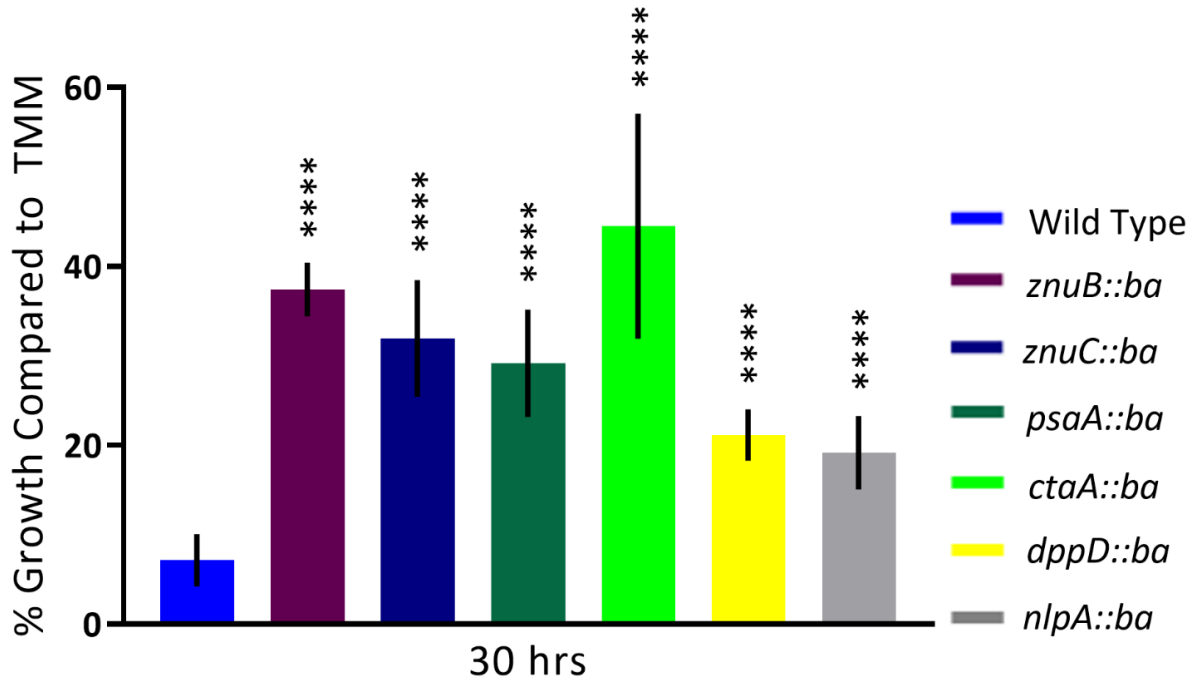
Percent increase growth of strains in TMM supplemented with ferric citrate with the addition of 10X citrate in comparison to growth in TMM at 30 hrs. All strains show increased growth in comparison to TMM and very similar to the growth observed in TMM with ferric sulfate Data represents three biological repeats each consisting of three technical repeats. ****P-value = <0.0001

3.2.2 Growth in bovine milk

Growth in pasteurized milk of Group B mutants (Figure 40) reveals that the *psaA::ba* mutant has a growth defect at 5 hrs (P-value = 0.0053). By 10 hrs this defect is no longer seen, which may be due to cultures reaching saturation at the 10 hr timepoint. The only mutant with a growth defect at 10 hrs is *purH::ba* (P-value = 0.0039). PurH is a purine biosynthesis protein, and as the name implies is involved in *de novo* purine biosynthesis. Purine metabolism is a necessary part of DNA synthesis and energy production. Disruption of purine metabolism can have wide reaching effects on regulatory proteins and hundreds of other genes, and consequently *purH* has previously been identified as a virulence factor in murine abscess infections [242].

Figure 40: Group B growth in bovine pasteurized milk
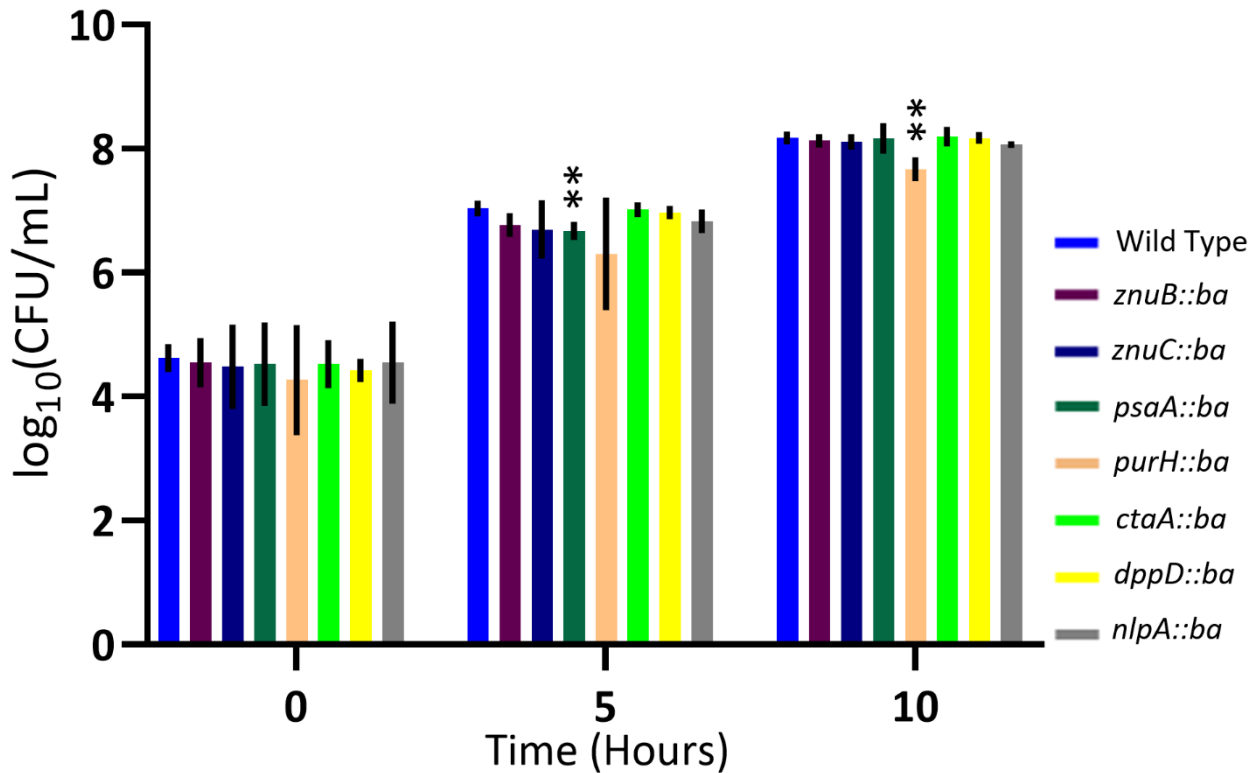
Growth in bovine pasteurized milk reported as the log transformation of CFU/mL at 0, 5, and 10 hrs. At 5 hrs the *psaA::ba* mutant displays a small but significant growth defect, while at 10 hrs a growth defect is seen in *fhuC:ba*. Data represents three biological repeats. Error bars represent 95% confidence intervals. **P-value = <0.01

To determine if pasteurization was masking or obscuring additional growth defects the mutants were then grown in unpasteurized bovine milk (Figure 41). *psaA::ba* again shows a slight defect in growth (P-value = 0.0324), this time at 10 hrs (the 5 hr timepoint also shows a lowered mean in comparison to the wild type, however there is higher variation between one of the replicates leading to no significance). These results could indicate that manganese availability may be limited in bovine milk and that PsaA is essential for overcoming that limitation. This is supported by previous research with *Streptococcus uberis* which determined that a homolog of *psaA* is essential in manganese acquisition during growth in bovine milk and *in vivo* bovine infections [243]. At 5 and 10 hrs *purH::ba* exhibits a significant growth defect (P-values 0.0011 and 0.0012 respectively). Although visually the means of these data points are substantially

lowered in unpasteurized milk than *purH::ba* values in pasteurized milk, the statistical significance is similar. Likely this is due to the low number of replicates (3) used to plot the data, making it hard to achieve high statistical power. The obvious visual decrease in growth of *purH:ba* in unpasteurized bovine milk may be due to interactions with viable host proteins which would not be present in pasteurized milk. Due to the-wide number of downstream effects *purH* can have, it is unlikely the defect seen in bovine milk (pasteurized or unpasteurized) is strictly due to iron or metal depravation.

Figure 41: Group B growth in unpasteurized bovine milk
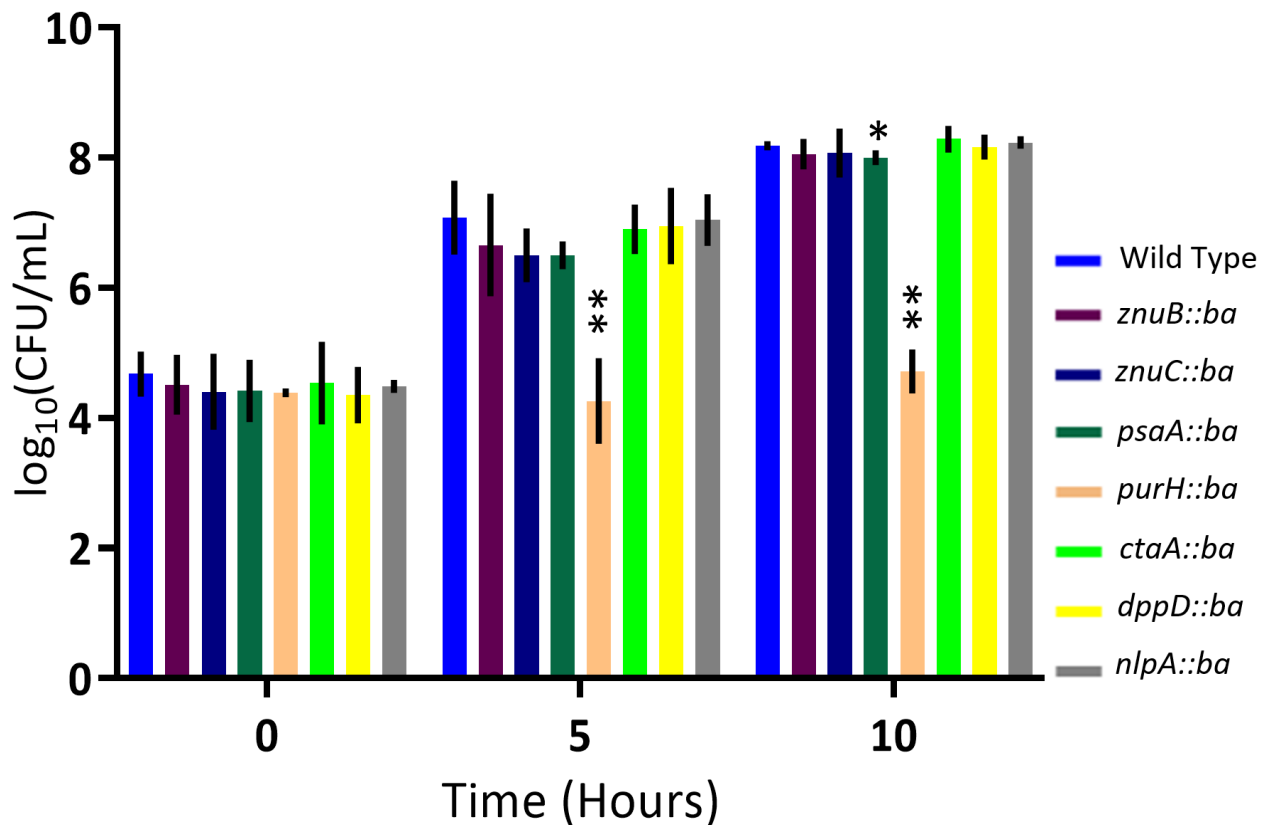
Growth in bovine unpasteurized milk reported as the log transformation of CFU/mL at 0, 5, and 10 hrs. At 5 and 10 hrs *purH::ba* displays a growth defect. *psaA::ba* also shows a slight defect at 10hrs. Data represents three biological repeats. Error bars represent 95% confidence intervals. *P-value = <0.05 **P-value = <0.01

3.2.3 Growth in human milk

Filtered human milk was also used to test the mutants for growth defects. Interestingly, the *purH::ba* mutant did not exhibit the same defect as seen in bovine milk. Several other mutants, however, exhibit significant growth defects (Figure 42). At 5 hrs *znuB::ba, znuC::ba,* and *psaA::ba* all display reduced growth (P-values = 0.0002, 0.0003, 0.0004 respectively). At 10 hrs this defect is still evident (P-values = 0.0004, <0.0001, <0.0001 respectively). Manganese has been shown to be a limiting factor for growth of *S. aureus* in human breastmilk, and *psaA* plays an essential role in oxidative stress resistance due to manganese acquisition [208,244]. The presence of *psaA, znuB,* and *znuC* in the same operon and all showing equal importance in

Figure 42: Group B growth in human milk
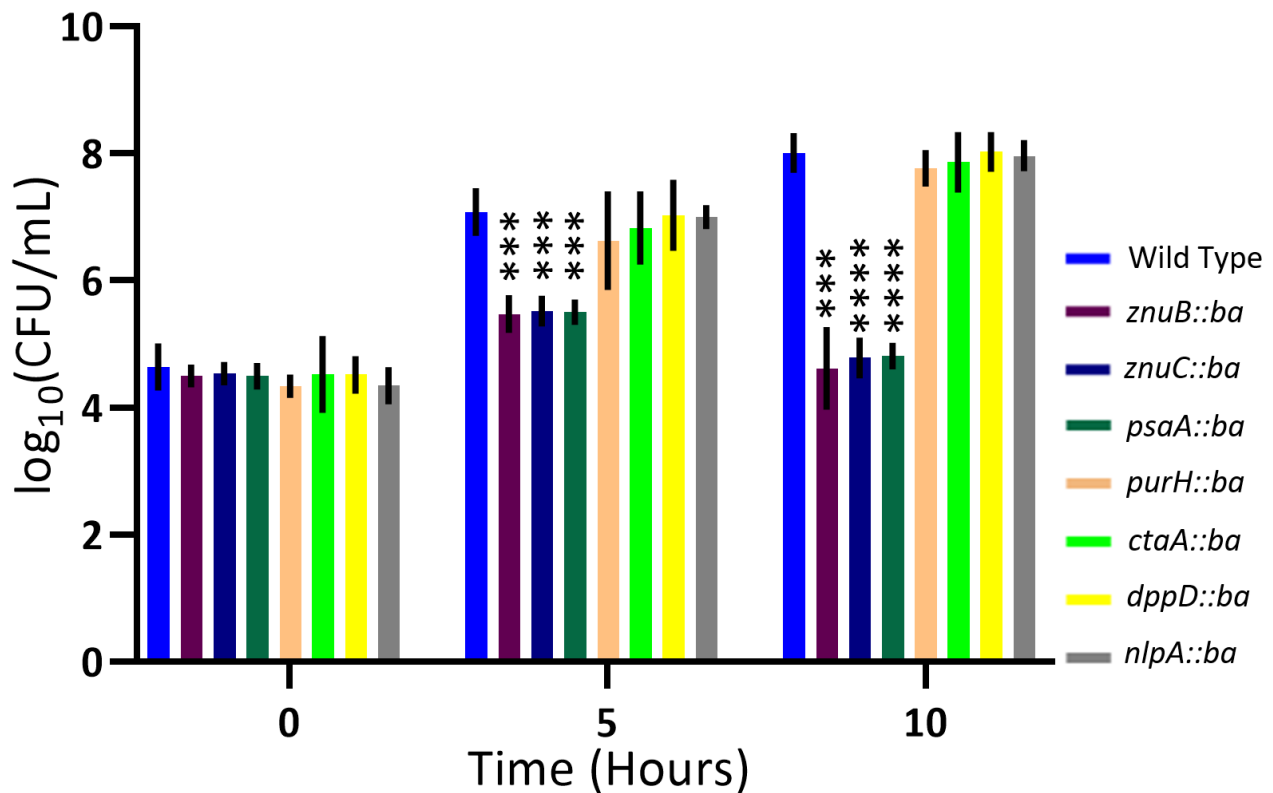
Growth in human milk reported as the log transformation of CFU/mL at 0, 5, and 10 hrs. At 5 and 10 hrs *znuB::ba, znuC::ba,* and *psaA::ba* have significant growth defects in comparison to the wild type. Data represents three biological repeats. Error bars represent 95% confidence intervals. ***P-value = <0.001 ****P-value = <0.0001

survival suggest that *psaA, znuB* and *znuC* may actually be functioning together in transporting essential metals into the bacterium.

To further investigate the function of *znuB*, wild-type and *znuB::ba* strains were grown in human milk supplemented with zinc, manganese, or iron as shown in Figure 43. Each metal was added at three different concentration as indicated (2X, 10X, or 100X the amount of each metal found naturally in human milk). Although this experiment has only been conducted once, it clearly shows differences in the utilization of the three metals. When 10X or 100X manganese was added, restoration of growth in the *znuB::ba* strain is seen and growth of the wild-type strain also improves. However, when zinc is added at any of the tested concentrations, no improvement occurs in growth for either strain indicating that zinc is not a growth limiting factor in human

Figure 43: Growth in human milk with additional metals
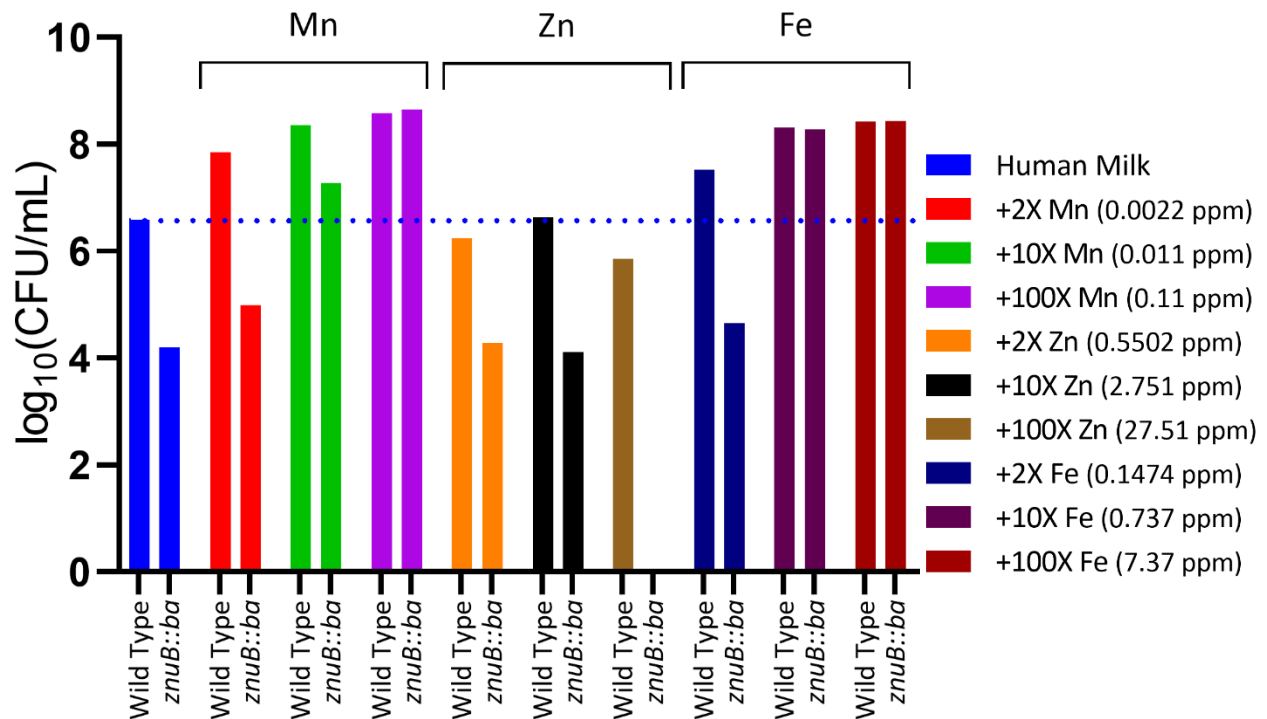
Growth of *znuB::ba* and wild-type strains in human milk supplemented with additional metals. Manganese, zinc, and iron were added at 2X, 10X and 100X concentrations of the normal amount of each metal found naturally in human milk. Parenthesis indicate the final concentration of each metal added (ppm). The addition of manganese and iron show restoration of growth for *znuB::ba* and increased growth of the wild-type strain in human milk. Data represents a single experiment.

milk. The addition of 10X or 100X iron also shows complete restoration of the *znuB::ba* growth defect in human milk, indicating that znuB may also function as an iron transporter. One caveat to this assumption is that none of the added metals are pure, therefore, the addition of 10X or 100X of iron could include trace amounts of manganese that may be influencing growth. Further investigation is required to fully understand the potential involvement of *znuB* in iron and manganese transport.

3.3 Metal differences between bovine milk, human milk and TMM

Using inductively coupled plasma spectroscopy (ICP-S) bovine milk, human milk and TMM samples were tested for elemental concentrations (Table 6). ICP-S results are reported in parts per million (ppm) and show several differences between the available metals in each type of growth medium. Lower levels of manganese in human milk may explain why mutants associated with this metal (*znuB::ba, znuC::ba,* and *psaA::ba*) showed such a strong growth defect in human milk compared to growth in bovine milk. In TMM, the concentration of manganese is also low and may account for the delayed growth phenotype attributed to these mutants in *in vitro* TMM cultures. Despite the delayed growth *znuB::ba, znuC::ba,* and *psaA::ba* mutants are able to grow in TMM which indicates that the limiting factor in human milk isn't the low amount of manganese present but rather the inaccessibility of this metal due to host metal-binding factors present in the milk.

Due to the clear differences in metal composition between human and bovine milk it's not surprising that different genes are needed to grow in human milk than in bovine milk. This is an important concept because much of the research conducted on mastitis is performed on bovine or animal mastitis models. While ethical and practical reasons generally limit the ability of researchers to use human subjects, it is important to understand that not all research conducted on

animals will apply to human mastitis and vice versa. These differences could make a large impact in our ability to find viable targets for treatment and potential vaccines.

Table 6: Element concentrations in bovine milk, human milk, TMM and TMM + FeSO4

Element	Bovine Milk (ppm) ± SD	Human Milk (ppm) ± SD	TMM (ppm) ± SD	TMM +FeSO4 (ppm) ± SD
B	0.5365 ± 0.0080	0.0349 ± 0.0073	0.0252 ± 0.0010	0.0275 ± 0.0016
Ca	1027.0000 ± 20.2978	115.0667 ± 1.0263	2.8036 ± 0.0441	3.1488 ± 0.0088
Cu	0.0352 ± 0.0017	0.1300 ± 0.0083	0.0015 ± 0.0006	0.0023 ± 0.0003
Fe	0.1595 ± 0.0006	0.0737 ± 0.0142	0.0150 ± 0.0046	0.9864 ± 0.0036
K	1345.0000 ± 24.2693	353.9667 ± 17.0418	1328.5625 ± 23.0637	1365.6000 ± 11.67
Mg	104.7333 ± 1.2503	31.1400 ± 2.1652	22.7625 ± 0.2156	25.9680 ± 0.0637
Mn	0.0156 ± 0.0001	0.0011 ± 0.0002	0.0018 ± 0.0002	0.0026 ± 0.0002
Na	304.8000 ± 6.3269	36.2400 ± 3.2218	^Error	^Error
P	905.8333 ± 9.0235	116.4667 ± 1.8771	88.5525 ± 0.6486	101.5800 ± 0.1408
S	272.5333 ± 2.8572	61.6867 ± 1.6276	64.5413 ± 1.4459	71.3040 ± 0.0926
Zn	2.7673 ± 0.0655	0.2751 ± 0.0118	0.0511 ± 0.0010	0.0560 ± 0.0006

Different element concentrations (ppm) in bovine milk, human milk, TMM (negative control) and TMM supplemented with ferric sulfate (positive control). ^Error = unable to calculate value, reading is too high. B = Boron, Ca = Calcium, Cu = Copper, Fe = Iron, K = Potassium, Mg = Magnesium, Mn = Manganese, Na = Sodium, P = Phosphorus, S = Sulfur, Zn = Zinc

The addition of ferric sulfate to the minimal TMM media results in minor increases in the concentrations of other metals in the media. Similar changes were seen with the addition of other iron sources (data not shown). However, these differences are minute in comparison to the difference in iron between minimal TMM (0.0150 ppm) and that of TMM with supplemented ferric sulfate (0.9864 ppm) or other iron sources which also increase the iron concentration to ~1 ppm. Therefore, it is highly unlikely that large changes of growth in mutants are due to any slight variations of other metals in the media.

SECTION 4: CONCLUSION

My results show manganese to be the greatest limiting factors for *S. aureus* growth in human milk. The genes *znuB* and *znuC* have been annotated to play a role in obtaining zinc and manganese, while *psaA* is essential in manganese acquisition. My data indicate these genes likely function together, and that previous annotations may be incorrect as *znuB* functions predominately in manganese acquisition not zinc. Notably, the measured levels of manganese in TMM media (which the mutants can grow in) is similar with levels in human milk (which the mutants cannot grow in). This indicates that the bioavailability of this metal in breast milk may be reduced by host nutritional immunity factors present in the milk and that without functional *znuBC* and *psaA* genes, these mutants are unable to overcome this nutritional barrier.

Deletion of the iron ABC transporter ATPase, *fhuC,* resulted in small but significant growth defects in both bovine and human milk. Conversely, the double knock-out for siderophore receptors, Δ*htsA/sirA::ba,* only exhibited a growth defect in human milk. Indicating that the function of HtsA and SirA have some overlap when it comes to iron import in human milk (likely involving human lactoferrin), yet in bovine milk these receptors are nonessential for growth. The acquisition of iron in bovine milk is potentially mediated through other means, possibly ferric citrate. However, FhuC mediated iron uptake likely also plays a role.

Both *htsA* and *sirA* siderophore receptors are important for the uptake of iron from human lactoferrin. This is likely due to these receptors binding iron-loaded siderophores SA and SB. However, the creation of a double siderophore knock-out for SA and SB has been unsuccessful and so it remains unclear if there is an interaction between iron-bound human lactoferrin and the receptors directly, or if this is a siderophore mediated event. Regardless of how it happens, these

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data show that both receptors (HtsABC and SirABC) are important in utilizing iron from human lactoferrin and the function of one can compensate if the other is knocked out.

In growth experiments with bovine lactoferrin isolated from colostrum, all of the strains, including the wild-type strain, grew poorly. Notably, among the strains completely unable to grow in this media were *fhuC::ba, ΔhtsA/sirA::ba,* as well as the gene for SB synthesis (*sbnE::ba*) and the SB receptor (*sirA::ba*). Metal transporter components (*znuB::ba, znuC::ba* and *psaA::ba*) also failed to grow. As mentioned previously, if apolactoferrin was present in higher concentrations in the bovine lactoferrin isolated from colostrum then more iron and other metals would be sequestered. According to the growth data, staphyloferrin B and its associated receptor play a dominate role in iron acquisition from bovine lactoferrin. Since the same growth defects are not observed when these strains are grown in bovine lactoferrin isolated from milk, there is likely an overlap of function between multiple genes. However, when more pressure is applied by increasing the amount of apolactoferrin ultimately only those strains with a functioning SB and receptor are able to overcome iron limitations by outcompeting the apolactoferrin. Enhanced zinc and manganese limitations due to apolactoferrin binding also highlight the importance of *znuB::ba, znuC::ba* and *psaA::ba* in overcoming additional metal restrictions.

Understanding which genes are needed for utilization of different iron sources has been difficult. The importance of genes can be masked by redundant functions between multiple genes and by limitations in my experimental design such as a small selection of mutants and not being able to achieve iron levels low enough in TMM to prevent bacterial growth. Despite these challenges, it is clear that growth in human and bovine milk requires specific and sometimes distinct genes for each growth environment. The genes I have discussed demonstrate the

importance of manganese and iron for *S. aureus* survival in milk and the lactating mammary gland and help shed light onto the elaborate lengths *S. aureus* employs to acquire these metals.

SECTION 5: METHODS

5.1 Strains and transposon mutants

The wild type strain was USA300_FPR3757 (NCBI reference sequence NC_007793). *S. aureus* transposon mutants (listed in Table 7) were generously provided by the Nebraska Transposon Mutant Library (NTML). NTML mutants were created using *bursa aurealis* (a mariner-based transposon with erythromycin resistance) in a strain derived from USA300 LAC [246]. Mutations were confirmed using the designated "upstream" and "buster" primers designated by the NTML with an additional gene-specific primer. All primers and the PCR protocol for checking authenticity of NTML mutants are found in Table 7.

Table 7: NTML strains

10.5 µL Reactions: 5 µL OneTaq Quick-Load 2XMM 0.5 µL Tn Upstream Primer 0.5 µL Tn Buster Primer 0.5 µL Gene Specific Primer 1 µL genomic NTML Mutant DNA 3 µL mgH20	PCR Program: 1. 95˚C – 3 min 2. 95˚C – 30 sec 3. 58˚C – 30 sec 4. 68˚C – 1 min 45 sec 5. Go to Step 2 – 30X 6. 68˚C – 3 min 7. 4˚C – ∞
NTML Name / Gene Name	Gene Specific Primer
Plus Orientation Tn (Upstream)	CTCGATTCTATTAACAAGGG
Minus Orientation Tn (Buster)	GCTTTTTCTAAATGTTTTTTAAGTAAATCAAGTAC
SAUSA300_0117 / *sirA*	TCCGCCAGCTAAGTTCCAAG
SAUSA300_0122 / *sbnE*	CTGCCACAACATTTGGCACA
SAUSA300_0618 / *psaA*	ACAACATTCCTAGTGGTGCG
SAUSA300_0619 / *znuB*	TAAAACGAAACCTGACGCCG
SAUSA300_0620 / *znuC*	TTATCGATACCAGTACGCGGC
SAUSA300_0633 / *fhuC*	CACGTTGTCTTTGACCACCAC
SAUSA300_0798 / *nlpA*	TATACTGCCCCTTTTGTGCCC
SAUSA300_0975 / *purH*	TGCTTGTTCTATCGTGTCACCG
SAUSA300_1015 / *ctaA*	ACAACCATCAGCTGATCCGG
SAUSA300_1032 / *isdE*	TGGAGCCGAATGTTGAAGCT
SAUSA300_1274 / *dppD*	TGCCTTGATTGGAGAGAGCG
SAUSA300_2136 / *htsA*	GCCCAAACATCACGGTCAAC
SAUSA300_2140 / *sfaD*	CCAGGATGTAGCGGATGACC
SAUSA300_2413 / *cntL*	CTAGGTCAACGGCTTGTGGA

5.2 Growth curves in milk and TSB

Growth curves of the various *S. aureus* mutants were conducted in human milk, bovine milk, and TSB following the same protocol. Pasteurized and unpasteurized whole bovine milk was purchased at local suppliers. Human milk was generously donated from a local mother. To account for normal flora and potential contamination in the human and unpasteurized bovine milk, samples were first centrifuged and filter sterilized. When using pasteurized bovine milk or TSB this step was skipped. Overnight cultures of the various *S. aureus* mutant strains were subcultured in TSB and diluted to an OD_{600} ~1. The culture was further diluted 1:100 in PBS, and again diluted 1:100 into the test media (human milk, bovine milk or TSB) making the starting concentration of bacteria 2-5 x 10^4. A spot plate of dilutions was made for each sample at 0, 5, and 10 hr timepoints on mannitol salt agar (MSA) plates and incubated overnight at 37˚C. The next day colonies were counted on the spot plates and CFU/mL calculations conducted. Data of three or more replicates for each test were compiled using GraphPad Prism 8 software and graphed as the log_{10} transformation of CFU/mL with error bars representing 95% confidence intervals. Statistical analyses were performed on GraphPad Prism 8 software using a two-way repeated measure ANOVA with the Geisser-Greenhouse correction and Dunnett's multiple comparisons test with individual variances computed for each comparison.

5.3 Growth curves in TMM

The wildtype and transposon mutants of *S. aureus* were grown overnight in TSB at 37˚C with shaking at 255 rpm. Approximately 2 mL of each sample was spun down and washed 3 times in TMM media, and then diluted in TMM media to an OD_{600} ~1. The cultures were further diluted 1:100 in TMM, and again a 1:100 dilution was made into TMM media. 100-well Honeycomb Bioscreen C plates were filled with 180 µL media (TMM or TMM with

supplementary iron sources, see Table 8) and 20 μL of selected bacterial strain to test (making the

starting concentration of bacteria 2-5 x 10^3). Each strain and media combination were tested in

triplicate at least three times unless otherwise stated.

Table 8: Media descriptions

Media	Item Number / Lot Numer	Concentration	Supplementation	Total Iron (ppm) ± SD
Tris Minimal Media (TMM)	NA / Batch # 10	NA	NA	0.0150 ± 0.0046
Bovine Transferrin[a]	T1283-100MG / SLBS2682V	50 mg/mL	22 μL/mL	1.0102 ± 0.0024
Bovine Lactoferrin from Milk[a]	L9507-10MG / SLBZ4922	6.66 mg/mL	155 μL/mL	0.9915 ± 0.0038
Bovine Lactoferrin from Colostrum[a]	L4765-MG / SLCC3075	50 mg/mL	30 μL/mL	0.9923 ± 0.0049
Human Transferrin[a]	T0665-100MG / SLBZ5500	50 mg/mL	21 μL/mL	1.0088 ± 0.0059
Human Lactoferrin[a]	L1294-100MG / SLCD2550	50 mg/mL	24 μL/mL	1.0272 ± 0.0036
Ferric Sulfate[b]	I146-500 / 175907	5 mg/mL	1.5 μL/mL	1.0201 ± 0.0036
Ferric Citrate[a] + (10X Sodium Citrate[c])	F6129-250G / SLBS9963 (0754-06 / C36H04)	4.39 mg/mL + (43.9 mg/ml)	16 μL/mL	0.9982 ± 0.0031

Iron sources used to supplement TMM media. All items were reconstituted in TMM and the amount of each source supplemented back into the media was calculated to give the final media an iron concentration of ~1ppm as determined by ICP-S.

[a]Item purchased from Sigma

[b]Item purchased from Fisher Scientific

[c]Item purchased from Mallinckrodt Chemicals

Growth curves were conducted using a Bioscreen C: Automated Microbiology Growth

Curve Analysis System set to 37°C for 30 hrs with continuous shaking (amplitude: medium;

speed: normal). The Bioscreen C was set to take an OD_{600} reading of each well every 15 min

(shaking ceased 10 sec before measurement) and this data was then plotted using GraphPad Prism

8 software. Statistical analyses were performed on GraphPad Prism 8 software using Brown-

Forsythe and Welch one-way ANOVA tests with Dunnett's T3 multiple comparisons test with

individual variances computed for each comparison. For graphs displaying data by media type

these comparisons were made against the TMM (negative control), and for graphs displaying data by mutant strain these comparisons were made against the wild type (standard reference).

5.4 Making low-iron tris-minimal media (TMM)

Low iron media was made by adapting a previously described protocol for Tris-Minimal Succinate Media [247]. Chelex® 100 sodium form beads (C7901-50G, from Sigma) in a glass column with wide pore membrane at the base were utilized in this protocol. The chelex beads require preparation before use by washing the column with 2 bed volumes of 1 N HCl, then rinsing the column with 5 bed volumes of Milli-Q H_2O, then washing with 2 bed volumes of 1 N NaOH, and again, rinsing with 5 bed volumes of Milli-Q H_2O.

Once the beads were prepared, 1.5 L Milli-Q grade water was chelexed 6X to remove contaminating iron and the pH adjusted to ~7.4. The TMM was made by adding 40 mL Tris Salt 25X (in 1 L Milli-Q H_2O dissolve: 145 g NaCl, 92.5 g KCl, 27.5 g NH_4Cl, 3.55 g Na_2SO_4, and 6.8 g KH_2PO_4), 12.1 g Tris Base, 16.6 g Succinate, 50 mL 20% Casamino Acids, and adjusted to 1 L with chelexed H_2O. This media was subsequently chelexed a total of 12X with intermittent washing of the column after every 4 media chelexations. The media was adjusted to pH ~7.4 and then supplemented with additional vitamins and metals as follows: 2 mL Tryptophan (10 mg/mL), 1 mL Cysteine (22 mg/mL), 1 mL Thiamine (16.9 mg/mL), 1 mL Nicotinic Acid (1.23 mg/mL), 1 mL Pantothenic acid (0.5 mg/mL), and 1 mL Biotin (0.01 mg/mL). As the process of chelexation removes other metals in addition to iron, the following essential metals were also added back into the media: 1 mL $MgCl_2$ (95.3 mg/mL), 1 mL $CaCl_2$ (11.1 mg/mL), 1 mL $MnCl_2$ (0.006 mg/mL), and 1 mL $ZnSO_4$ (0.25 mg/mL). Note: Chelexed-H_2O was used as the diluent for all supplements added into the media after chelexation.

The media was filter sterilized, aliquoted, and frozen at -20˚C. Testing on ICP-S revealed the finished media contained between 0.005-0.015 ppm iron. All growth curve data presented in this thesis was obtained using TMM-10 (batch #10) (Table 9). Media left at room temperature long term tended to leach iron from the plastic container, thus only freshly thawed media was utilized during experiments to ensure consistency between replicates. Recall, that in experimental growth curves, additional iron sources (Table 8) were added to achieve a final iron concentration of approximately 1 ppm.

Table 9: TMM-10 ICP-S results

Element	Amount (ppm) ± SD
Boron (B)	0.0252 ± 0.0010
Calcium (Ca)	2.8036 ± 0.0441
Copper (Cu)	0.0015 ± 0.0006
Iron (Fe)	0.0150 ± 0.0046
Potassium (K)	1328.5625 ± 23.0637
Magnesium (Mg)	22.7625 ± 0.2156
Manganese (Mn)	0.0018 ± 0.0002
Sodium (Na)	Unable to Calculate (Value Too High)
Phosphorus (P)	88.5525 ± 0.6486
Sulfur (S)	64.5413 ± 1.4459
Zinc (Zn)	0.0511 ± 0.0010

Final elemental concentrations (ppm) in TMM media used for growth curves.
Data is the average of 3 samples tested.

5.5 Inductively coupled plasma spectroscopy (ICP-S)

ICP-S testing was conducted in the Environmental Analytical Lab (EAL) located on BYU campus. Samples were run on ICP-S according to the EAL sample protocol, without microwave digestion. Milk samples first had to be highly centrifuged and filtered at 0.2 µM so as to not clog the machinery. For more information or questions regarding the EAL or ICP-S process please visit the EAL website at https://pws.byu.edu/eal.

5.6 Making *S. aureus* knock-outs

5.6.1 Creation of knock-out insertion

First, a region ~1500-2000 bp in of DNA both upstream and downstream of the target gene was amplified using PCR. All primers and PCR instructions for amplification of these upstream and downstream regions are found in Table 10. The forward primer of the upstream region included an attB1 tail (attB1: GGGGACAAGTTTGTACAAAAAAGCAGGCT) while the reverse primer of the downstream region included an attB2 tail (attB2: GGGGACCACTTTGTAC AAGAAAGCTGGGT) for future insertion into the pKOR1 plasmid. The reverse primer of the upstream, and the forward primer of the downstream region both included complementary bases providing an area of overlap between the upstream and downstream amplicons (this overlapping design allows stitch PCR to be performed and create a large contiguous section of DNA with the target gene cleanly removed).

After each upstream and downstream region were individually amplified using PCR, the upstream and downstream region were "stitched" together using in 2 rounds of PCR following the protocol in Table 9. Finished products were ran on a 0.75% gel and the bands were cut out, cleaned and concentrated using Monarch DNA Gel Extraction Kit (New England Biolabs, #T1020S).

Next, a BP Clonase reaction was performed to insert the stich PCR product into the pKOR1 plasmid. Instructions of Gateway BP Clonase II Enzyme Mix (Invitrogen, #11789020) were followed with minor modifications. In brief: at room temperature, 10 μL reactions were set up combining ~50-150 ng of each attB-PCR product with ~150 ng donor vector (pKOR1) in TE Buffer. BP Clonase II enzyme was thawed on ice and 2 μL was added to each reaction. Reactions were incubated at 25˚C overnight and terminated by the addition of 1 μL Proteinase K with an additional 10 min incubation at 37˚C.

Table 10: Upstream and downstream region amplification for clean deletion knock-outs

25 μL Reactions:	PCR Program:
12.5 μL Q5 Ultra II Enzyme	1. 98°C – 30 sec
1 μL Forward Primer	2. 98°C – 10 sec
1 μL Reverse Primer	3. 55°C – 30 sec
1 μL genomic *S. aureus* DNA	4. 72°C – 70 sec
9.5 μL mgH20	5. Go to Step 2 – 30X
	6. 72°C – 2 min
	7. 4°C – ∞

Target Gene, Region	Primers 5'-3'
fhuC, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTGATGAAGCGTGGTTGGTAGC Reverse: TCTTCTGTTGAACCTGTAGCGAACGCCCACAGATAAGTCTTCAT
fhuC, Downstream	Forward: ATGAAGACTTATCTGTGGGCGTTCGCTACAGGTTCAACAGAAGA Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTCAAATGGCCATTCGTCACCC
sfaD, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTGGATGCCCCTCTGGTAC Reverse: CCCTAGGAAAGCGAAGCCATTCAACAGAATGCGGGTGTAAGT
sfaD, Downstream	Forward: ACTTACACCCGCATTCTGTTGAATGGCTTCGCTTTCCTAGGG Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTCGATAAAAAGGGCTTGGGACA
htsA, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCGCTTTGTTTGCTTCCAG Reverse: GGAGTGGAACGATGAGAGGTCGTTGACCGTGATGTTTGGGC
htsA, Downstream	Forward: GCCCAAACATCACGGTCAACGACCTCTCATCGTTCCACTCC Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTGTAGTGAACTTGGGGCCACA
sbnE, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTGCAATACGGCGCATTACCAG Reverse: GCTTTAGCCGCAATGGTCGTCGATAGCCGCATACGCTG
sbnE, Downstream	Forward: CAGCGTATGCGGCTATCGACGACCATTGCGGCTAAAGC Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTGATACGCCTCGATGATGCC
sirA, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTTGCGCCACCTAATACTGCAA Reverse: GTCATACATCACGAAGGAGGCTCTTGGAACTTAGCTGGCGGA
sirA, Downstream	Forward: TCCGCCAGCTAAGTTCCAAGAGCCTCCTTCGTGATGTATGAC Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTGCTTGTCTCCGATTAGCCC
cntL, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTAGCAGACATTGATCCACCGT Reverse: ACGAGCGTGTATTACAAGACGAACAGCGGAAGATAAGTGGCA
cntL, Downstream	Forward: TGCCACTTATCTTCCGCTGTTCGTCTTGTAATACACGCTCGT Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTTGGTGCGTATTGTATGGGCA
isdE, Upstream	Forward: GGGGACAAGTTTGTACAAAAAAGCAGGCTCACTGCCAAAGATGAACGCA

	Reverse: TGCTGTAATACCGAATGGCACTGAACTGGAAGATTGACAGCTGG
isdE, Downstream	Forward: CCAGCTGTCAATCTTCCAGTTCAGTGCCATTCGGTATTACAGCA
	Reverse: GGGGACCACTTTGTACAAGAAAGCTGGGTCCGACATGGTGCCCGTATAA

Table 11: Stitch PCR protocol

Round 1	PCR Program:
<u>18 µL Reactions:</u>	1. 98˚C – 30 sec
10 µL Q5 Ultra II Enzyme	2. 98˚C – 10 sec
1 µL Upstream Amplicon	3. 69˚C – 30 sec
1 µL Downstream Amplicon	4. 72˚C – 1 min 20 sec
6 µL mgH20	5. Go to Step 2 – 7X
	6. 72˚C – 2 min
	7. 4˚C – ∞
Round 2	PCR Program:
<u>20 µL Reactions:</u>	1. 98˚C – 30 sec
18 µL Reaction from Round 1	2. 98˚C – 10 sec
1 µL Upstream-forward Primer (aatB1 Primer)	3. 69˚C – 30 sec
1 µL Downstream-reverse Primer (attB2 Primer)	4. 72˚C – 2 min 50 sec
	5. Go to Step 2 – 30X
	6. 72˚C – 2 min
	7. 4˚C – ∞

5.6.2 Transformation into *Escherichia coli*

Transformation was conducted adding 3 µL of BP Clonase reaction products into 50 µL One Shot TOP10 Chemically Competent *E. coli* Cells (Invitrogen, C4040-03). Cells were incubated on ice 30 min and then heat-shocked 1 min in a 42˚C water bath. Cells were then incubated on ice 2 min, after which, 500 µL warmed LB broth was added, followed by a 37˚C incubation for 1 hr with shaking at 250 rpm. 100 µL of each sample were plated onto LB plates with Ampicillin (100 µg/mL) and incubated overnight at 37˚C. The remainder was plated out the next day if needed.

Colonies were checked for successful transformants using the "*E. coli* PCR Protocol" from Table 12. To save time on this, and future PCR reaction, new primers were created to test for the missing target gene but with a shorter product length than the original forward-upstream and reverse-downstream primers would produce. These new primers are listed in Table 12.

Table 12: Checking transformations PCR protocols and primers

E. coli PCR Protocol	PCR Program:
	1. 95˚C – 5 min
10 µL Reactions:	2. 95˚C – 30 sec
5 µL OneTaq Quick-Load 2XMM	3. 58˚C – 30 sec
0.5 µL Forward Primer	4. 68˚C – 1 min 45 sec
0.5 µL Reverse Primer	5. Go to Step 2 – 30X
Touch of colony	6. 68˚C – 3 min
4 µL mgH20	7. 4˚C – ∞
S. aureus PCR Protocol	PCR Program:
	1. 95˚C – 3 sec
10 µL Reactions:	2. 95˚C – 30 sec
5 µL OneTaq Quick-Load 2XMM	3. 58˚C – 30 sec
0.5 µL Forward Primer	4. 68˚C – 1 min 45 sec
0.5 µL Reverse Primer	5. Go to Step 2 – 30X
1 µL *S. aureus* DNA (after lysostaphin/boil)*	6. 68˚C – 3 min
4 µL mgH20	7. 4˚C – ∞

*To help break down the *S. aureus* cells, a touch of the colony was suspended in 10 µL PBS with 3 µL Lysostaphin (1 mg/mL) and incubated 1 hr at 37˚C, then boiled 8min in a 100˚C water bath

Target Gene, Region	Primers 5'-3'	Product Size
Δ*sfaD*	F: GCAAGAAATGTTGTTGGCGC R: TTAATGGCGAGACTCCTGAGGG	594 bp
Δ*htsA*	F: ACCCTGCAATCATCCTTGGC R: TGGTAGATTGGAAAGATGGCCG	1454 bp
Δ*sbnE*	F: GCGATACTGCAAGGTTTAGCG R: GACACCGTCTTGATATGACGC	616 bp
Δ*sirA*	F: CAATGATTGTTCTCGACGCCC R: GTTGAACCATAGGTGTTTGCCC	583 bp
Δ*fhuC*	F: ACGGACATGGCTATTATACGCG R: AATCCATCACGTCTAGATCGGC	873 bp
Δ*isdE*	F: ACTAGTTGACGAAAAGGCGC R: CCAATGATATTCGCCTCTGCC	1263 bp
Δ*cntL*	F: CTTCCAGATGTTGATGCGCC R: GTTTAAGGTGAAGGTGTCTGGC	943 bp

5.6.3 Transformation into *S. aureus* RN4220 (cloning intermediate)

For each target knock-out, a successful DH5α colony was chosen and grown up overnight in LB + 10 µg/mL chloramphenicol at 37˚C with shaking. Plasmid was isolated using I-Blue Mini Plasmid Kit (IBI Scientific, IB47172) and concentrations determined by nanodrop spectrophotometry. Electrocompetent RN4220 cells were thawed on ice and 20 ng-2µg (no more than 5 uL) isolated plasmid was added. Cells were transferred to chilled cuvette with 0.1 cm

electrode gap. Electroporation was conducted on a Bio-Rad MicroPulser Electroporator using the internal "Sta" setting for *S. aureus* (1.8 kV, time constant of 2.5 ms). 250 μL ice cold TSB was immediately added to the cells and then incubated at 37°C with shaking at 350 rpm for 1 hr. 100 μL was plated on TSA with chloramphenicol (10 μg/mL) and incubated overnight at 37°C. The remainder was also plated out the next day if needed. Colonies were checked for successful transformants using the "*S. aureus* PCR Protocol" and primers located in Table 12.

5.6.4 Transformation into *S. aureus* USA300

For each target knock-out, a successful RN422 colony was chosen and grown up overnight in TSB at 37°C with shaking. Plasmid was isolated following the "Isolating *S. aureus* Plasmid" protocol (below) and concentrations were determined by nanodrop spectrophotometry. *S. aureus* USA300 cells were thawed on ice and 20 ng-2 μg (no more than 5 μL) isolated plasmid was added. Cells were transferred to chilled cuvette with 0.2 cm electrode gap. Electroporation was conducted on a Bio-Rad MicroPulser Electroporator using the internal "Sta" setting for *S. aureus* (1.8 kV, time constant of 2.5 ms). 250 μL ice cold TSB was immediately added to the cells and then incubated at 37°C with shaking at 350 rpm for 1 hr. 100 μL was plated on TSA with chloramphenicol (10 μg/mL) and incubated overnight at 37°C. The remainder was also plated out on next day. After overnight incubation plates were left at room temperature for up to a one week and checked regularly for developing colonies. Colonies were checked for successful transformants using the "*S. aureus* PCR Protocol" and primers in Table 12.

5.6.5 Allelic recombination and plasmid eviction

Once a successful transformant in USA300 was obtained, the colony was cultured in 20 mL + 10 μg/mL chloramphenicol and incubated overnight at 30°C with 230 rpm shaking. 5 mL of TSB + 10 μg/mL chloramphenicol was prewarmed to 43°C and then inoculated with 5 μL

overnight culture and incubated 24 hrs at 43˚C with shaking at 230 rpm. 5 mL of TSB + 7.5 µg/mL chloramphenicol was prewarmed to 43˚C and then inoculated with 5 µL overnight culture and incubated 24 hrs at 43˚C with shaking at 230 rpm. The culture was streaked out to single colonies on TSA + 10 µg/mL chloramphenicol plates and incubated 24 hrs at 43˚C. A single colony was selected to inoculate 5 mL TSB + 10 µg/mL chloramphenicol and incubated 24 hrs at 30˚C with shaking at 230 rpm. The culture was then diluted 1:10,000 with sterile H20. 10 µL and 100 µL aliquots were used to plate on TSA + 1 µg/mL Anahydrotetracycline plates, which were incubated at least 36 hrs 30˚C. Colonies were checked for successful transformants using the "*S. aureus* PCR Protocol" and primers in Table 12.

5.7 Isolating *S. aureus* plasmid

For plasmid isolations the IBI Scientific High-speed Plasmid Mini Kit was utilized following the instructions with modifications as needed. In brief, a single colony from an RN4220 transformation reaction was selected and grown in 5 mL TSB + 10 µg/mL chloramphenicol overnight at 37˚C with shaking. 2 mL of the culture was spun down (14-16,000 x g, 1 min) and the supernatant was discarded. The pellet was resuspended in 200 µL PD1 Buffer and 25 µL Lysostaphin (1 mg/mL). Suspension was incubated at 37˚C for at least 1 hr or until clearing occurred. 200 µL of PD2 Lysis Buffer was then added and gently mixed by inverting the tube 10 times and incubated at room temperature for 2-3 minutes. 300 µL PD3 Neutralization Buffer was added and mixed by inverting the tube 10 times. Sample was centrifuged 14-16,000 x g for 10 min. DNA binding, wash, and elution steps were followed according to the given IBI protocol. Concentrations were determined by nanodrop spectrophotometry.

5.8 Generating electrocompetent *S. aureus*

100mL TSB media was inoculated with 1mL of overnight culture *S. aureus* and incubated at 255 rpm at 37˚C to an OD_{600} ~0.5. Once grown, the bacteria were chilled 15 min on ice and split into 50 mL conical tubes to be centrifuged (4000 x g, 10 min, 4˚C). Supernatant was discarded, and each pellet was washed with 50 mL ice-cold sterile deionized water and then centrifuged again (4000 x g, 10 min, 4˚C). Supernatant was discarded and each pellet was washed with 25 mL ice-cold sterile deionized water and then combined into one 50 mL conical tube and centrifuged (4000 x g, 10 min, 4˚C). Supernatant was discarded and the pellet resuspended in 16 mL ice-cold sterile 10% glycerol and centrifuged (4000 x g, 10 min, 4˚C). This was repeated using 10 mL ice-cold sterile 10% glycerol for the next wash. Supernatant was discarded and the pellet resuspended in 1 mL ice-cold sterile 10% glycerol and transferred to a 1.5 mL microcentrifuge tube and centrifuged (5000 x g, 5 min, 4˚C). Supernatant was discarded and the pellet resuspended in 800 µL ice-cold sterile 10% glycerol. 40 µL aliquots were immediately frozen and stored at -80˚C.

SECTION 6: SUPPLEMENTARY FIGURES

The following growth curves are another representation of the data presented in Figures 4-20, and Figures 25-39. However, rather than displaying growth of all mutants in one media type, these tables display the growth of one mutant in all the different media tested. Mutants from Group A (mutants which function in iron acquisition) are shown in Figures 44-52, while Group B (mutants involved with additional metals and cellular processes) are shown in Figures 53-59. Significant differences were determined by comparing the ending 30 hr OD600 value for each media against the negative control (TMM). All data represents three biological repeats each consisting of three technical repeats with the exception of bovine lactoferrin isolated from milk which represents one biological repeat consisting of three technical repeats.

Figure 44: Wild type growth in media
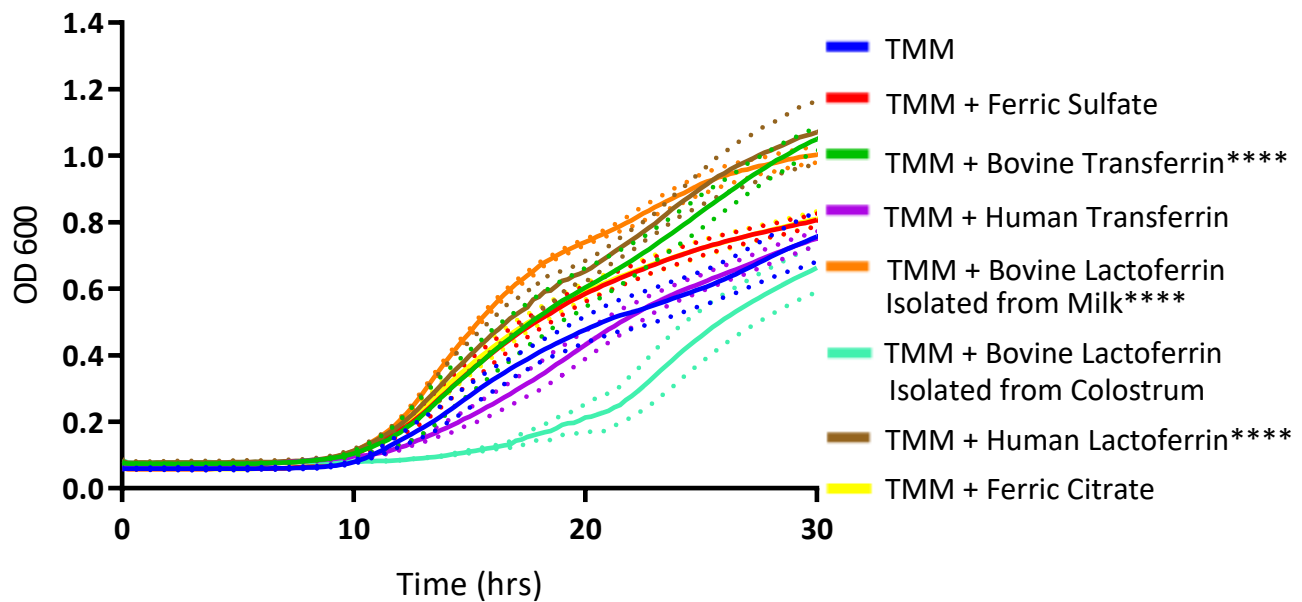
Growth of wild type USA300 in all TMM media. Notably, no significant difference in growth is found between the negative (TMM) and positive (ferric sulfate) controls showing how efficient *S. aureus* is at acquiring iron when all genes are functional. ****P-value = <0.0001

Figure 45: *sfaD::ba* growth in media
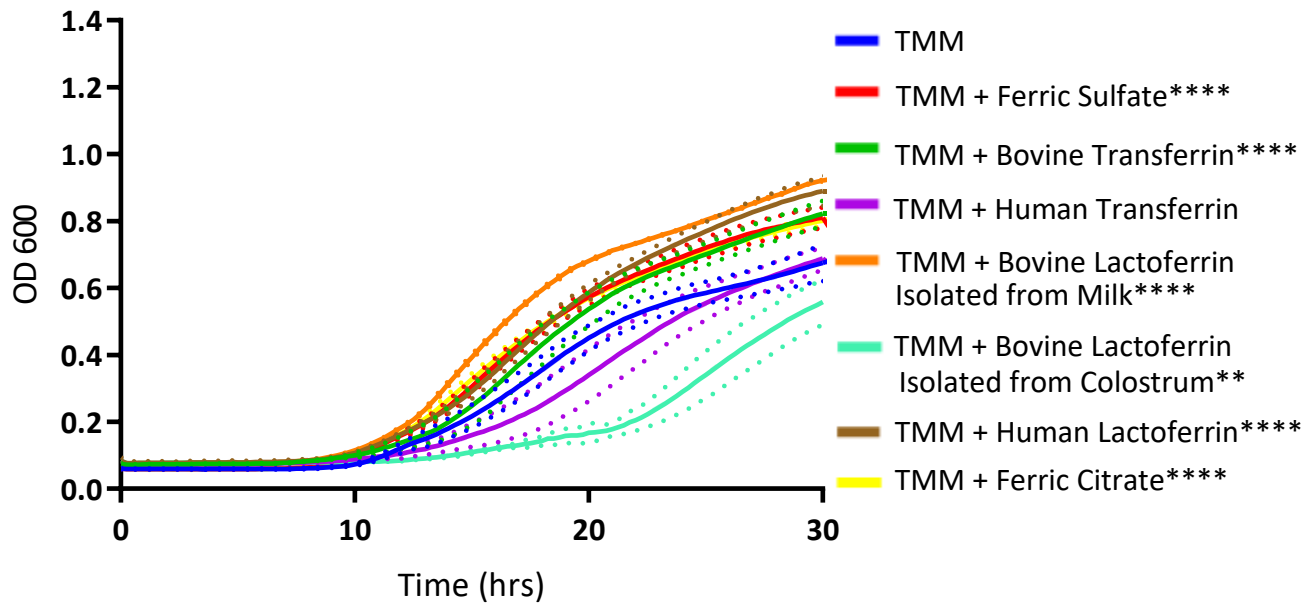
Growth of *sfaD::ba* in all TMM media. **P-value = <0.01 ****P-value = <0.0001

Figure 46: *htsA::ba* growth in media
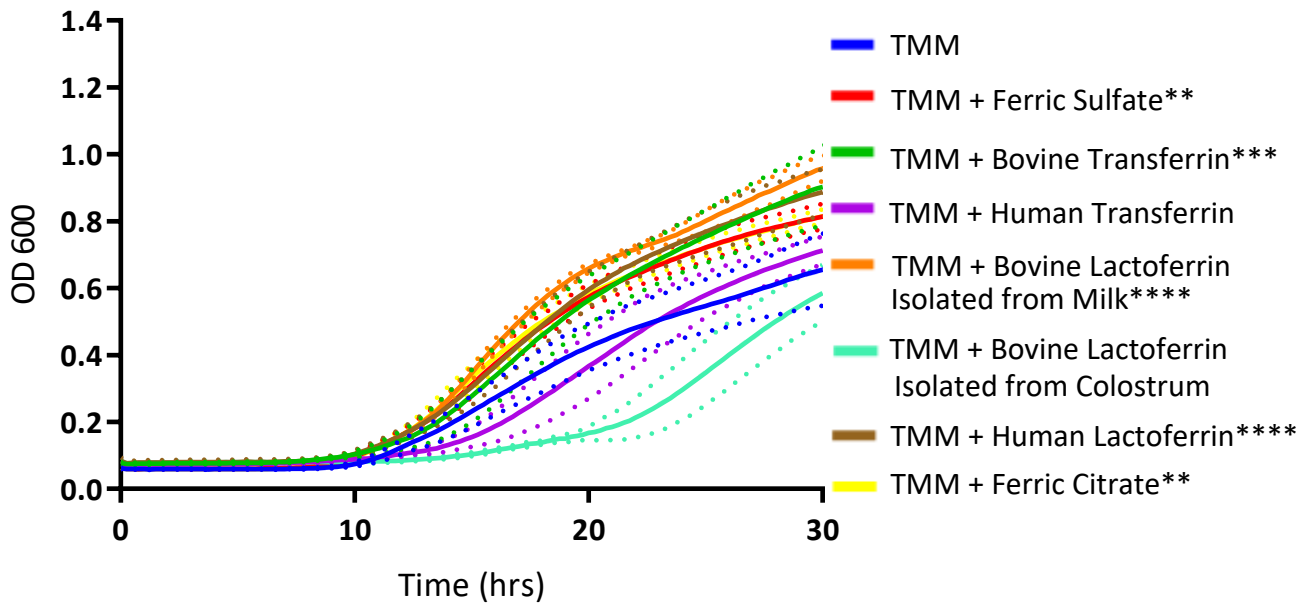
Growth of *htsA::ba* in all TMM media. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Figure 47: *sbnE::ba* growth in media

■	TMM
■	TMM + Ferric Sulfate****
■	TMM + Bovine Transferrin**
■	TMM + Human Transferrin**
■	TMM + Bovine Lactoferrin Isolated from Milk***
■	TMM + Bovine Lactoferrin Isolated from Colostrum****
■	TMM + Human Lactoferrin****
■	TMM + Ferric Citrate****
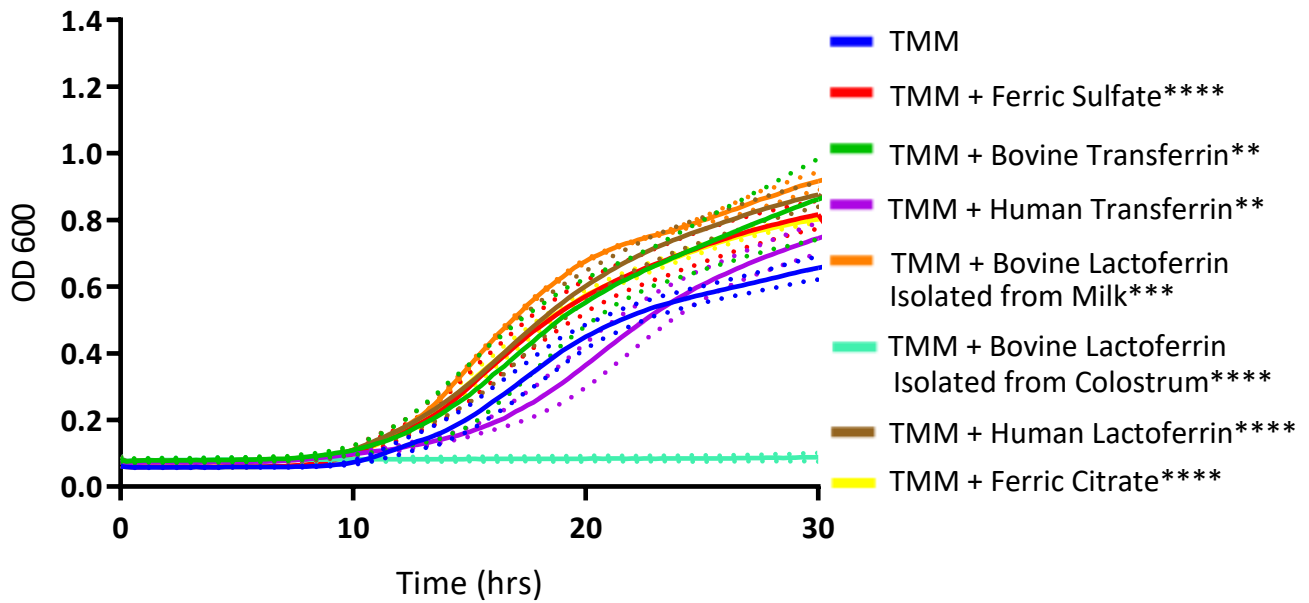
Growth of sbnE::ba in all TMM media. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Figure 48: *sirA::ba* growth in media

■	TMM
■	TMM + Ferric Sulfate****
■	TMM + Bovine Transferrin****
■	TMM + Human Transferrin****
■	TMM + Bovine Lactoferrin Isolated from Milk****
■	TMM + Bovine Lactoferrin Isolated from Colostrum****
■	TMM + Human Lactoferrin****
■	TMM + Ferric Citrate****
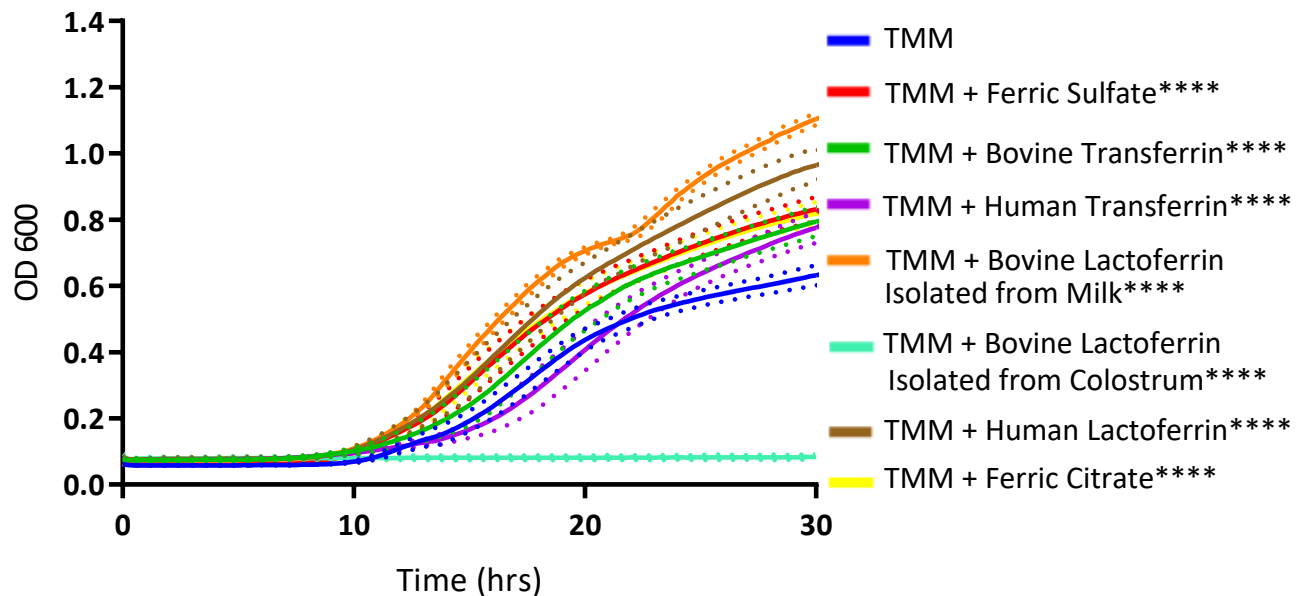
Growth of *sirA::ba* in all TMM media. ****P-value = <0.0001

Figure 50: *fhuC::ba* growth in media
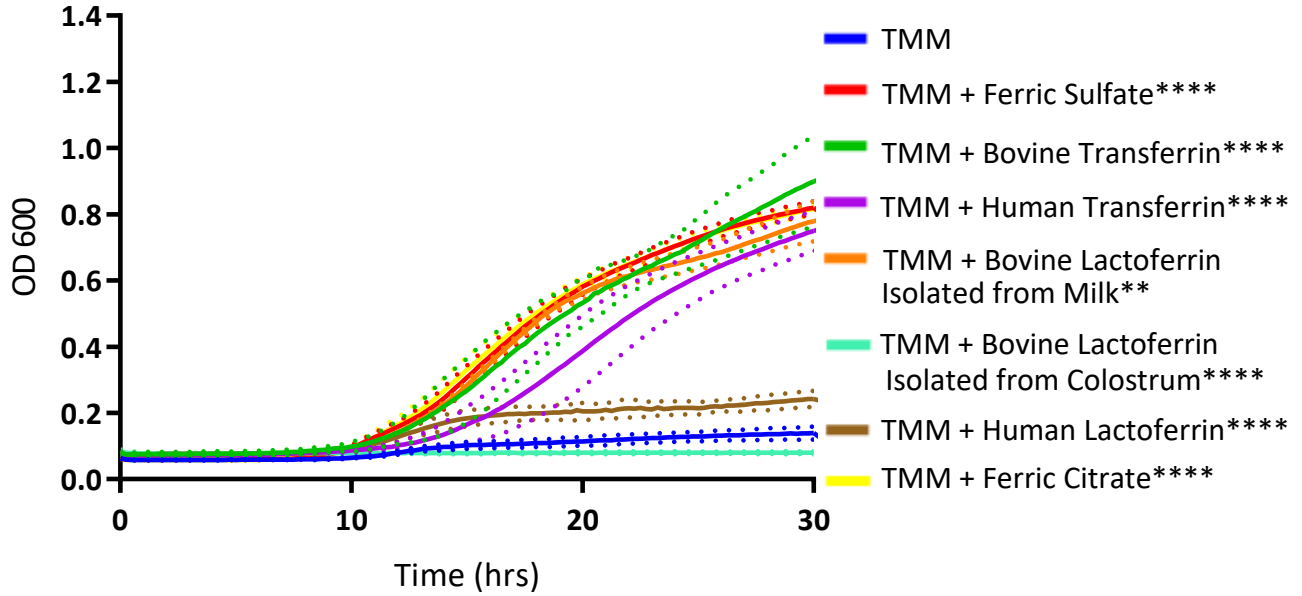
Growth of *fhuC::ba* in all TMM media. **P-value = <0.01 ****P-value = <0.0001

Figure 49: *isdE::ba* growth in media
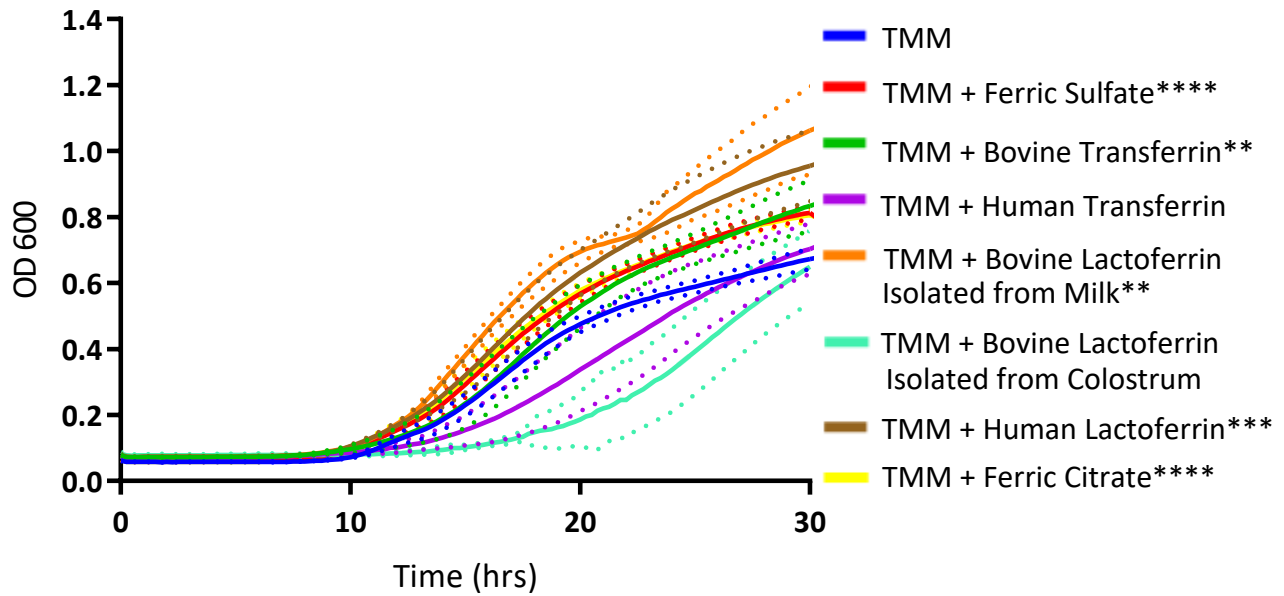
Growth of *isdE::ba* in all TMM media. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Figure 51: *cntL::ba* **growth in media**
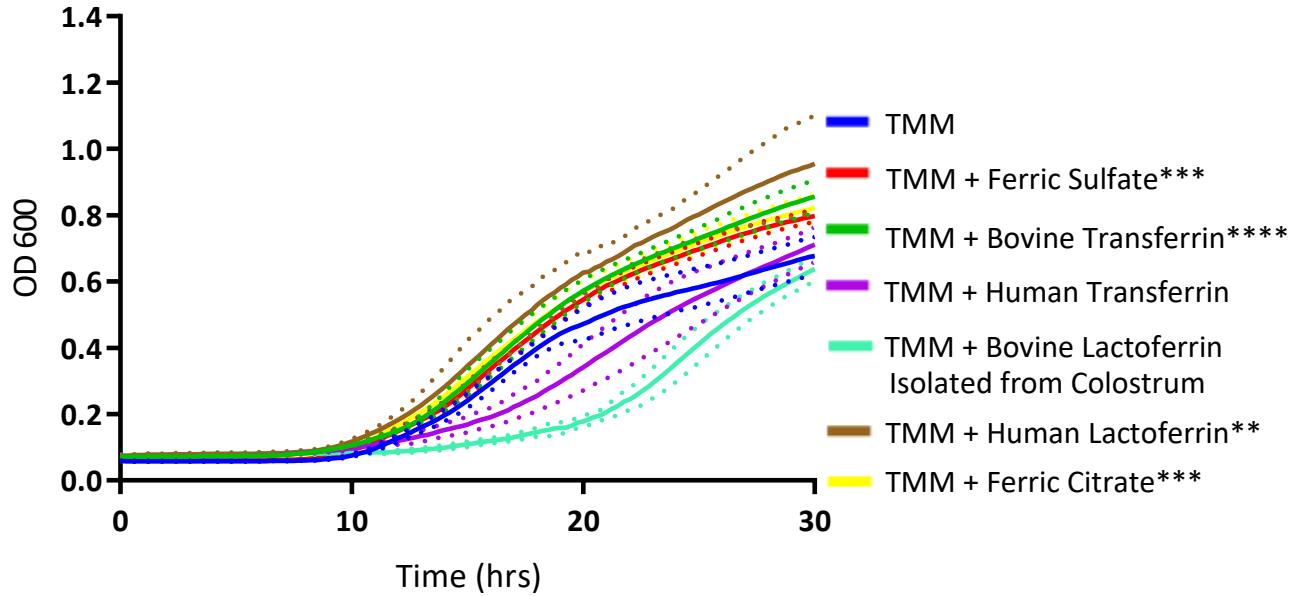
Growth of *cntL::ba* in all TMM media. **P-value = <0.01 ***P-value = <0.001 ****P-value = <0.0001

Figure 52: Δ*htsA/sirA::ba* **growth in media**
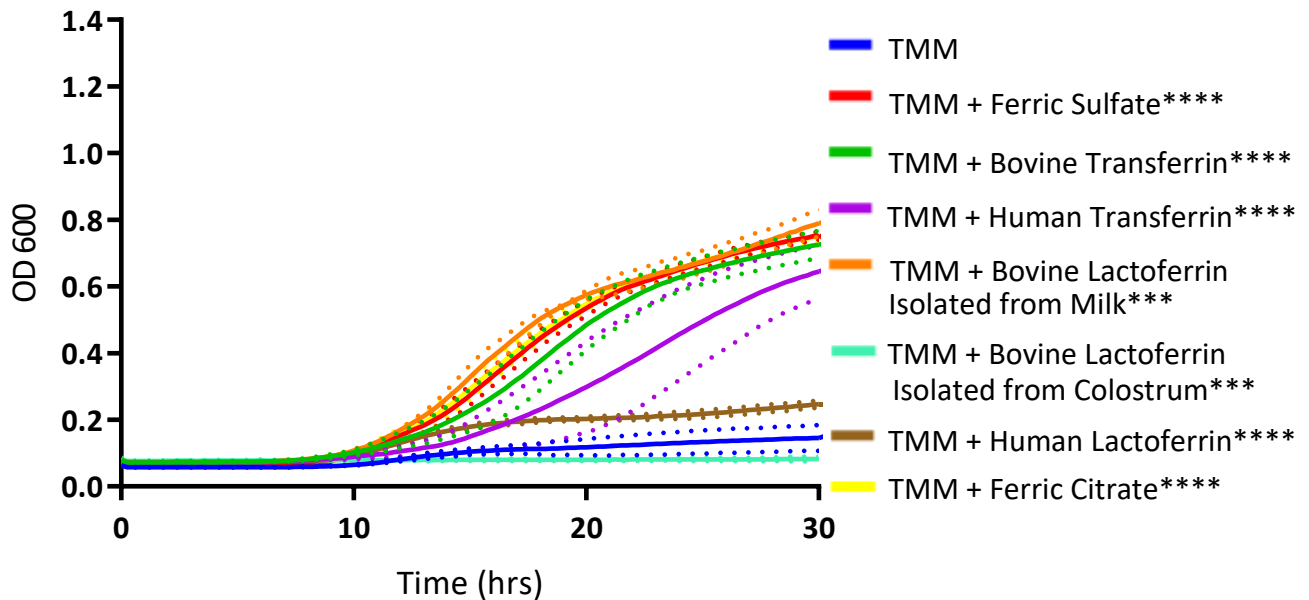
Growth of Δ*htsA/sirA::ba* in all TMM media. ***P-value = <0.001 ****P-value = <0.0001

Figure 53: *znuB::ba* **growth in media**

Growth of *znuB::ba* in all TMM media. Note: due to the limited samples (3) of *znuB::ba* growth in bovine lactoferrin isolated from milk statistical power was low even though visually it is much higher than the TMM control. **P-value = <0.01 ****P-value = <0.0001

Figure 54: *znuC::ba* **growth in media**
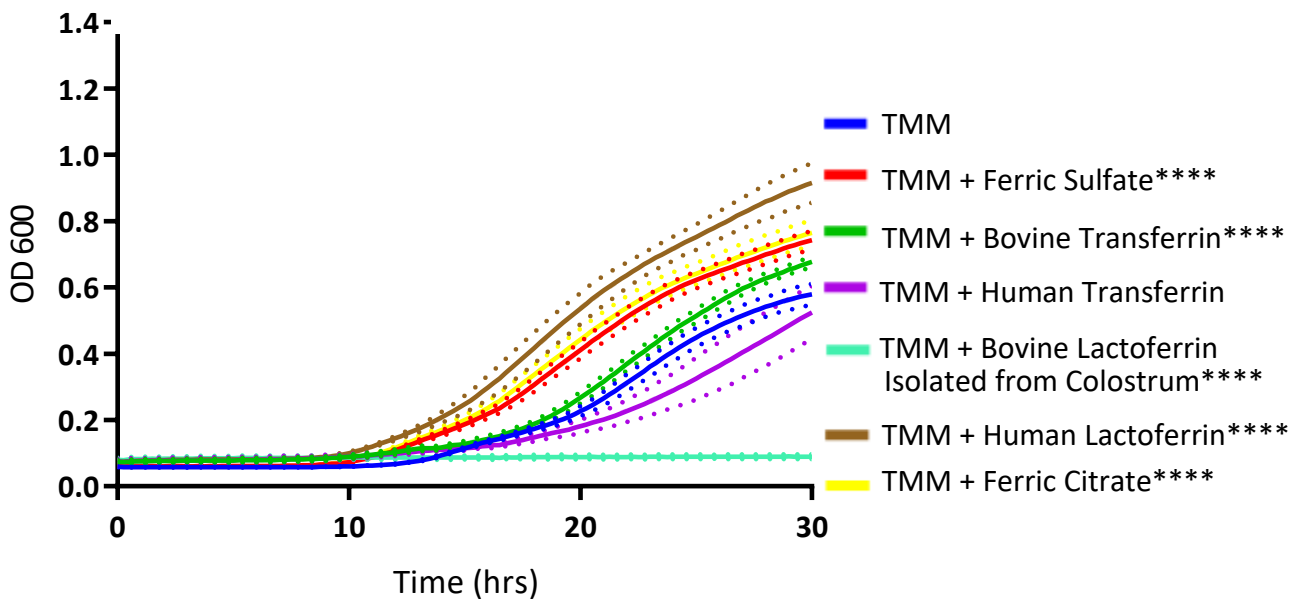
Growth of *znuC::ba* in all TMM media. ****P-value = <0.0001

Figure 55: *psaA::ba* **growth in media**
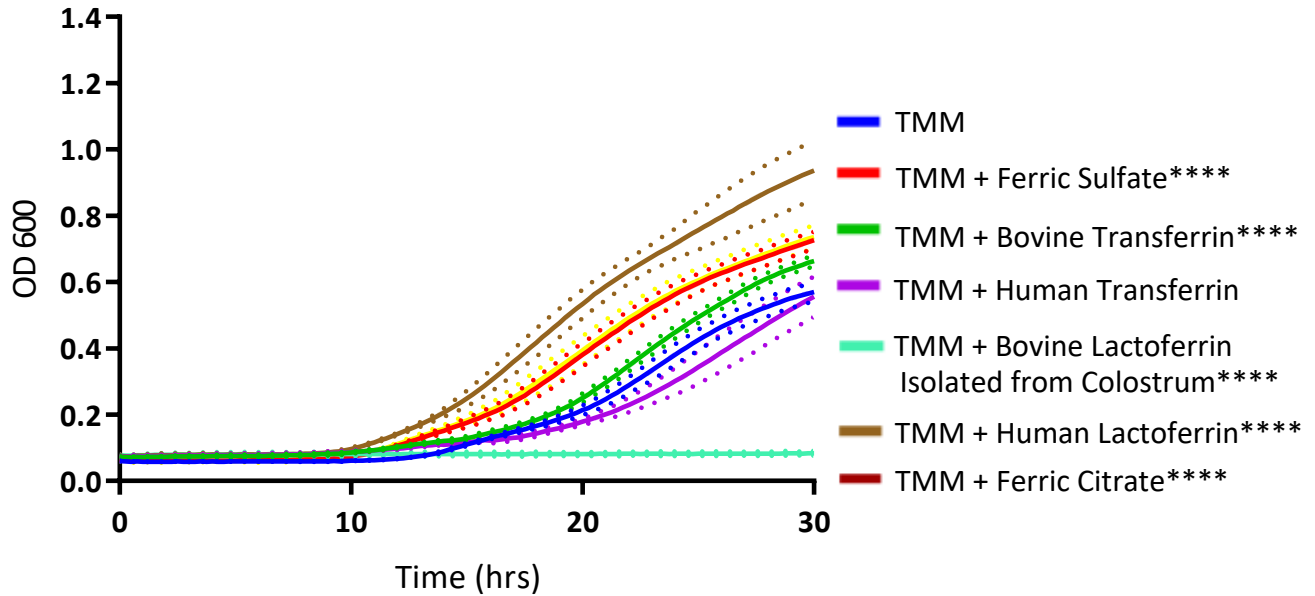
Growth of *psaA::ba* in all TMM media. ****P-value = <0.0001

Figure 56: *purH::ba* **growth in media**
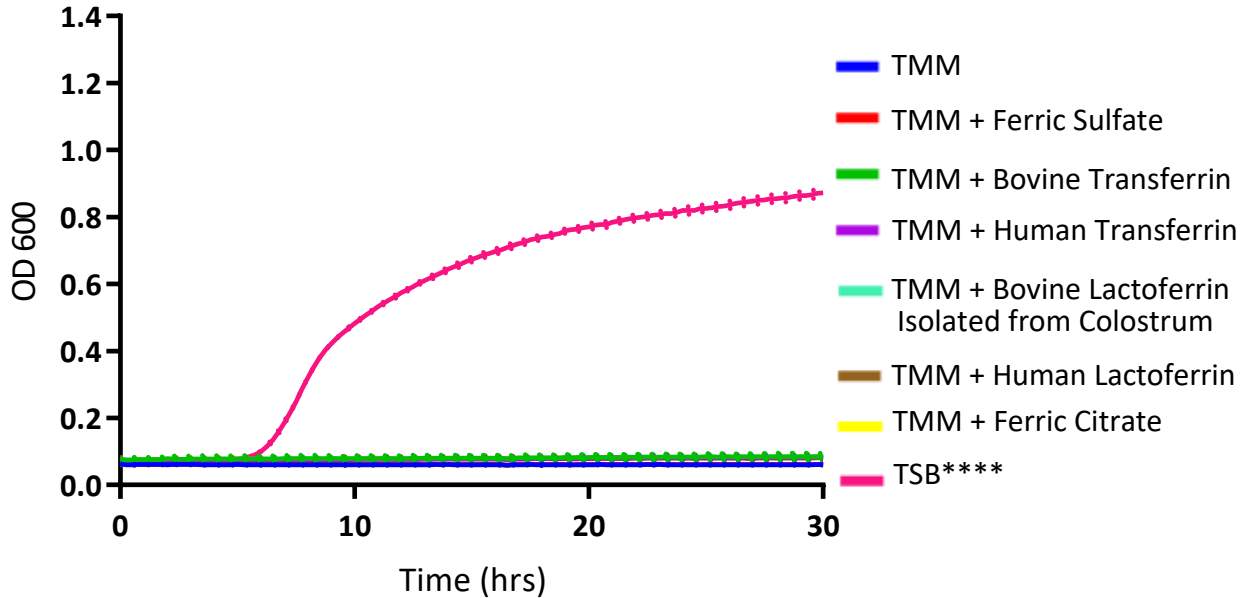
Growth of *purH::ba* in all TMM media. A control, using TSB, is also included. Since the mutant can grow fine in TSB, but is completely unable to grow in all TMM media when given the same starting amount of bacteria. ****P-value = <0.0001

Figure 58: *ctaA::ba* growth in media
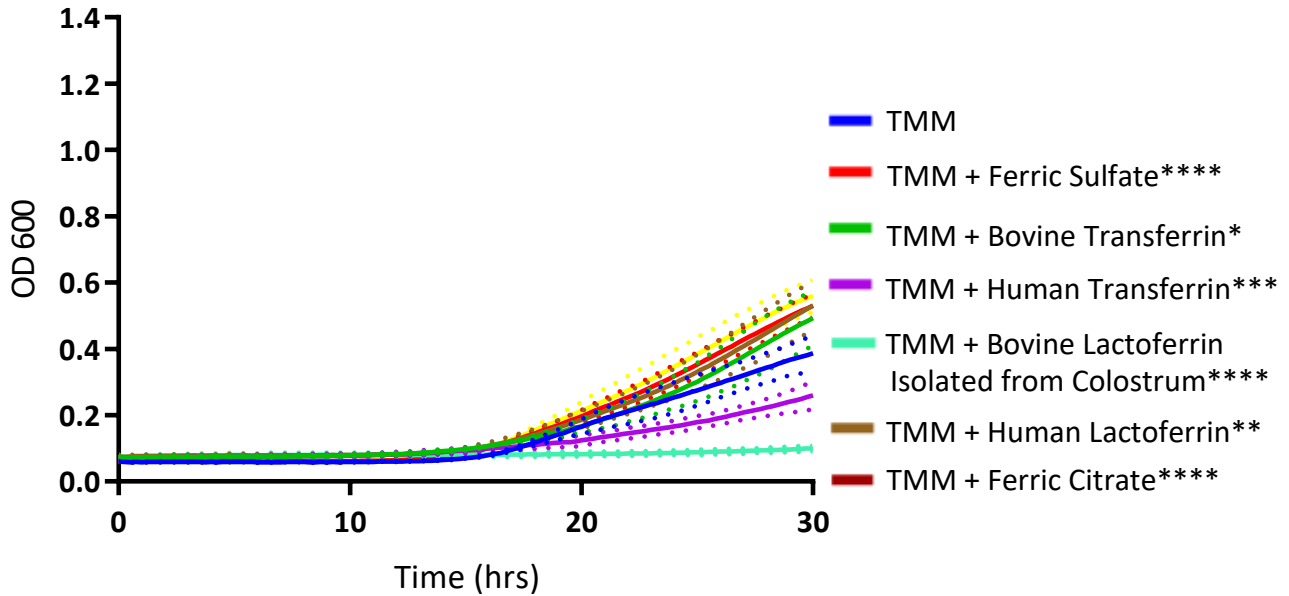
Growth of *ctaA::ba* in all TMM media. *P-value = <0.05 **P-value = <0.01 ***P-value = <0.001 ****
P-value = <0.0001

Figure 57: *dppD::ba* growth in media
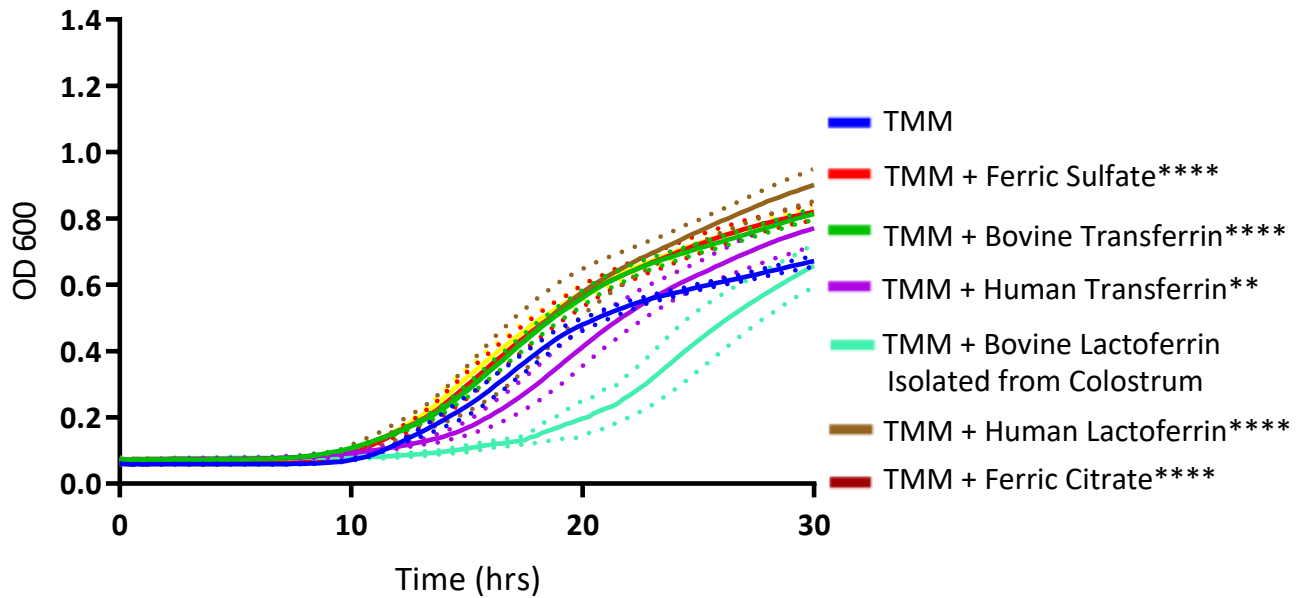
Growth of *dppD::ba* in all TMM media. **P-value = <0.01 ****P-value = <0.0001

Figure 59: *nlpA::ba* growth in media

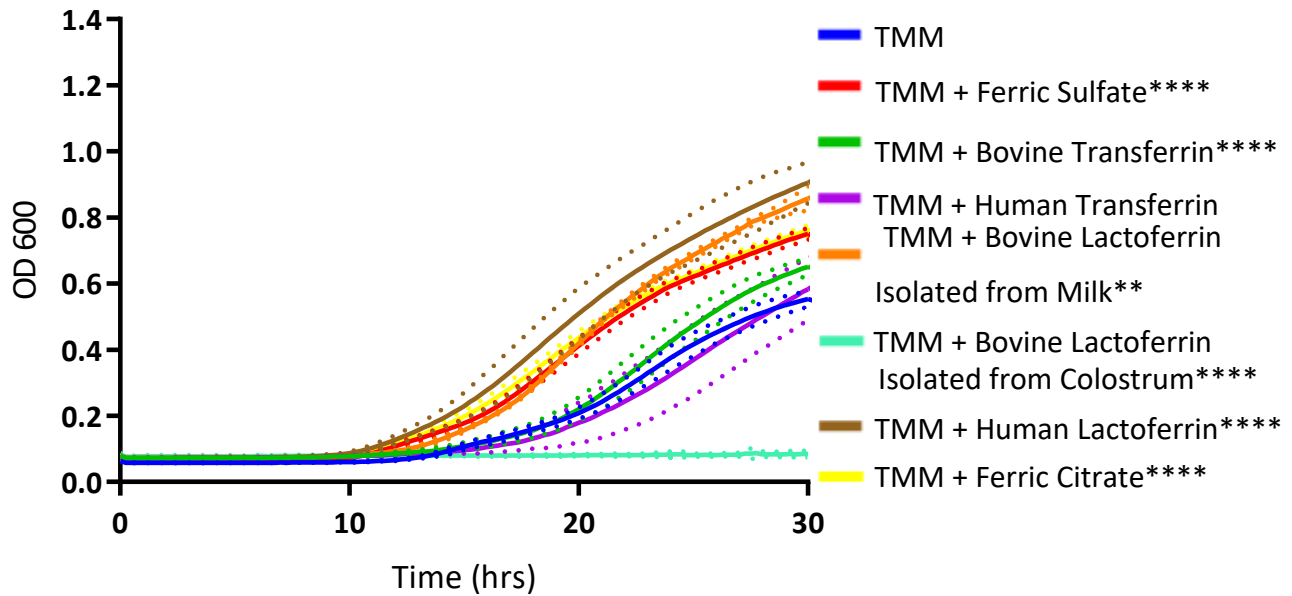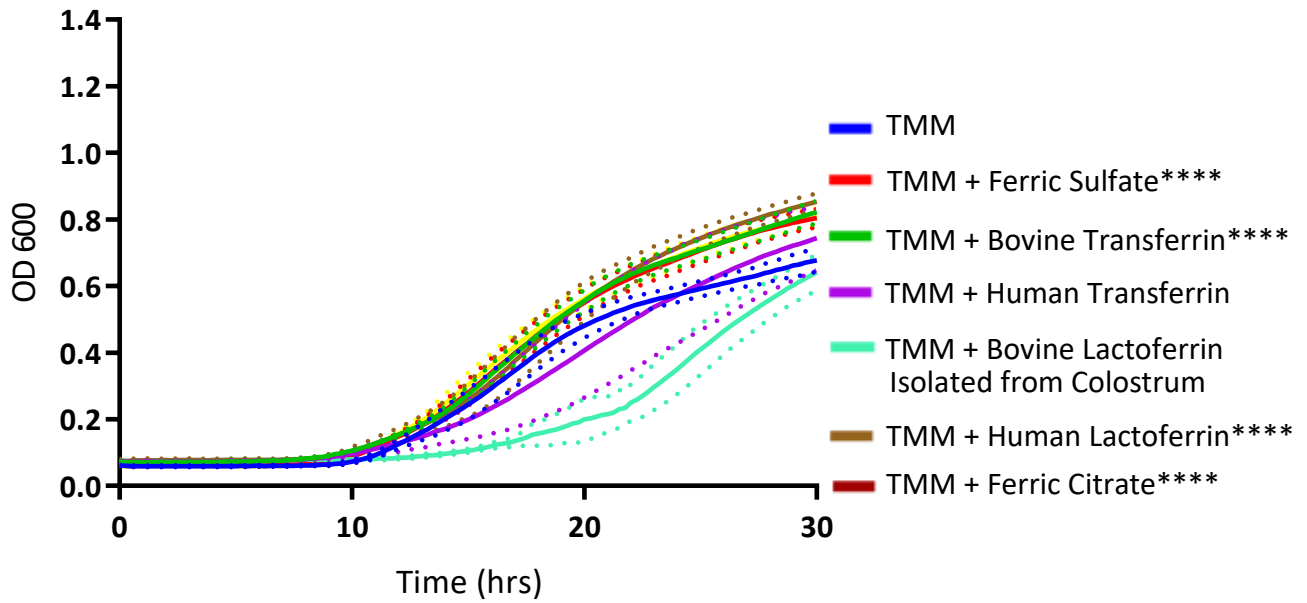Growth of *nlpA::ba* in all TMM media. ****P-value = <0.0001

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