Functional Importance of Proline Transporters in Staphylococcus aureus

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Functional Importance of Proline Transporters in 
*Staphylococcus aureus*

By

Dianne L. Wellems

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Functional Importance of Proline Transporters in *Staphylococcus aureus*

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University of Nebraska, 2017

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Abstract

*Staphylococcus aureus* can survive and colonize a multitude of environmental niches including the human host. *S. aureus* is responsible for over 11,000 deaths yearly, and many studies correlate the survival and proliferation of *S. aureus* to its acquisition and utilization of proline.

Proline is an important amino acid as it serves as both an osmotic protectant and carbon source. It was determined that *S. aureus* had a severe growth defect when grown in osmotically stressed conditions without proline. Additionally, in an abscess environment, where it is hypothesized that the primary carbon sources (i.e. glucose) are depleted, *S. aureus* will acquire proline from the extracellular milieu as a secondary carbon source. The transport of proline occurs through three transporters, a high-affinity proline permease, PutP and two low-affinity proline transporters, OpuD and ProP. In this study, we sought to investigate the function and regulation of the three transporters. It was determined that CcpA negatively regulated the expression of the three transporters, while σB (SigB) negatively regulated the expression of *putP*. Finally, we found that osmotic stress upregulates the expression of *opuD* and *proP*, but downregulates *putP* expression.
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Dianne Louise Wellems
Chapter 1: Introduction

*S. aureus* is an important opportunistic pathogen and is responsible for over 11,000 deaths annually [1]. The formation of a skin and soft tissue abscess is a hallmark of a *S. aureus* infection. The ability to form an abscess requires adaptation to the unique micro-niches within the human host [2, 3]. Surviving in the human host requires the utilization of a variety of virulence factors to colonize, adhere, and manipulate the host immune system [2]. Moreover, the environment within an abscess varies in pH, with an increase in pH associated with both chronic skin infections and a decrease in wound healing time [3-5]. Additionally, studies have shown that the environment within the abscess is also metabolically limited with proteins and peptides serving as the primary carbon source after host-mediated depletion of glucose [2-6]. Adapting to the limited carbon sources and changes in pH determines whether *S. aureus* can proliferate in the environment. Furthermore, it is hypothesized that the host responds to the invading pathogen by excreting sodium chloride to the site of the infection, creating an osmotically stressed environment [3, 5, 7]. Thus, mechanisms have evolved to protect bacterial cells against these environmental stressors [8, 9].

One response to osmotic stress is the accumulation of osmolytes [10]. Osmolytes are non-toxic at high concentrations, highly soluble, and have no net charge at physiological pH [11]. Many classes of osmolytes exist, from polyols (i.e. glycerol, sorbitol, and glucose), and a wide variety of amino acids [8, 11]. Of the amino acids, glycine betaine and proline are found to be the most osmotically protective [8, 11-13]. Multiple studies in *S. aureus* and other bacterial species associate the accumulation of proline with survival under osmotically stressed conditions [3, 8-11, 14-23]. A study observing the effects of osmotic shock in *S. aureus*, found intracellular proline concentration increasing from approximately 35 nmol to 400 nmol after exposure to a 1.72 M NaCl solution [19]. Furthermore, the addition of 2 M NaCl was found to upregulate the expression
of rocD and rocF, the genes involved in the metabolism of proline in S. aureus [24]. The acquisition of proline in S. aureus occurs through three known proline transporters, PutP, a high-affinity proline permease, and two low-affinity proline transporters: ProP and OpuD [25-29]. Indeed, a mutation in high-affinity proline transporter gene, putP, resulted in attenuation of growth in an abscess model, and a decrease in concentration of intracellular proline [30]. This thesis will discuss the unique functions and transcriptional regulation of the three proline transporters and their potential roles in the adaptation and survival of S. aureus in an abscess.
**Glycolysis: Survival under Nitric Oxide Stress**

One of the first responses to an invading pathogen from the host innate immune system is the production of nitric oxide (NO) from macrophages [31-33]. Nitric oxide (NO) is a broad-spectrum antimicrobial agent capable of disrupting metabolic pathways (i.e. the tricarboxylic acid cycle, pyruvate metabolism, and lipid biosynthesis), aerobic respiration, and DNA replication [34-38]. *S. aureus* can circumvent the disruption of metabolic pathways of NO by fermenting glucose via an NO-insensitive lactate dehydrogenase (Ldh) into lactate and regenerating NAD+, thus restoring glycolytic flux and redox balance [38-40]. Indeed, mutations in *ldh* and glycolytic genes resulted in attenuation of growth under NO stress [31, 38, 41, 42].

As *S. aureus* continues to persist within the abscess, it will form bacterial aggregates, surrounded by lysed host cells and phagocytes [2, 33]. It is hypothesized that this environment will be depleted of glucose as the M-1 macrophages will rapidly consume glucose through H1F-1α (a transcription factor that mediates the upregulation of glycolysis) activation [2, 6, 33]. As *S. aureus* lacks a glyoxylate shunt and β-oxidation system, required for the catabolism of lipids, it must rely on the acquisition of peptides and amino acids for use as carbon sources [43]. Moreover, previous studies have determined that *S. aureus* will grow in the absence of glucose under NO stress when supplemented with lactate and amino acids [31, 42]. Therefore, the acquisition of secondary carbon sources, in the absence of glucose, is critical for *S. aureus* to evade the host innate immune system and further manifest itself in the host.
Proline Metabolism

Amino acid catabolism is crucial for survival within the human host in the absence of glucose [33]. Of the amino acids, proline is critical as it serves as a precursor for glutamate, the key metabolite that connects carbon and nitrogen metabolism (Figure 1) [44, 45]. Frank et al. traced radiolabeled proline ($^{14}$C) and detected carbon residues in glutamate and aspartate in an *E. coli* proline auxotroph. As the auxotroph is unable to synthesize proline, the detected residues are derived from the catabolism of the radiolabeled proline [17]. In *Bacillus subtilis*, a model gram-positive bacterium, the loss of proline catabolic biosynthetic genes is associated with a growth lag or abolishment of growth [21, 45]. Moreover, the relationship between glutamate and proline is essential. In the plant species *Arabidopsis*, it was found that an increase in the activity of P5C dehydrogenase has been associated with the intracellular accumulation of proline [32]. Glutamate is a critical amino acid serving as a precursor for 2-oxoglutarate, an intermediate in the tricarboxylic acid cycle (TCA) [46, 47]. Additionally, both glutamate and glutamine serve as valuable nitrogen donors for the synthesis of a variety of amino acids and nucleotides [45, 48, 49].

In *E. coli* and *B. subtilis*, glutamate can be catabolized by the conserved ArgJBCDFGH pathway to ornithine, and enzymatically converted into arginine or proline [50-52]. Additionally, *E. coli* and *B. subtilis* contain a pathway to synthesize proline directly from glutamate via P5C dehydrogenase [45, 49, 51]. However, the metabolism of proline in *S. aureus* differs from the conserved pathways found in *B. subtilis* and *E. coli*. As a proline and arginine auxotroph, *S. aureus* catabolizes proline into glutamate via PutA and RocA, but *S. aureus* does not have the enzymatic capability to synthesize proline using glutamate as a precursor (Figure 1) [16, 53]. While the *S. aureus* genome encodes the conserved argJBCDFGH pathway, the growth conditions that de-repress this operon are not known [54]. Townsend et al. were unable to detect the $^{14}$C label in
arginine or proline when the media was supplemented with 14C glutamate. However, 14C arginine was incorporated into proline [53]. Additionally, S. aureus had a growth defect in media lacking proline, unless it was supplemented with excess arginine [53].

To further confirm if proline served as a precursor for glutamate, our laboratory discovered that proline dehydrogenase (putA) and P5C dehydrogenase (rocA) mutants had a significant decrease in growth yield when grown in complete defined medium (CDM) lacking glutamate (CDM-E) [55]. As predicted when 13C-labeled proline and arginine were supplemented in CDM, Halsey et al. found that 13C-labeled proline and 13C-labeled arginine were incorporated into glutamate. However, no 13C derived from glutamate was detected in proline or arginine. These data indicate that proline and arginine are the primary sources for glutamate synthesis in S. aureus [55]. Furthermore, our laboratory determined that a glutamate dehydrogenase (gudB) mutant was unable to grow in the absence of glucose [55]. Our conclusions indicate that glutamate, and those amino acids that can be converted to glutamate (proline, arginine, and histidine), serve as the major carbon sources in CDM lacking glucose.

In S. aureus, the conserved catabolite control protein A (CcpA) regulates the expression of genes required for the utilization of secondary carbon sources. Once the preferred carbon source is depleted, CcpA repression of secondary carbon sources is alleviated [56-59]. Furthermore, it was determined that CcpA repress the transcription of proline catabolic and anabolic genes in the presence of glucose [54, 58, 59]. Transcriptional analysis further revealed CcpA-dependent downregulation of rocA (proline dehydrogenase), rocD (ornithine aminotransferase), and rocF (arginase) (Figure 1) [58, 59]. Additionally, electrophoretic mobility shift assays (EMSA) determined that CcpA binds to cre elements within the promoters of rocF, rocD and binds weakly to the proC promoter (See Figure 1) [59]. In conclusion, proline can be
used as a carbon source only in the absence of glucose, and it can be synthesized from arginine, or *S. aureus* will utilize the three proline transporters to acquire it.
Figure 1: The metabolic pathways of proline, glutamate, and arginine in JE2

The metabolism of proline to glutamate proceeds from A proline dehydrogenase (PutA) to B P5C dehydrogenase (RocA). Glutamate may enter the TCA cycle as 2-oxoglutarate through C glutamate dehydrogenase (GudB). 2-oxoglutarate can be catabolized into glutamate through D glutamate synthetase (GOGAT), and glutamate can also be catabolized into glutamine by E glutamine synthetase (GlnA). In B. subtilis and E. coli, glutamate can serve as a precursor for ornithine synthesis via the ArgJBCDFGH pathway [50-52]. While S. aureus encodes F the ArgJBCDFGH pathway, this is not transcriptionally active [54]. For the catabolism of proline into arginine, proline is converted into A pyrroline 5-carboxylate by PutA, and then non-enzymatically converted into glutamate-5-semialdehyde. Glutamate-5-semialdehyde is catabolized into ornithine by ornithine transferase (RocD), where it proceeds enzymatically through the Urea cycle to citrulline and arginosuccinate by ArgF GH into arginine. S. aureus can synthesize proline from arginine via catabolism by H RocF into Ornithine, through RocD. Finally, proline-5-carboxylate is catabolized by I P5C reductase (ProC) into proline [45, 51, 60]. The ArgJBCDFGH pathway is dashed to represent a multi-enzymatic pathway.

This figure was adapted from the following sources [45, 49-51, 54, 59, 60].
Primary and Secondary Response: The Relationship of K+ and Proline Transporters

Primary Response

Upon first exposure to osmotic stress, *S. aureus* requires a system to immediately adapt to the loss of water and subsequent decrease in turgor pressure. The first system is composed of high and low-affinity potassium transporters that will rapidly transport potassium ions (K+) into the cell [10, 14]. For example, the transport of K+ was found to rapidly increase on first exposure to osmotic stress, with transcriptional analysis finding upregulation of the transporters in the presence of 2 M NaCl [24, 61]. Furthermore, mutations in the K+ transporters resulted in a growth defect and loss of cytoplasmic pH homeostasis [62]. As K+ accumulates and reaches a final concentration, there is a corresponding increase of internal proline concentration [63]. In *B. subtilis*, intracellular proline concentration began to rise 2 hours after exposure to 400 mM NaCl, which corresponded to the peak of K+ concentration [61]. However, it was observed that K+ transport decreases when the environment exceeds 500 mM NaCl [11]. These data indicate that proline transport is preferential, as proline can be accumulated to a high concentration in environments with increasing salinity. Experiments by da Costa et al. determined that proline could account for approximately 20% of bacterial dry weight after exposure to osmotic stress [64]. Additionally, proline can be accumulated under increasing NaCl concentrations, unlike K+ transport which is hypothesized to be limited to increasing NaCl concentrations [63, 64].

*B. subtilis* and *S. aureus* rely on compatible solutes, glycine betaine and proline, to neutralize the charges from the increasing K+ concentration [10, 61]. A study in *B. subtilis* transport experiments found proline biosynthesis had increased before proline was transported from the medium [9]. Lastly, the activation of proline transport is hypothesized to be dependent on the intracellular accumulation of K+, as the activation of proline transport occurs after K+ ions accumulate [65, 66].
Secondary Response: Proline Transporters

*S. aureus* has three known proline transporters. OpuD and ProP function as low-affinity transporters, whereas PutP has been defined as a high-affinity proline permease [28, 30, 67]. The high-affinity system (presumably PutP) has a Vmax of 1.1 nmol/min/mg, versus a Vmax of 22 nmol/min/mg in the low-affinity system (presumably OpuD and ProP) [53, 67]. To evaluate the behavior of the high-affinity transporter, Schwan et al. grew *S. aureus* in defined media with varying concentrations of proline (1740-1.74 µM). The *putP* mutant had a growth defect only in media containing low concentrations of proline (1.74 µM) [68]. Furthermore, it was found that as proline concentration decreased, from 17.4 µM to 1.74 µM, *putP* transcription increased [68]. Moreover, the transcription of *putP* was found to be negatively regulated by σB (SigB), a stress-inducible sigma factor found in several gram-positive bacteria [28, 69]. Schwan et al. determined that the transcription of *putP* decreased sharply after exposure to 1M NaCl, which is hypothesized to activate *sigB* expression [28]. However, this decrease in transcription is no longer observed in a *sigB* mutant suggesting that proline transport is regulated by the stress response [28]. Moreover, PutP has also been found to be important during soft tissue infections. It was determined that *putP* transcription increased 45-fold post-infection in the liver and spleen tissue, indicating that *putP* may be important for survival in a soft tissue infection [28]. Lastly, the bacterial burden of a *putP* mutant was significantly reduced in an endocarditis model of infection [30].

*S. aureus* has two low-affinity proline transporters, ProP and OpuD. Despite sharing similarity to the ProP homolog in *E. coli*, little is known about ProP in *S. aureus*. In *E. coli*, the low-affinity system is repressed in the presence of increasing NaCl concentrations [70]. In contrast, hyperosmotic conditions (1M NaCl) stimulate the expression of the low-affinity transporter
system (presumably OpuD and ProP) in *S. aureus* [26, 71]. However, while *opuD* expression is induced by osmotic stress, the expression of *opuD* decreases when NaCl concentrations exceed 400 mM [27]. Therefore, while *proP* expression is likely induced under increasing NaCl concentrations, *opuD* is repressed. As the second known low-affinity transporter, expression of *opuD* has been found to increase as proline concentrations increase [27]. When grown in high concentrations of proline, an *opuD* mutant had a decrease in intracellular proline from 802 nmol/mg to 489 nmol/mg in comparison to the wild-type strain [64]. In summary, *opuD* and *proP* are expressed under osmotic stress, with *opuD* expressed in high concentrations of proline [26, 27]. In contrast to the low-affinity proline transporter, the transcription *putP* increase when proline levels decrease and is repressed under increasing osmotic stress [28]. Therefore, the three proline transporters are essential for *S. aureus* to colonize and adapt to environments that vary in osmotic pressure and proline concentrations.
**Proline in Protein Stability**

Bacterial cells have evolved to form a system capable of adapting to osmotic stress in hostile environments, allowing them to colonize a large variety of environmental niches, including the human host [11, 32]. The system is composed of three factors: 1) the salt in the cytoplasm 2) compatible solutes, and 3) the specific, and non-specific transporters [11]. The first factor is the accumulation of organic osmolytes (i.e., K+ or amino acids), with amino acids being most cited, to maintain cytoplasmic salt concentration, and to stabilize proteins [8, 11, 72]. The second factor is the accumulation of compatible solutes, which are compounds that have no net charge at physiological pH, and can accumulate to high intracellular concentrations without disrupting cellular processes [8, 10, 11]. Compatible solutes can serve to balance both the charges from the rapid accumulation of K+ during the primary response and prevent water loss in hyper or hypotonic environments [8, 11]. While many compatible solutes exist, proline is the most ubiquitous and most widely used in nature [32, 73]. Indeed *S. aureus* grown in media not supplemented with glycine betaine or proline had a growth defect as NaCl concentrations increased, while the presence of either compatible solute (glycine betaine or proline) enabled growth up to 3.06 M NaCl [13]. Finally, the third factor is composed of specific and non-specific transporters, which in *S. aureus* can be a combination of K+ transporters, glycine betaine transporters, and the three known proline transporters: PutP, ProP, and OpuD [8, 11, 14, 67].

Studies observing changes of the amino acid pool in *S. aureus*, after exposure to osmotic stress, found proline to rapidly accumulate [13, 19, 73]. Proline is accumulated because it is a net-neutral osmolyte and readily interacts with proteins improving solubility and stability, therefore preventing protein denaturation in environments that vary in osmolarity [11, 15, 29]. Furthermore, protein-folding experiments by Auton et al. determined that proline assists with protein folding and therefore improves stability [74]. In the presence of proline, the exposed side
chains of an unfolded and unstable protein do not favor interactions with the solvent and correctly fold into a functional protein [74]. However, when the protein is in the presence of urea, a denaturing osmolyte, it generates a solvent-protein interaction that facilitates protein unfolding [74]. In conclusion, the acquisition and utilization of proline prevents proteins from denaturing when \textit{S. aureus} is exposed to environments that may compromise cellular integrity.

Though the acquisition of proline in \textit{S. aureus} is known to be associated with survival under osmotically stressed conditions, the behavior and regulation of the proline transporters are largely unknown. In the case of ProP, no studies have investigated the behavior and function in \textit{S. aureus}. Our study was designed to address the role of transcriptional regulation of CcpA and SigB on \textit{putP}, \textit{opuD}, and \textit{proP} in \textit{S. aureus} JE2. As previously stated, SigB negatively regulates the expression of high-affinity proline transporter, \textit{putP} [28]. Additionally, Wetzel et al. did not find SigB affecting the expression of the low-affinity proline transporter, \textit{opuD} [27]. However, the role of SigB regulation on \textit{proP} is unknown. As CcpA was found to regulate proline catabolism genes, we investigated if CcpA affects the expression of the three proline transporters [58, 59]. Previous studies on proline transport in \textit{S. aureus} have utilized various strains of \textit{S. aureus} and only investigated one proline transporter [26-28, 30, 67, 68]. Lastly, we were able to investigate all three transporters and their expression and regulation in one strain, \textit{S. aureus} JE2.
Chapter 2: Materials and Methods

Strains, Media, and Primers

*S. aureus* USA 300 JE2 proline transporter mutants (*proP::ϕNΣ, putP::ϕNΣ, and opuD::ϕNΣ*) and the wild type, *S. aureus* USA 300 JE2, used in this study were provided by the Nebraska Transposon Mutant Library (NTML) (Table 1) [75]. The *S. aureus* *sigB* mutant strain, JE2 *sigB::tetL* strain, was transduced via phage from a *S. aureus* strain previously described [76]. The *S. aureus* *ccpA* mutant strain, *S. aureus* JE2 *ccpA::tetL*, was provided as previously described [54]. *S. aureus* strains were cultured in Complete Defined Media (CDM), which is comprised of components listed in Table 2, and supplemented with 1M or 2M sodium chloride (Fisher Scientific), proline (Sigma-Aldrich), or 14 mM glucose (Sigma-Aldrich) as previously described [77]. All CDM is noted as CDM without the addition of 14 mM glucose unless otherwise noted.

*S. aureus* strains were grown aerobically (10:1 flask to volume ratio, 250 rpm, 37°C) from a single colony in Tryptic Soy Broth (TSB) (Becton Dickinson, Franklin Lakes, NJ). In order to establish starting optical density (OD<sub>600</sub>), 1 mL of media from overnight cultures was removed, and OD<sub>600</sub> was measured on Bio-Rad Smart Spec<sup>TM</sup> 3000. The starting inoculum was prepared by removing 1 mL of TSB from overnight flasks, centrifuging sample at 3,000 rpm for 2.5 minutes, and washed twice with 0.9% Sodium Chloride Irrigation Solution (Baxter Healthcare Corporation). Finally, media was inculcated to a starting OD<sub>600</sub> of 0.05.

Osmotic Shock Experiment:

For this experiment, CDM was prepared without proline (CDM-P), and 60 mLs of CDM-P was added to a 1 L flask. Media was inoculated to an OD<sub>600</sub> of 0.05, and grown until mid-exponential phase (CDM-P OD<sub>600</sub> ~0.75-1.0). Then 5-10mLs of culture were removed and pelleted
for RNA isolation and free amino acid analysis (See RNA Isolation section). For the osmotic shock, four stock solutions containing either 5 M NaCl with 3.75 g proline, 5 M NaCl, 3.75 g proline, or 0.576 g proline with 5M NaCl were prepared and warmed to 37°C before use. Then 40mL of each of the four solutions was added to each culture to a final concentration of CDM-P with 2 M NaCl 2X proline, 2 M NaCl, 2 X Proline, or 2 µM 2X Proline in 100 mLs. 2X proline refers to twice the concentration normally found in CDM, 0.15 g, and 2 µM proline refers to 0.023 g [77]. OD was measured every 30 minutes for 2 hours after osmotic shock, with 10-15 mLs of media removed for RNA isolation and free amino acid analysis.

**RNA Isolation, Northern blots, and rtPCR:**

*S. aureus* JE2 was grown overnight in TSB, and cultures were inoculated to an OD<sub>600</sub> of 0.05 into CDM, CDM with 1M NaCl, or CDM+ Glucose (CDM+G). Cultures were grown aerobically in a 10:1 flask to volume ratio at 37°C at 250 rpm. 10-20 mLs of bacterial culture were collected at early exponential phase, (CDM +/- 1M NaCl: OD<sub>600</sub> ~0.2-.4, CDM+G OD<sub>600</sub> ~0.8-1.0), mid-exponential (CDM +/- 1M NaCl: OD<sub>600</sub> ~.75-1.0, CDM+G OD<sub>600</sub> ~2.0-2.4) and late exponential phase (CDM +/- 1M NaCl: OD<sub>600</sub> ~1.8-2.1, CDMG OD<sub>600</sub> ~3.5-4.0). Samples were pelleted at 4,000 rpm for 15 min at 4°C. Then the supernatant was discarded, and pellets were re-suspended in cold Qiagen RNA Lysis buffer (RLT) containing 1% β-mercaptoethanol and transferred to 2.0 mL HydroLogix™ Tubes containing 0.1 mm dia Glass Beads provided by BioSpec. Samples were lysed in Omni Bead Ruptor from Omni International, for 40 seconds on speed 6. Samples were then pelleted at 13,300 rpm for 20 min at 4°C. The supernatant was collected and placed in a sterile 1.7 mL tube containing 500 µL cold 100% ethanol. Samples were stored at -80°C.

RNA was isolated from using RNeasy mini kit per manufacturer’s instructions provided by Qiagen, Inc. The RNA was quantified using a NanoDrop Spectrophotometer using ND-1000
software, and approximately 3-5. μg of isolated RNA was used for northern analysis. In order to create labeled DNA probes, the primers listed in Table 3 were amplified with digoxigenin-labeled dUTP DNA probes with the DIG PCR Mix provided by Roche. The northern blot was hybridized and washed with DIG buffers and washes provided by Roche. Northern blots were labeled with Anti-Digoxigenin-AP Fab fragments from Roche, and ECF substrate provided by GE Healthcare was used for detection. The northern blots were imaged using the Typhoon FLS 7000 imaging system provided by GE Healthcare.

To perform rtPCR, RNA was isolated and quantified as described above. Next, cDNA was generated per manufacturer's instructions provided by Qiagen QuantiTech Kit. Next, the primers listed in Table 3 were added to form the primer mix for the amplification of three respective proline transporters (proP, opuD, and putP). The rtPCR was set up according to manufacturer's instructions provided by LightCycler 480 SYBR Green 1 Master by Roche. Samples were loaded into a white 96-well plate and quantified using LightCycler 480 II by Roche.

**Free Amino Acid Analysis**

For free amino acid analysis, 1mL of media is extracted from flasks and pelleted for 5 minutes at 10,000 rpm. 600 μL of the supernatant is then transferred to a clean tube and stored at -20°C. If both RNA and Free Amino Acid Analysis are performed simultaneously, 1 mL of the supernatant is removed from the pelleted vial and stored in -20°C until all samples are collected.

The 600 μL sample was subsequently filtered using a 3000 MW cutoff column Vivapsin 500 provided by Sartorius and prepared per manufacturer’s instructions. Samples were analyzed at the Protein Structure Core Facility with Hitachi L-8800 at the University of Nebraska Medical Center.
### Table 1: Strain used in this study

<table>
<thead>
<tr>
<th>Strain Used</th>
<th>Reference</th>
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<tbody>
<tr>
<td>S. aureus JE2</td>
<td>[75]</td>
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<tr>
<td>S. aureus JE2 putP::ϕNΣ</td>
<td>[75]</td>
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<tr>
<td>S. aureus JE2 proP::ϕNΣ</td>
<td>[75]</td>
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<tr>
<td>S. aureus JE2 opuD::ϕNΣ</td>
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<tr>
<td>S. aureus JE2 sigB::tetL</td>
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<td>S. aureus JE2 ccpA::tetL</td>
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### Table 2: Complete Defined Media Components

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<tr>
<td>KH₂PO₄</td>
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<td>Biotin</td>
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<td>Pyridoxamine dihydrochloride</td>
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Table 3 Primers used in this study

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<th>Target on Chromosome</th>
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<td>GGTAGCCGTCTCGTATG</td>
<td>opuD Northern 5’ Forward</td>
</tr>
<tr>
<td>2</td>
<td>GGAACCTAGCAAGACCCAGAAA</td>
<td>opuD Northern 3’ Reverse</td>
</tr>
<tr>
<td>3</td>
<td>CTCCAGATAAGCGTGAAACTC</td>
<td>proP Northern 5’ Forward</td>
</tr>
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<td>4</td>
<td>CGCGTCGTACCACCAAATA</td>
<td>proP Northern 3’ Reverse</td>
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<tr>
<td>5</td>
<td>CTGTTTTCTGTATCTGGGTGTAAA</td>
<td>putP Northern 5’ Reverse</td>
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<td>6</td>
<td>GCTAGAATCGACAGCAAGTAAGA</td>
<td>putP Northern 3’ Reverse</td>
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<td>TGGGGATAGGTTTGGTTTTT</td>
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<td>ACATGGTGAGTGCAAAGAAA</td>
<td>proP rtPCR 5’ Forward</td>
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<td>GCTCAATGTACCGCTGTTG</td>
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<td>TTTGGATGGCTGGATTAAA</td>
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<td>14</td>
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Chapter 3: S. aureus Requirement of Proline under Osmotically Stressed Conditions

Amino Acid Accumulation in Chemically Defined Media

S. aureus is able to survive numerous environmental stresses, most notably growing in high salinity [78]. We found comparable results with S. aureus grown in Complete Defined Media (CDM) supplemented 1M sodium chloride (NaCl) (Figure 2). In these conditions, S. aureus had a growth lag but was able to obtain a similar growth yield to CDM without 1M NaCl. As previous studies correlated the acquisition of proline with survival under osmotic stress, we hypothesized that under increasing salinity, S. aureus would acquire proline from the surrounding environment [19, 27, 67]. To address this hypothesis, S. aureus JE2 was grown in CDM with and without 1M NaCl. Amino acid consumption was compared between CDM without 1M NaCl (CDM), and CDM with 1M NaCl (CDM 1M NaCl) (Figure(s) 3A-D). When grown in CDM, arginine, proline, and glutamate were exhausted from the media within 8 hours (Figure 3A). The exhaustion of these three amino acids is correlated with the late exponential phase of growth (Figure 3B).

While S. aureus grown in CDM has exhausted proline, glutamate, and arginine by late exponential phase, S. aureus grown in CDM 1M NaCl had exhausted glutamate and proline from the media (Figure 3C-3D). Anderson et al. also noted an intracellular accumulation of proline and glutamine in S. aureus under osmotic stress, with a 1,100% and 3,500% increase after exposure to 10% NaCl solution, respectively [19]. However, arginine is not exhausted from the media by the late exponential phase (Figure 3D). In conclusion, our data suggests that S. aureus prefers to accumulate proline and glutamate under osmotic stress.
Figure 2: JE2 grown in CDM compared to CDM 1M NaCl

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM (solid triangle) and CDM 1M NaCl (solid square). The mean and standard deviation were calculated from experiments repeated four independent times.
3A: Amino Acid consumption of JE2 in CDM per OD

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM. The OD was compared to the concentration of amino acids. Media samples were taken for free amino acid analysis at 0, 4, 8 and 10-hour time points.

---

**Figure 3B: Arginine and glutamate consumption of JE2 in CDM and CDM 1M NaCl**

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM (solid line) and CDM 1M NaCl (dashed line). 0M represents CDM and 1M represents CDM 1M NaCl. Media samples were taken for free amino acid analysis at 0, 4, 8 and 10-hour time points.
Figure 3C: Proline consumption of JE2 in CDM and CDM 1M NaCl

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM (solid line) and CDM 1M NaCl (dashed line). 0M represents CDM and 1M represents CDM 1M NaCl Media samples were taken for free amino acid analysis at 0, 4, 8 and 10-hour time points. Figure 3C is the same growth curve as figure 3B. However, figure 3C examines the proline consumption.

3D: Amino acid consumption of JE2 in CDM 1M NaCl per OD

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM 1M NaCl. The OD was compared to the concentration of amino acids. 1M NaCl represents CDM 1M NaCl. Media samples were taken for free amino acid analysis at 0, 4, 8 and 10-hour time points.
Concentration of proline is correlated with growth under osmotic stress

Based on our observation that proline and glutamate are accumulated under osmotic stress, we hypothesize that limited proline concentrations will negatively affect growth in an osmotically stressed environment. In order to confirm the relationship between proline concentrations and growth, *S. aureus* was grown in CDM with varying concentrations of proline, with or without the presence of 1M NaCl (Figure(s) 4A- B). In CDM, an increase in proline concentration was associated with an observed increase in growth rate and growth yield (Figure 4A). However, it was determined that concentrations of proline lower than 1.3 mM have an observed growth lag, with CDM with no proline (CDM-P) growing to the same yield as 1.74 µM proline. These data support previous studies noting the concentration of proline associated with growth rate and growth yield [67, 68].

In order to confirm if concentrations of proline affected the growth of *S. aureus* under osmotic stress, *S. aureus* was grown CDM with 1M NaCl (CDM 1M NaCl). We observed that when *S. aureus* was grown in CDM without proline containing 1M NaCl (CDM-P 1M NaCl), it had severe growth defect (Figure 4B). Additionally, when *S. aureus* was grown in CDM 1M NaCl with 1.74 µM proline, it had the same phenotype as CDM-P 1M NaCl (see figure 4B). Proline concentrations above 650 µM were observed to have a growth lag in the presence of 1M NaCl but could obtain a similar growth yield to CDM. These results demonstrate that there is a correlation between the concentrations of proline and growth yield. Moreover, under osmotic stress, *S. aureus* is not able to grow without the addition of proline.
Figure 4A: Effects of limited proline concentration on JE2 in CDM

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM. Data in Figure 4A are representative of growth performed three independent times, except CDM-P which was performed twice. Concentration of 1.3mM proline is standard proline concentration in CDM [77].

Figure 4B: Effects of limited proline concentration on JE2 in CDM 1M NaCl

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM 1M NaCl. Data in Figure 4B is representative of growth experiments performed three independent times, except CDM-P which was performed twice. Growth in Figure 4A and 4B were performed simultaneously. Concentration of 1.3mM proline is standard proline concentration in CDM [77].
Chapter 4: Proline Transporters

Multiple studies associate the survival of *S. aureus* in a stressed environment to the transport and utilization of proline [3, 28-30, 68, 79]. To evaluate the function of each proline transporters (ProP, OpuD, and PutP) in regards to growth in CDM and CDM 1M NaCl, we utilized the three transporter strains (JE2 *opuD::φNΣ, proP::φNΣ*, and *putP::φNΣ*) from the Nebraska Transposon Mutant Library (figure 5A-B). We hypothesized that *putP::φNΣ* would not have a growth defect in CDM 1M NaCl, as *putP* is repressed in the presence of osmotic stress (figure 5B) [68]. As CDM contains 1.3 mM proline, we further hypothesized that the loss of one of the low-affinity transporters, *opuD::φNΣ* or *proP::φNΣ*, can be compensated by the activity of the remaining low-affinity transporter (Figure 5B). Therefore, it was not surprising to find that none of the transporter mutants displayed any growth lag or defect in CDM or CDM with 1M NaCl.
Figure 5A: Growth of JE2 TML proline transporters in CDM

*S. aureus* JE2 and proline transporter mutants from the Nebraska Transposon Mutant Library (TML) (*opuD::φNΣ, proP::φNΣ, putP::φNΣ*) were grown aerobically (10:1 flask to volume ratio) in CDM. The mean and standard deviation from Figure 5A are calculated from growth experiments performed three independent times.

Figure 5B: Growth of JE2 TML proline transporters in CDM 1M NaCl

*S. aureus* JE2 and proline transporter mutants from the Nebraska Transposon Mutant Library (TML) (*opuD::φNΣ, proP::φNΣ, putP::φNΣ*) were grown aerobically (10:1 flask to volume ratio) in CDM 1M NaCl. Both data from figure 5A and 5B were experiments performed simultaneously, with JE2 CDM serving growth marker for growth in CDM. The mean and standard deviation from Figure 5B are from growth experiments performed from three independent experiments.
**Northern Blot and rtPCR Results**

To evaluate the expression of the three transporters in CDM compared to CDM 1M NaCl, Northern blot analysis and rtPCR was performed. We observed that in CDM 1M NaCl, the expression of *opuD* and *proP* increased from early to late exponential growth, with the expression of *opuD* having the highest fold change (Figure 6). However, the expression of *putP* did not change in CDM 1M NaCl when compared to expression in CDM.

The northern transcriptional analysis found that *opuD* had the highest expression in early exponential growth in CDM and decreased in expression by late exponential phase (Figure 7A). As previously discussed, proline is exhausted from the media by late exponential phase. Therefore the reduction in *opuD* expression correlates with the exhaustion of proline from CDM. As it was determined that the expression *opuD* in CDM 1M NaCl is greater than in CDM, this indicates that the expression of *opuD* is induced by osmotic stress. The expression of *proP* differed from *opuD*, in that *proP* was expressed more during mid-exponential growth (Figure 7B). The expression of *proP* is similar to *opuD* in CDM 1M NaCl as both are induced by osmotic stress. As expected, there was no detectable expression of *putP* in CDM 1M NaCl, as previously literature noted repression of *putP* expression during osmotic stress (Figure 7C) [68]. In CDM, *putP* was only expressed in early exponential phase, indicating that *putP* might be regulated in mid and late exponential growth. These data demonstrate a regulation of *putP* in mid to late exponential phase. Additionally, we determined that *proP* and *opuD* are induced by osmotic stress. Overall, we were able to confirm previous data supporting that the low-affinity transporters, OpuD and ProP, are induced by osmotic stress, while the high-affinity transporter, PutP, is repressed.
Figure 6: rtPCR analysis of JE2 proline transporters grown in CDM and CDM 1M NaCl

Expression of the three transporters (opuD, putP, and prop) was measured from RNA isolated in early exponential, mid-exponential and late exponential from JE2 grown aerobically (10:1 flask to volume ratio) in CDM and CDM 1M NaCl. cDNA probes for opuD, putP and prop were used in the rtPCR analysis. The error bars represent standard deviation from the mean, calculated from three independent experiments. SigA was used as the transcriptional control.
Figure 7A: JE2 opuD RNA expression in CDM and CDM 1M NaCl

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM and CDM 1M NaCl. RNA was isolated at early exponential, mid-exponential and late exponential. Samples were hybridized with opuD DNA probe. The loading control is included below the northern blot.

Figure 7B: JE2 proP RNA expression in CDM and CDM 1M NaCl

*S. aureus* JE2 was grown aerobically (10:1 flask to volume ratio) in CDM and CDM 1M NaCl. RNA was isolated at early exponential, mid-exponential and late exponential. Figure 7B is the blot from 7A. Blot from 7A was stripped and re-hybridized with a proP DNA probe. The loading control is included below the northern blot.
S. aureus JE2 was grown aerobically (10:1 flask to volume ratio) in CDM and CDM 1M NaCl. RNA was isolated at early exponential, mid-exponential and late exponential. Samples were hybridized with putP DNA probe. The loading control is not included below the northern blot.
**Osmotic Shock Experiment**

In order to determine when there is expression of these three transporters, *S. aureus* JE2 was grown in CDM without proline (CDM-P) and exposed to varying proline and salt concentrations. First, *S. aureus* JE2 was grown in CDM-P to mid-exponential phase (figure 8A). Then *S. aureus* was subjected to four solutions: 2X proline and 2M NaCl (2X 2M), 2X concentration of proline (2X), 2 M NaCl (2M), or 2 µM proline and 2 M NaCl (2µM 2M) (figure 8B). 30 minutes after exposure to the solutions, all samples had a decrease in OD as they were diluted from the solutions. 1 hour following exposure only the solutions containing proline were able to restore growth. However, *S. aureus* JE2 exposed to the 2M NaCl solution, without proline, was unable to recover from the osmotic stress, and the OD declined.

Through previous northern blot analysis, we determined that both *proP* and *opuD* expression are induced, and *putP* expression was repressed by the presence of osmotic stress (Figure(s) 7A-C). We further hypothesized that the expression of the low-affinity transporters, *opuD* and *proP*, would be highest in the 2M, 2X, and 2X 2M solutions, whereas expression of the low-affinity transporters would decrease in the 2µM 2M solution. However, *opuD* expression was weaker in the 2X 2M compared to 2µM 2M (figure 9A). The minimal expression of *opuD* was unexpected as OpuD is a low-affinity transporter, and excess proline (2X) would most likely induce the expression of *opuD*. Finally, only the 2M and 2µM 2M solutions consistently induced the expression of *opuD*. Note that two hours following addition of the various solutions, *opuD* expression was not detected in all conditions except the flask containing 2M NaCl.

Similar to *opuD*, we hypothesized that *proP*, as it is a low-affinity transporter and induced by osmotic stress, would be induced by the 2X concentration of proline and 2M NaCl solution. As predicted, *proP* was expressed in the 2M NaCl solution (figure 9B). However, *proP* was only
expressed in the 2X 2M solution 30 minutes following the addition of the solution. The expression of proP decreased in the 2X 2M solution 1 hour after exposure and was expressed after one hour only in the 2µM 2M and 2M solutions. The lack of expression in the 2X proline solution was unexpected, as OpuD and ProP are low-affinity transporters, and high concentrations of proline should induce expression of these genes.

As PutP is a high-affinity transporter and repressed in the presence of osmotic stress, we hypothesized putP would be minimally expressed in either of the 2X or 2M solutions. However, putP was expressed in both the 2X 2M and 2µM 2M solutions, despite the addition of a 2M NaCl solution. The expression of putP decreased 1.5 hours after shock (figure 9C). Our experiments further support the conclusion that osmotic stress upregulates the expression of proP and opuD, and downregulates putP. Although, in the presence of an osmotic stressor with proline (2X 2M or 2µM 2M), putP expression increased. These results indicate that osmotic stress may override the physiological regulation of the proline transporter.
**Figure 8A: JE2 growth in CDM-P prior to addition of 2 M NaCl**

*S. aureus* was grown aerobically (10:1 flask to volume ratio) in CDM-P until mid-exponential phase. Once at mid-exponential phase (OD$_{600}$ ~ 0.8-1.0) flasks were exposed to 2X concentration of proline with 2M NaCl, 2X concentration of proline, 2M NaCl solution, or 2 µM concentration of proline and 2M NaCl. Data in figure 8A are representative of growth performed two independent times. Figure 8B below documents the growth after osmotic shock.

**Figure 8B: JE2 growth in CDM-P following addition of 2 M NaCl**

*S. aureus* JE2 grown aerobically (10:1 flask to volume ratio) in CDM-P and exposed to an addition of 2X concentration of proline with 2M NaCl, 2X concentration of proline, 2M NaCl solution, or 2 µM concentration of proline and 2M NaCl. Time point 0 is from hour 6 on graph 8A to represent growth before the osmotic shock. There is an initial decline in OD as the addition of the solutions dilutes the flasks. However, all solutions were able to recover growth except 2M NaCl. Data in figure 8B are representative of growth performed two independent times.
Figure 9A: JE2 opuD RNA expression following addition of 2M NaCl

*S. aureus* JE2 grown aerobically (10:1 flask to volume ratio) in CDM-P and exposed to an addition of 2X concentration of proline with 2M NaCl, 2X concentration of proline, 2M NaCl solution, or 2 μM concentration of proline and 2M NaCl. RNA was isolated every 30 minutes after exposure and hybridized with *opuD* DNA probe. Lanes 1-14 and lanes 15-17 are separate gels run simultaneously. Lane 18 was from a separate experiment. The loading control is included below the northern blot.

Figure 9B: JE2 proP RNA expression following addition of 2M NaCl

*S. aureus* JE2 grown aerobically (10:1 flask to volume ratio) in CDM-P and exposed to an addition of 2X concentration of proline with 2M NaCl, 2X concentration of proline, 2M NaCl solution, or 2 μM concentration of proline and 2M NaCl. RNA was isolated every 30 minutes after exposure and hybridized with *proP* DNA probe. The loading control is included below the northern blot.
Figure 9C: JE2 putP RNA expression following addition of 2M NaCl

*S. aureus* JE2 grown aerobically (10:1 flask to volume ratio) in CDM-P and exposed to an addition of 2X concentration of proline with 2M NaCl, 2X concentration of proline, 2M NaCl solution, or 2 µM concentration of proline and 2M NaCl. RNA was isolated every 30 minutes after exposure and samples were hybridized with putP DNA probe. The loading control is included below the northern blot.
**Amino Acid Analysis of Osmotic Shock Experiment**

Our data from Northern blot analysis confirmed that *opuD* and *proP* are expressed under osmotic stress (2X 2M, 2M, and 2µM 2M). Surprisingly, *putP* expression was detected in 2µM 2M and 2X 2M, despite previous data demonstrating downregulation of *putP* under osmotic stress. To assess our hypothesis that osmotic stress may override the normal physiological regulation of the transporters, we analyzed the free amino acid concentration from the 2X 2M and 2X solutions (See Figures 10A-C).

In the 2X proline solution, we observed recovery from osmotic shock and growth, indicating that proline may be transported despite minimal expression of *opuD*, *proP* and *putP* (figure 8B). We evaluated the amino acid concentration in 2X solution 30 minutes and 1 hour after exposure. As expected, no proline was detected at the 0-hour mark or before shock (BS), as *S. aureus* was grown in CDM-P. We observed a minimal change in proline concentration 30 minutes and 1 hour after osmotic shock, yet glutamate and arginine were exhausted from the media (Figure(s) 10A-C). In these conditions, proline was not acquired from the media, but arginine and glutamate were most likely serving as secondary carbon sources. Thus, the three proline transporters were not required and therefore were not expressed.

In the 2X 2M sample, proline was rapidly acquired from the media. However, the concentration of glutamate decreased slightly in the 2X 2M, compared to 2X. Furthermore, there is a decrease in glutamate consumption compared to the 2X solution. We also observed a minimal change in arginine levels. We determined that under osmotic stress *S. aureus* does not prefer the acquisition of arginine. In the presence of an osmotic stressor, 2M NaCl with excess proline 2X, *S. aureus* rapidly acquired proline from the media utilizing the proline transporters.
Proline consumption was measured 30 minutes and 1 hour after osmotic shock in the 2X 2M and 2X samples in figure 10A. Glutamate (10B) and Arginine (10C) concentration were also measured. Time point 0 Hour is the initial amino acid concentration in CDM-P before JE2 was added. Before Shock (BS) is the concentration of amino acids before the addition of the four solutions.
Chapter 5: Regulation of Transporters

As previously discussed in Chapter 2, in the presence of glucose, CcpA negatively regulates the expression of *S. aureus* proline catabolic genes (*putA, rocF, and rocD*) [58, 59]. As M1-macrophages rapidly consume glucose, an abscess environment is hypothesized to be glucose deplete, therefore alleviating CcpA mediated repression and allowing for the expression of proline catabolic genes [2, 6, 33]. Another important feature of CcpA is that it regulates the production of virulence factors and contributes to survival in host tissues [58]. Indeed, a *ccpA* mutant had a significant reduction in bacterial load in the liver and was defective at forming an abscess [59]. In order to be fully virulent, *S. aureus* requires both glycolysis and gluconeogenesis [80]. Therefore, *S. aureus* relies on the acquisition of amino acids for a carbon source in the absence of glucose [33].

Another factor that may regulate the expression of the proline transporters is σB (SigB), a sigma factor activated in *S. aureus* in response to stress (i.e. heat shock, NaCl, pH, NO, or starvation) [69, 81-84]. Previous studies on proline transporters have found that SigB negatively regulates the expression of *putP* in *S. aureus* and positively regulates the expression of *opuE* (a low-affinity proline transporter) in *B. subtilis* [28, 85]. Although *opuE* in *B. subtilis* share some homology to *opuD* in *S. aureus*, the expression of *opuD* was not determined to be affected by SigB [27]. However, the regulation and expression of *sigB* vary in different *S. aureus* strain backgrounds [82]. While a study found *sigB* expression was negatively regulated by the presence of 1M NaCl, other *S. aureus* strains had increased *sigB* expression in 1M NaCl [82]. Presently, no studies have investigated the role of CcpA and SigB on the expression of the proline transporters in *S. aureus* JE2.
CcpA Regulation of the Proline Transporters

We hypothesized that putP expression would be negatively regulated by CcpA, as putP is hypothesized to utilize proline as a carbon source [25, 86]. Therefore, the expression of putP would only be necessary for glucose deplete conditions. We further hypothesized that CcpA would have no effect on the expression of opuD or proP, as the low-affinity transporter are hypothesized to be only responsive to osmotic stress [26, 27]. Therefore, only in the absence of glucose would putP be expressed. To determine if CcpA mediates expression of the transporter genes S. aureus ccpA::tetL and S. aureus JE2 were grown in CDM, and CDM supplemented with 14 mM glucose (CDM+G). Then the RNA expression of the transporters was analyzed during early and late exponential growth (Figure(s) 11A-D).

The expression of both opuD and putP had increased expression in JE2 ccpA::tetL (Figure(s) 11A and 11B). However, the corresponding RNA expression in JE2 had decreased expression in the CDM+G. Surprisingly, there was a decline in putP expression in CDM in JE2 in comparison to expression of JE2 ccpA::tetL (Figure 11B). It is noted by late exponential phase there was no expression detected in opuD or putP. In early exponential phase, proP had decreased expression in CDM+G compared to the JE2 ccpA::tetL, which had increased expression (Figure 11C). By late exponential phase, the expression of proP was minimal in JE2, but there was an increase in expression in the ccpA::tetL strain (Figure 11D). Our data suggests that the expression of putP, proP, and opuD are negatively regulated by CcpA. Furthermore, we hypothesize that SigB may be affecting putP expression in CDM.
Figure 11A: opuD expression in JE2 and ccpA::tetL

S. aureus strains were grown aerobically (10:1 flask to volume ratio) in CDM+G, and CDM. RNA was collected from JE2 (gray) and ccpA::tetL (black) at early and late exponential phase and hybridized with an opuD DNA probe. Lanes 1-4 (early exponential) and 5-8 (late exponential) are separate images merged from the same membrane. The loading control is included below the northern blot.

Figure 11B: putP expression in JE2 and ccpA::tetL

S. aureus strains were grown aerobically (10:1 flask to volume ratio) in CDM+G, and CDM. RNA was collected from JE2 (gray) and ccpA::tetL (black) at early and late exponential phase and hybridized with a putP DNA probe. Lanes 1-4 (early exponential) and 5-8 (late exponential) are separate images merged from the same membrane. The loading control is included below the northern blot.
Figure 11C: proP early exponential phase in JE2, ccpA::tetL and sigB::tetL

S. aureus JE2, ccpA::tetL, and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM+G, and CDM with or without 1M NaCl. RNA was collected from cells during early exponential growth. Samples were then hybridized with a proP DNA probe. The loading control is included below the northern blot.

For comparing growth in ccpA::tetL to JE2, use lanes 1,3,5,7 for early exponential growth. For comparing growth in sigB::tetL to JE2 use lanes, 1-4, and 9-12 for early exponential growth.

Figure 11D: proP late exponential phase in JE2, ccpA::tetL and sigB::tetL

S. aureus JE2, ccpA::tetL, and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM+G, and CDM with or without 1M NaCl. RNA was collected from cells during late exponential growth. Samples were then hybridized with a proP DNA probe. The loading control is included below the northern blot.

For comparing growth in ccpA::tetL to JE2, use lanes 1,3,5,7 for late exponential growth. For comparing growth in sigB::tetL to JE2 use lanes, 1-4, and 9-12 for late exponential growth.
SigB Regulation of the Proline Transporters

A previous study on the expression of putP observed SigB downregulates putP expression in osmotically challenged environments [28]. Furthermore, transcription of putP was detectable only in a SigB mutant background in an osmotically challenged environment [28]. While putP expression was found to be negatively regulated by SigB, the regulation of the low-affinity proline transporters in S. aureus is largely unknown. We had previously determined opuD and proP expression to be upregulated in response to osmotic stress. Therefore, we hypothesized that expression of opuD and proP is SigB-dependent.

Utilizing Northern blot analysis, JE2, and JE2 sigB::tetL were grown in CDM, and CDM+G, with or without 1M NaCl (Figure(s) 12A-B). RNA was isolated at early and late exponential time points (Figure 13A-D). The osmotically induced opuD did not appear to have a difference in expression in early or late exponential phase between JE2 sigB::tetL and JE2 (Figure 13A-B). Interestingly in JE2 sigB::tetL, opuD expression increased in CDM+G. The expression of putP in early exponential phase had no noticeable difference between the JE2 sigB::tetL and JE2 (Figure 13C). However, by late exponential there was minimal expression of putP in JE2, while the expression of putP in JE2 sigB::tetL was upregulated in CDM and CDM 1M NaCl (Figure 13D). These data confirm previous findings that SigB regulates the expression of putP and support our observation that putP was downregulated in JE2 in CDM [14].

The expression of proP in early exponential phase also had a minimal difference between JE2 sigB::tetL and JE2 in CDM and CDM 1M (Figure 11C). As CcpA negatively mediates expression of proP, there was minimal expression in CDM+G and CDM+G 1M. However, by late exponential phase, proP expression in JE2 sigB::tetL was upregulated in CDM+G 1M NaCl and CDM 1M (Figure 11D). These data suggest that JE2 putP expression is negatively regulated by SigB by
late exponential growth. However, in early exponential growth, \textit{putP} does not appear to be regulated by SigB. Finally, the expression of \textit{opuD} does not appear to be affected by SigB.
Figure 12A: JE2, ccpA::tetL, sigB::tetL grown in CDM, CDM+G

*S. aureus* JE2, ccpA::tetL, sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM+ glucose, and CDM. Figure 12A is representative of experiments repeated three independent times.

Figure 12B: JE2, ccpA::tetL, sigB::tetL grown in CDM+ 1M NaCl, CDM+G 1M NaCl

*S. aureus* JE2, ccpA::tetL, sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM+ G with 1M NaCl, and CDM+ 1M NaCl. Figure 12B is representative of experiments repeated three independent times.
**Figure 13A: opuD expression in early exponential phase in JE2 and sigB::tetL**

JE2 and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM with or without glucose and/or 1M NaCl, and had RNA collected from the early exponential phase. Samples were hybridized with an opuD DNA probe. Lanes 1-4 and 5-8 are separate images merged from the same membrane. The loading control is included below the northern blot.

**Figure 13B: opuD expression late exponential phase in JE2 and sigB::tetL**

JE2 and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM with or without glucose and/or 1M NaCl. RNA collected from late exponential phase and hybridized with opuD DNA probe. Lanes 1-3, are from a separate gel run simultaneously with lanes 4-8. However, lanes 4, and are 5-8 separate images merged from the same membrane. The loading control is included below the northern blot.
JE2 and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM with or without glucose and/or 1M NaCl. RNA collected from early exponential phase and hybridized with putP DNA probe. Lanes 1-4 and 5-8 are separate images merged from the same membrane. The loading control is included below the northern blot.

**Figure 13C: putP expression early exponential phase in JE2 and sigB::tetL**

JE2 and sigB::tetL were grown aerobically (10:1 flask to volume ratio) in CDM with or without glucose and/or 1M NaCl. RNA collected from late exponential phase and hybridized with putP DNA probe. Lanes 1-4 and 5-8 are separate images merged from the same membrane. The loading control is included below the northern blot.

**Figure 13D: putP expression late exponential phase JE2 and sigB::tetL**
Chapter 6: Discussion

The World Health Organization (WHO) recently posted a list of pathogens identified as serious health threats with one being *S. aureus* [87]. The Center for Disease Control (CDC) estimates antibiotic resistance leads to over two million hospitalizations and 23,000 deaths yearly. Therefore, it is imperative to research new therapeutic discoveries [1]. A better understanding of how pathogens can adapt to environmental changes and colonize susceptible niches is a promising area of research.

As discussed, *S. aureus* was found to require a functioning metabolic cycle for survival and proliferation in an abscess environment and circumvent the NO stress [33]. While glucose is initially present in an abscess, after HF1-α mediated depletion of glucose from M1 macrophages, *S. aureus* relies on the acquisition of amino acids for a carbon source, with proline being a critical amino acid [2]. Proline, a ubiquitous amino acid, can be acquired from the host surrounding tissue and environment to serve as a carbon source [55, 86]. Proline also functions as an osmoprotectant to cope with changes in salinity and pH of the various micro-niches of the host [32]. The acquisitions of proline in *S. aureus* occurs through three known proline transporters; OpuD, ProP and PutP [25-27]. We are the first known study to investigate the regulation and behaviors of the three proline transporters in one strain. Moreover, we sought to investigate the relationship of the three proline transporters and their respective roles during osmotic stress and potential role in an abscess environment.

This study found that *S. aureus* cannot grow without proline in the presence of an osmotic stressor, and demonstrated a relationship between the concentration of proline and growth yield. When *S. aureus* was grown in CDM with limited (1.74 µM) or no proline (CDM-P) in the presence of 1M NaCl it had severely attenuated growth (Figure 4A-B). However, in the presence of 650 µM
to 2.0 mM proline, growth is restored (Figure 4A). The amino acid analysis further confirmed the uptake of both glutamate and proline from the media in the presence of 1M NaCl (Figure 3 A-D). We also note that more time points are needed to determine the uptake of amino acids between early to late exponential growth. Glutamate and arginine were also acquired from the media when *S. aureus* was exposed to a high concentration of proline (2X), and only when an osmotic stressor was added (2X 2M) would *S. aureus* transport proline and glutamate (Figure(s) 10 A-C). It was noted that arginine was not depleted from the media by late exponential growth in CDM 1M NaCl, while arginine is exhausted in CDM by exponential growth (Figure 3A, 3C). Whether proline is serving as a precursor for arginine synthesis under osmotic stress, and why glutamate is more rapidly acquired during osmotic stress, may be an area for further study. Additionally, we hypothesize that transport of K+ and the utilization and transport of proline is connected, and we further posit that CDM with excess K+ would not have a growth lag in 1M NaCl. However, future studies on the relationship between potassium acquisition and proline utilization are necessary.

In growth conditions under osmotic stress, it was observed that the transporter mutants in CDM and CDM 1M NaCl had no phenotypic difference compared to the wildtype, *S. aureus* JE2 (Figure 5A-B). It is hypothesized that the low-affinity transporters can compensate for each other, as both proP and opuD were found to be transcriptionally active in CDM (Figure 7A-C). Furthermore, Northern blot and rtPCR data confirmed upregulation of proP and opuD in CDM 1M NaCl and CDM 2M NaCl. However, the expression of opuD had the largest fold change in late exponential growth compared to proP (Figure 6A). The expression of opuD in CDM 1M NaCl mirrored the expression in CDM; in CDM, expression decreased from early to late exponential phase, while expression increased in CDM 1M NaCl (Figure 7A). It was determined that opuD and proP expression is induced by osmotic stress, and not the presence of proline, as both transporters
were expressed in the 2M NaCl solution (Figure 9 A-B). Future studies evaluating the impact of an *opuD* and *proP* double mutant in osmotically stressed conditions may be another area of research.

Our data also supported previous studies that the high-affinity proline transporter gene, *putp*, is repressed in the presence of osmotic stress, as the expression of *putP* was not detected in CDM 1M or CDM-P with the 2M NaCl solution (Figure(s) 7C, 9C). As previously discussed, the expression of *putP* is negatively regulated by osmotic stress and SigB [28]. It was determined in our study that *putP* is regulated by SigB in CDM in early exponential and late exponential growth (Figure 13 C-D). However, *S. aureus* grown in CDM-P and subsequently exposed to solutions containing proline with an osmotic stressor (2X 2M, 2µM 2M) induced *putP* expression. We further hypothesize that the requirement of proline in osmotically stressed conditions may override the regulation of *putP*.

Our results confirm that in the presence of glucose, CcpA negatively regulates the expression of the three transporters. Previous studies support that only after glucose is depleted can the genes necessary for utilization of proline be expressed [58, 59]. We observed no expression of either of the three transporters in CDM+G in JE2, while there was corresponding expression in *ccpA::tetL* (Figure 11A-D). Therefore, when *S. aureus* is in the presence of glucose, *putP*, *opuD*, and *proP* will be repressed. We further posit that after M1-macrophage dependent glucose depletion, *S. aureus* will utilize the three transporters to acquire proline from the surrounding environment.

We further sought to investigate the role of SigB in *S. aureus* JE2 on the three proline transporters (Figure(s) 13A-D). As previously mentioned, *putP* was found to be negatively regulated by SigB (Figure 13C-D). Additionally, our study found *proP* expression to be regulated by SigB by late exponential phase, as the JE2 *sigB::tetL* had *proP* expression in CDMG 1M NaCl
We determined that the expression of *opuD* was not affected by SigB regulation in early or late exponential phase (Figure 13A-B).

We further hypothesize that the regulatory effects of CcpA and SigB on these three proline transporters may overlap in different environments. When grown in CDM+G, we had expected an increase in expression of *opuD, proP, and putP* in JE2 *ccpA::tetL*, as CcpA downregulates expression in JE2. However, *opuD* expression was also detected in the JE2 *sigB::tetL* in CDM+G with minimal expression in JE2 (Figure 13A). Additionally, the expression of *proP* in late exponential phase had minimal expression in the JE2 strain, however when grown in CDM G+ 1M NaCl, JE2 *ccpA::tetL* was strongly upregulated, as was JE2 *sigB::tetL* (Figure 11D). Expression in *putP* may also have overlapping regulation by CcpA and SigB; when grown in CDM, *putP* expression was minimal except in the JE2 *ccpA::tetL* and in the JE2 *sigB::tetL* (Figure(s) 11B, 13C-D). However, future studies are needed to investigate the role of CcpA and SigB under osmotic stress.

We posit it that the colonization and survival of *S. aureus* in a host depend on its three proline transporters. Upon colonization of the host, *S. aureus* is dependent on fermentation of glucose to resist NO stress, and CcpA negatively regulates the expression of both the genes required for the transport and catabolism of proline. However, once there is host-mediated depletion of glucose via HIF-1α, CcpA mediated repression will be alleviated, and *S. aureus* will acquire and utilize proline as both as osmoprotectant and carbon source. However, as the pH in the abscess fluctuates, SigB will negatively regulate the expression of *putP* and *S. aureus* will depend on OpuD and ProP to acquire proline. We further hypothesize that as *proP* expression is negatively regulated by SigB by late exponential phase, OpuD will be the primary proline transporter in a late stage abscess.
Finally, the proline transporters may be critical for establishing an abscess as previous studies on skin and soft tissue infections identify putP as essential for survival in animal models [28, 30, 68]. In a S. aureus skin abscess, putP expression increased 39.5-fold after 4 hours to 29-fold after 8 hours in murine model[28]. However, expression decreased 1.5-fold after 18 hours [28]. Schwan et al. further investigated putP expression in human abscesses and found similar expression in a young abscess, but found limited expression of putP in an older abscess [28]. We posit that this is indicative of SigB regulation on putP expression in a late stage abscess. Furthermore, a putP mutant has a significant decrease in the viable count, and higher LD₅₀ than the parental strain [30, 68]. Interestingly, the expression of opuD in a skin abscess increased 1.5-fold after 4 hours and a two-fold increase after 18 hours in a murine model [27]. The expression level of opuD is opposite that of putP, as putP expression decreases by 18 hours, while opuD increases [27, 28]. The activities of the low and high-affinity transporters may be complementing each other to facilitate optimal proline uptake [27, 28]. Therefore, future studies on the behavior of the three proline transporters in an animal model may have the potential to discover new drug targets on the metabolism and adaptation of S. aureus.
References

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