Amino Acid Catabolism in Staphylococcus aureus

Cortney Halsey
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Amino Acid Catabolism in *Staphylococcus aureus*

By

Cortney R. Halsey

A DISSERTATION

Presented to the Faculty of

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Pathology and Microbiology

Under the Supervision of Dr. Paul D. Fey

University of Nebraska Medical Center

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Paul Dunman, Ph.D.             Rakesh Singh, Ph.D.
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Amino Acid Catabolism in *Staphylococcus aureus*

Cortney R. Halsey, Ph.D.

University of Nebraska Medical Center, 2016

Advisor: Paul D. Fey, Ph.D.

Abstract

*Staphylococcus aureus* must rapidly adapt to a variety of carbon and nitrogen sources during invasion of a host. Within a staphylococcal abscess, preferred carbon sources such as glucose are limiting, suggesting *S. aureus* survives through the catabolism of secondary carbon sources. *S. aureus* encodes pathways to catabolize multiple amino acids including those that generate pyruvate, 2-oxoglutarate, and oxaloacetate. To assess amino acid catabolism, *S. aureus* JE2 and mutants were grown in complete defined medium containing 18 amino acids but lacking glucose (CDM). A mutation in glutamate dehydrogenase (*gudB*), which generates 2-oxoglutarate from glutamate, abrogated growth in CDM suggesting that glutamate and those amino acids generating glutamate, particularly proline, serve as the major carbon source in this media. Nuclear Magnetic Resonance (NMR) studies confirmed this hypothesis. Furthermore, a mutation in acetate kinase (*ackA*) also abrogated growth of JE2 in CDM suggesting that ATP production from pyruvate-producing amino acids is also critical for growth. In addition, although a functional respiratory chain was absolutely required for growth, the relative oxygen consumption rate and intracellular ATP concentration were significantly lower during growth in CDM when compared to growth in glucose-containing media. Finally, transcriptional analyses demonstrated that genes coding for the enzymes that synthesize glutamate from proline, arginine and histidine are repressed by CcpA and
carbon catabolite repression. We also demonstrated that in the absence of CcpA repression, the global transcriptional regulator, CodY, functions to positively regulate proline catabolism by activating putA expression. The arginine transcriptional regulator, ArgR1, also functions to positively regulate putA expression, while the arginine transcriptional regulators, ArgR2 and AhrC, are functioning to repress arginine biosynthesis via argG; presumably in the presence of arginine. These data demonstrate that pathways important for glutamate catabolism and ATP generation via Pta/AckA are important for growth in niches where glucose is not abundant.
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Chapter 1

Amino Acid Metabolism in *Bacillus subtilis* and current knowledge in *Staphylococcus aureus*
Introduction

Metabolism is central to microbial life, as all bacteria require carbon and energy for growth and replication. While lifestyles and specific niches occupied by organisms vary, the fundamental metabolic tasks are similar across divergent species of bacteria. All organisms must regulate the uptake of nutrients and coordinate energy, carbon, and nitrogen metabolism to sustain cell maintenance and growth (1, 2).

Pathogenic bacteria such as *Staphylococcus aureus* transiently derive their carbon and energy sources through destruction of host tissue via the synthesis of a wide assortment of virulence factors (1). The specific carbon and nitrogen sources available during host invasion are unclear as *S. aureus* has the ability to establish an infection in a wide variety of niches within the human host, including the heart, bone, kidney, blood, and soft tissue. Recent studies have demonstrated that *S. aureus* adapts to the changing carbon sources and oxygen availability found within specific organ systems. For example, *S. aureus* defective in anaerobic fermentation were less virulent than wild type in a mouse model of kidney infection (3), while mutants defective in aerobic respiration were unable to mediate infection in the heart or liver. However, kidney colonization and subsequent abscess formation was unaffected in the mutants defective in aerobic respiration (4). Furthermore, mutants defective in CcpA, the global carbon catabolite repressor, are less effective at infecting the liver where glucose is abundant, but colonization in the kidney is not affected (5). Collectively, these
data suggest that *S. aureus* uses distinct metabolic pathways to adapt to nutrient availability and environmental changes within specific host niches.

*S. aureus* lacks the ability to utilize phospholipids, triglycerides, and potentially other short chain fatty acids as carbon due to the lack of a β-oxidation system and glyoxylate shunt pathway. However, it has been presumed *S. aureus* has the ability to utilize peptides/amino acids as carbon sources as it encodes multiple oligopeptide permeases and free amino acid transporters, as well as proteases that have the ability to degrade host proteins (6-9). Bioinformatics analysis suggests that pathways are present to catabolize multiple amino acids including those that generate the central metabolic intermediates pyruvate, 2-oxoglutarate, and oxaloacetate (Table 1, Figure 1.1). Taken together, these data suggest *S. aureus* encounters environments within the host where peptides and free amino acids may be abundant carbon sources, fueling growth.

Recent work from Spahich and colleagues found that the addition of lactate plus peptides support growth of *S. aureus* under moderate nitric oxide (NO) stress (10). These studies demonstrated that lactate quinone oxidoreductase (Lqo) assimilates the lactate excreted during the fermentation of glucose as a result of NO stress. Lqo generates pyruvate and subsequently acetate via Pta/AckA in an NAD-independent manner and requires an electron acceptor such as oxygen or nitrate (11). Peptides are required under these conditions to serve as a carbon backbone to facilitate gluconeogenesis, suggesting a requirement for amino acid catabolism during pathogenesis.
Figure 1.1. Amino acid catabolic pathways in *B. subtilis* and *S. aureus*. Genes in blue are encoded by *B. subtilis*. Genes in black are encoded by *B. subtilis* and *S. aureus*. Genes in red are encoded by *S. aureus.*
### Table 1.1 Predicted amino acid catabolism in *Staphylococcus aureus* FPR375

<table>
<thead>
<tr>
<th>Amino acids yielding pyruvate</th>
<th>Reaction</th>
<th>Enzyme</th>
<th>FPR3757</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>(1) Alanine yielding pyruvate</td>
<td>Alanine Dehydrogenase (EC 1.4.1.1)</td>
<td>SAUSA300_1331 (ald)</td>
</tr>
<tr>
<td>Serine</td>
<td>(1) Serine yielding pyruvate</td>
<td>Serine Dehydratase (EC 4.3.1.7)</td>
<td>SAUSA300_2469 (sdhAA) SAUSA300_2470 (sdhAB)</td>
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<tr>
<td>Glycine</td>
<td>(1) Glycine yielding Serine (via 5,10 methylenetetrahydrofolate and glycine cleavage enzyme)</td>
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<td>SAUSA300_2067</td>
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<td></td>
<td></td>
<td>Glycine Dehydrogenase (EC 1.4.4.2)</td>
<td>SAUSA300_1496 SAUSA300_1497</td>
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<td></td>
<td>Glycine Cleavage System H protein</td>
<td>SAUSA300_0791 (gcvH)</td>
</tr>
<tr>
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<td></td>
<td>Glycine Cleavage System T protein (EC 2.1.2.10)</td>
<td>SAUSA300_1498 (gcvT)</td>
</tr>
<tr>
<td></td>
<td>(2) Serine yielding pyruvate</td>
<td>Serine Dehydratase (EC 4.3.1.7)</td>
<td>SAUSA300_2469 (sdhAA) SAUSA300_2470 (sdhAB)</td>
</tr>
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<td>Threonine</td>
<td>(1) Threonine yielding Glycine (via 5,10 methylenetetrahydrofolate and glycine cleavage enzyme)</td>
<td>Threonine Aldolase (EC 4.1.2.5)</td>
<td>SAUSA300_1214</td>
</tr>
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<td></td>
<td>(2) Glycine yielding Serine (via 5,10 methylenetetrahydrofolate and glycine cleavage enzyme)</td>
<td>Serine Hydroxymethyl Transferase (EC 2.1.2.1)</td>
<td>SAUSA300_2067</td>
</tr>
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<td></td>
<td></td>
<td>Glycine Dehydrogenase (EC 1.4.4.2)</td>
<td>SAUSA300_1496 SAUSA300_1497</td>
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<td>Glycine Cleavage System H protein</td>
<td>SAUSA300_0791 (gcvH)</td>
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<td></td>
<td></td>
<td>Glycine Cleavage System T protein (EC 2.1.2.10)</td>
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<td></td>
<td>(3) Serine yielding pyruvate</td>
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<td>SAUSA300_2469 (sdhAA) SAUSA300_2470 (sdhAB)</td>
</tr>
<tr>
<td>Cysteine</td>
<td>(1) Cysteine yielding Alanine</td>
<td>Cysteine Desulferase (EC 2.8.1.7)</td>
<td>SAUSA300_0620 (cysS)</td>
</tr>
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<td>(2) Alanine yielding pyruvate</td>
<td>Alanine Dehydrogenase (EC 1.4.1.1)</td>
<td>SAUSA300_1331 (ald)</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Amino acids yielding 2-oxoglutarate</th>
<th>Reaction</th>
<th>Enzyme</th>
<th>FPR3757</th>
</tr>
</thead>
<tbody>
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<td>Glutamate</td>
<td>(1) Glutamate yielding 2-oxoglutarate</td>
<td>Glutamate Dehydrogenase (EC 1.4.1.3)</td>
<td>SAUSA300_0861 (gudB)</td>
</tr>
<tr>
<td>Glutamine</td>
<td>(1) Glutamine yielding Glutamate</td>
<td>Glutamate Synthase (EC 1.4.1.13)</td>
<td>SAUSA300_0445 (gltB) SAUSA300_0448 (gltD)</td>
</tr>
<tr>
<td>Amino acids yielding oxaloacetate</td>
<td>Amino acid</td>
<td>Reaction</td>
<td>Enzyme</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>------------</td>
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<td>--------</td>
</tr>
<tr>
<td></td>
<td>Aspartate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Aspartate yielding Oxaloacetate</td>
<td>Aspartate Aminotransferase</td>
<td>SAUSA300_1916 (aspA)</td>
<td></td>
</tr>
<tr>
<td>(2) Aspartate yielding Oxaloacetate</td>
<td>Aspartate Aminotransferase</td>
<td>SAUSA300_1916 (aspA)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Asparagine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) Asparagine yielding Aspartate</td>
<td>Asparaginase</td>
<td>SAUSA300_1368 (asnA)</td>
<td></td>
</tr>
<tr>
<td>(2) Aspartate yielding Oxaloacetate</td>
<td>Aspartate Aminotransferase</td>
<td>SAUSA300_1916 (aspA)</td>
<td></td>
</tr>
</tbody>
</table>
Amino acids that cannot be catabolized (predicted)
Tryptophan
Isoleucine
Leucine
Lysine
Methionine
Phenylalanine
Tyrosine
Valine
**Amino acid catabolism**

Bacteria take advantage of a wide range of nutrient sources available within specific environments. The human body is a reservoir of nutrients for pathogenic bacteria, which can be divided into numerous microenvironments. Extracellular bacterial pathogens often face environments with frequently changing nutrients; therefore, they must be able to scavenge those available nutrient sources to fuel central metabolic pathways for generation of energy and macromolecule production (12, 13).

Amino acids are essential building blocks for protein synthesis in all living organisms. In many bacteria, the conversion of peptides to free amino acids and the subsequent utilization of those amino acids in central metabolic pathways is essential for growth and survival. The hydrolysis of exogenous peptides via peptidases allows for free amino acids to be utilized in processes such as protein synthesis, generation of energy, and carbon metabolism (14). Evidence suggests that amino acid catabolism in anaerobic and fermentative bacteria is important for obtaining energy in nutrient-limiting environments. For example, in several species of lactic acid bacteria, arginine catabolized via the arginine deiminase pathway (ADI) provides energy for growth via substrate-level phosphorylation (15, 16). Furthermore, it has been known since the 1930’s that in several nonpathogenic *Clostridium* species, amino acid metabolism via Stickland fermentation is a primary source of energy when the bacteria are grown with amino acids as sole sources of carbon and nitrogen (17-20). Stickland fermentation couples the oxidation of one amino acid (typically alanine, valine,
leucine, serine, isoleucine, or threonine) to the reduction of another (typically glycine, proline, or aspartic acid), resulting in organic acid production, redox balance, and ATP synthesis via substrate-level phosphorylation (Figure 1.2; (21, 22). Recent work from Bouillaut and colleagues demonstrated that Stickland fermentation of proline in the pathogenic Clostridium difficile is required for optimal growth, even in rich media with abundant carbon sources (22). Taken together, these data provide evidence of the importance of amino acid utilization in central metabolism in several bacterial species.

To date little is known about S. aureus amino acid catabolism. As previously mentioned, bioinformatics analysis predicts S. aureus has the ability to catabolize amino acids into three central metabolic intermediates (Table 1). The glucogenic amino acids alanine, serine, glycine, threonine, and cysteine can serve as precursors for the synthesis of pyruvate, while aspartate and asparagine are substrates for the TCA cycle intermediate, oxaloacetate. Furthermore, glutamate, glutamine, histidine, arginine, and proline can serve as precursors for 2-oxoglutarate synthesis. Genes for complete catabolic pathways were not identified for tryptophan, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, and valine. The following sections will describe the current knowledge of amino acid catabolic pathways in B. subtilis and how these pathways may be involved in S. aureus amino acid catabolism. Figure 1.1 is an overview of the pathways discussed in the following sections.
Figure 1.2. **Stickland Fermentation.** Oxidation (A) and reduction (B) of pairs of amino acids. The recycling of the electrons and protons facilitates redox balance while generating ATP.
2-oxoglutarate generating amino acids:

The TCA cycle intermediate, 2-oxoglutarate, is positioned at the intersection of carbon and nitrogen metabolism. It can be utilized in carbon metabolism and energy generation via the TCA cycle, while also acting as the major carbon skeleton for nitrogen-assimilatory reactions through its conversion to glutamate via glutamate synthase (GOGAT). Intracellular 2-oxoglutarate may serve as an indicator to the cell of the available carbon supply, as it has been demonstrated that with the addition of glucose, there is a quick and significant increase in intracellular 2-oxoglutarate (23). In *B. subtilis*, GOGAT expression is induced in the presence of 2-oxoglutarate, while being suppressed by high glutamate concentrations. Thus, the 2-oxoglutarate/glutamate ratio is regulating nitrogen assimilation (24). Glutamate and those amino acids that serve as substrates for glutamate (proline, arginine, and histidine) can fuel 2-oxoglutarate synthesis via the enzyme glutamate dehydrogenase in the absence of CcpA repression (23, 25).

*Glutamate.*

Glutamate and glutamine serve as the major amino group donors for all nitrogen-containing compounds, with glutamate providing 80-88% of the nitrogen in nitrogenous compounds. In *Escherichia coli*, glutamate is the most abundant metabolite in the cell, accounting for 40% of the metabolite concentration (26). Glutamate is synthesized exclusively in *B. subtilis* from the carbon backbone of 2-oxoglutarate via glutamate synthase (GOGAT) and can also be degraded into 2-oxoglutarate directly via glutamate dehydrogenase, thus linking carbon and
nitrogen metabolism (12, 27). *B. subtilis* encodes two glutamate dehydrogenases via the rocG and gudB genes. However, in most laboratory strains of *B. subtilis* only RocG is enzymatically active (25, 28). GudB is inactive due to a 9 bp direct repeat, causing a duplication of three amino acids in the active center of the enzyme (29). Interestingly, inactivation of rocG causes spontaneous mutations to occur in GudB, resulting in enzymatic activity (28). In *S. aureus*, only gudB is present and currently is not well characterized.

In *B. subtilis*, rocG is subject to regulatory signals from both carbon and nitrogen metabolism. In the presence of glucose, CcpA strongly represses rocG transcription through its interaction with the rocG promoter (28, 30). The rocG gene is induced by the transcription factors AhrC and RocR in presence of arginine, ornithine, and/or proline, allowing for the use of these compounds as carbon and nitrogen sources (31-33). The alternative sigma factor, $\sigma^L$, is also required for transcription of rocG. Interestingly, $\sigma^L$ is also strongly repressed by CcpA in the presence of glucose (34, 35). These layers of regulation, which are dependent on the carbon and nitrogen sources available, ensure that an active RocG is only present when the degradation of glutamate to 2-oxoglutarate is required.

*B. subtilis* also utilizes glutamate as a substrate for both proline and arginine synthesis. Proline can be synthesized via three enzymatic reactions carried out by $\gamma$-glutamate kinase (ProB/ProJ), $\gamma$ glutamyl-phosphate reductase (ProA), and $\Delta^{1}$-pyrroline-5-carboxylate (P5C) reductase (Prol/ProH/ProG) (36). However, *S. aureus* does not encode orthologus genes for this pathway and
apparently cannot synthesize proline from glutamate. The absence of proline synthesis via glutamate has been demonstrated by studies showing *S. aureus* grown in the presence of radiolabeled glutamate does not result in the generation of radiolabelled proline (37).

*B. subtilis*, among several other bacterial species, also utilizes glutamate as a substrate for arginine synthesis via the highly conserved *argJBCDFGH* operon. In this pathway, glutamate is catabolized in five enzymatic steps to ornithine, which is then further catabolized into arginine via the urea cycle (38). However, *S. aureus* is an arginine auxotroph despite the presence of all of the *arg* genes within its chromosome (39, 40). While, transcription of *argGH* has been detected by northern blot analysis in a *ccpA* mutant, *argJBCDF* transcript has not been detected in any conditions tested. Furthermore, *S. aureus* grown in the presence of $^{13}$C-glutamate yields no accumulation of $^{13}$C-arginine (41). These data suggest three major differences in *S. aureus* glutamate catabolism compared to *B. subtilis*. First, *S. aureus* only encodes *gudB* for catabolism of glutamate into the TCA cycle intermediate, 2-oxoglutarate. Additionally, the *S. aureus* genome does not encode orthologues to the *B. subtilis* *proB/proJ, proA, or proI/proH/proG* genes involved in proline synthesis via glutamate degradation. Lastly, *S. aureus* does not appear to utilize glutamate as a substrate for arginine biosynthesis via the *argJBCDFGH* pathway.

**Glutamine.**

Glutamine is an optimal source of nitrogen for many bacteria. In the absence of glutamine, ammonium can be used as a source of nitrogen through
assimilation with glutamate via glutamine synthetase (GS) and GOGAT. In this cycle, GS (glnA) is expressed in the absence of glutamine and functions to convert glutamate and ammonium into glutamine (42). GOGAT, encoded by gltAB, produces two glutamate molecules from glutamine and 2-oxoglutarate via reductive amination. In this reaction, glutamine provides an amide group to 2-oxoglutarate, resulting in the synthesis of glutamate. GOGAT is only synthesized in the presence of its substrates, 2-oxoglutarate and glutamine (12, 25, 27). S. aureus encodes both GS and GOGAT genes, however, few studies have been performed to elucidate how glutamate/glutamine metabolism functions in S. aureus.

Proline.

In B. subtilis, proline can be utilized as a sole source of carbon or nitrogen through its oxidation into glutamate via the putBCP operon (43). Proline dehydrogenase (PutB) catalyzes the conversion of proline into Δ¹-pyrroline-5-carboxylate (P5C), which is then spontaneously hydrolyzed to γ-glutamate-5-semialdehyde. The NAD-dependent Δ¹-pyrroline-5-carboxylate dehydrogenase (PutC) then oxidizes γ-glutamate-5-semialdehyde further into glutamate. The high affinity proline transporter, PutP, is well suited for scavenging proline from scarce nutritional environments (43-45). The expression of the putBCP operon is dependent on the proline-responsive activator protein, PutR. PutR requires only submillimolar concentrations of proline for induction of putBCP (43, 46). CodY, a global transcriptional regulator of metabolic genes, represses putBCP transcription by displacing PutR (46). The presence of proline dehydrogenase
(putA), $\Delta^{1}$-pyrrole-5-carboxylate dehydrogenase (rocA), and putP in the *S. aureus* genome indicates the ability to transport and catabolize proline. However, unlike *B. subtilis*, these genes are not found in an operon and there does not appear to be an orthologous putR gene present within the genome.

In *S. aureus*, proline can also serve as a substrate for arginine biosynthesis when grown on non-preferred carbon sources. It has been known since the 1930’s that most strains of *S. aureus* exhibit multiple amino acid auxotrophies when grown in media containing glucose, including arginine and proline (39, 47). However, genomic analyses have shown that the *S. aureus* genome contains genes necessary for the synthesis of all 20 amino acids (40). It has been recently understood that some of these pathways, such as the interconversion of proline and arginine, are repressed by CcpA and are only induced when growing on non-preferred carbon sources (5, 37, 41). Nuxoll and colleagues demonstrated that alleviation of CcpA repression mediates arginine biosynthesis via proline degradation by proline dehydrogenase and the urea cycle. Moreover, *S. aureus* grown in the presence of $^{13}$C-proline yields accumulation of $^{13}$C-arginine (41).

**Arginine.**

Arginine catabolism has been extensively studied as many bacteria contain more than one arginine catabolic pathway (38). *B. subtilis* can utilize arginine as a sole nitrogen and carbon source through its conversion to glutamate via the arginase pathway, generating two molecules of glutamate per one molecule of arginine. The arginase pathway is encoded by two operons,
rocABC and rocDEF, and converts arginine into glutamate in three enzymatic steps. First, arginine is cleaved by arginase, encoded by rocF, into ornithine. Ornithine is converted to γ-glutamate-5-semialdehyde via ornithine aminotransferase (RocD). In the last step of the arginase pathway, the conversion of γ-glutamate-5-semialdehyde into glutamate is catalyzed by RocA. While the protein encoded by rocB is of unknown function, rocC and rocE are hypothesized to encode amino acid permeases (32, 38, 48). Induction of these operons requires the positive transcriptional regulator RocR and the presence of arginine, ornithine, citrulline, or proline. A second regulatory protein, AhrC, also controls the expression of the arginase pathway. In the presence of arginine, AhrC acts as a positive regulator of rocF and rocD transcription, while repressing the arginine biosynthetic arg operon (48). S. aureus encodes all the genes necessary to convert arginine to glutamate via the arginase pathway. As previously mentioned, the S. aureus genome does not appear to encode an orthologous rocR gene. However, it does encode an ahrC gene, though to date its function in the regulation of arginine catabolism has not been demonstrated.

As previously discussed, S. aureus is an auxotroph for arginine and proline. However, the interconversion of arginine and proline can be mediated with the alleviation of CcpA repression. It has been demonstrated by Li et al. that S. aureus can utilize arginine as a substrate for proline synthesis in the absence of glucose via the Δ¹-pyrroline-5-carboxylate reductase pathway, which utilizes RocF and RocD, along with Δ¹-pyrroline-5-carboxylate reductase (ProC). Furthermore, it was demonstrated that when grown in the presence of
radiolabeled arginine, *S. aureus* accumulates radiolabeled proline, indicating arginine is serving as a precursor to proline biosynthesis (5, 37).

Arginine can also be catabolized via the highly conserved arginine deiminase (ADI) pathway. ADI catalyzes the conversion of arginine into ornithine, ammonia, and carbon dioxide, as well as the production of 1 mol of ATP per 1 mol of arginine consumed. ADI is comprised of three enzymes: arginine deiminase (ArcA), ornithine carbamoyl transferase (ArcB) and carbamate kinase (ArcC), which are encoded by the *arc* operon. The *arc* operon also encodes an arginine/ornithine antiporter (ArcD) and two regulatory proteins, ArgR and ArcR, which are dependent on arginine availability and anaerobic conditions respectively (38, 49). Recently, increased attention has been drawn to the ADI pathway in endemic *S. aureus* USA300 strains due to the acquisition of a second copy of the *arc* operon found on the arginine catabolic mobile element (ACME), which is hypothesized to contribute to the success of this particular methicillin-resistant community associated *S. aureus* (CA-MRSA) strain in colonizing acidic environments such as the skin (50, 51).

Transcription of the *arc* operon is subject to CcpA repression and is often arginine dependent via the ArgR family of transcriptional regulators, ArgR and AhrC (52-55). Expression of the *arc* operon is also induced under anaerobic growth conditions and dependent upon ArcR, which is also encoded within the *arc* operon (49). In lactic acid bacteria it has been demonstrated that ADI functions to mediate pH homeostasis and survival under low-pH stress via ammonia synthesis (55, 56). In *S. aureus* USA300, it has been demonstrated
that the acquisition of ACME has increased the pathogen’s ability to colonize the skin, partially due to the contributions of ADI to pH homeostasis (51). Furthermore, ammonia from ADI in *Staphylococcus epidermidis* contributes to biofilm development, as well as pH homeostasis in the presence of organic acids (57). The ATP generated via ADI also has the ability to provide *S. aureus* with energy in nutrient limiting conditions, thus making ADI a critical component for successful host colonization.

Arginine serves as a substrate for NO synthesis, a highly reactive free radical gas, which is produced via the NO synthase enzymes (NOS). NOS is well characterized in mammals and involved in a wide variety of cellular processes, including protection against pathogens (58). A number of bacterial NOS enzymes have also been identified in gram-positive species, including *S. aureus*, with diverse functions in virulence, biofilm development, toxin synthesis, and antibiotic resistance (59-61). Currently in *S. aureus*, there is evidence that NOS contributes to hydrogen peroxide resistance and may be involved in staphyloxanthin production (62). Overall, arginine is involved in several metabolic pathways important for survival within the host, including pH homeostasis, energy production, and carbon and nitrogen metabolism, all contributing to the virulence and success of the pathogen.

*Histidine.*

The histidine utilization (Hut) system is a widely distributed pathway amongst bacteria in which histidine is degraded into glutamate. The first three steps in this pathway appear to be universal, in which histidase (HutH) removes
ammonia from histidine, yielding urocanate. The hydration of urocanate to imidazolone propionate (IP) is catalyzed by urocanase (HutU), followed by the ring cleavage of IP by IP hydrolase (HutI) to yield formiminoglutamate (FIG). Two pathways can utilize FIG; one generates glutamate in a two-step reaction via FIG deiminase and formyl glutamate hydrolase (FGase) (63, 64). Another pathway, found in B. subtilis, involves the hydrolysis of FIG to glutamate by FIG hydrolase (HutG) (65).

Histidine synthesis requires an input of 20 high-energy phosphate bonds, thus being one of the most expensive amino acids for the cell to synthesize (66). Therefore, it is of no surprise that its degradation is stringently regulated. The regulation of the hut pathway has been studied extensively in several bacteria including Klebsiella pneumoniae, several Pseudomonas species, and B. subtilis. The details of the regulatory mechanisms controlling histidine utilization differ considerably among these bacteria; however, most species share several common features including the following: (i) Exogenous histidine must be present at higher concentrations than internal pools generated by histidine synthesis for expression of hut genes. (ii) Only if cells are limited in some essential requirement that degradation of histidine can fulfill, such as a carbon source, will the Hut enzymes be maximally expressed. (iii) The affinities of the histidine degradative enzymes and the tRNA synthetases for histidine are such that internal pools of histidine are not depleted below levels that would halt protein synthesis (67).
In *B. subtilis*, three proteins (HutP, CcpA, and CodY), acting at three different sites within the operon, regulate *hut* transcription. The *hutP* gene, located just upstream of *hutHUIG*, encodes an antiterminator protein that binds to a termination stem-loop structure located between *hutP* and the enzyme-encoding genes of the operon in the presence of histidine, allowing for read through of the termination signal (68, 69). The *hut* operon is also strongly repressed by availability of carbon sources via CcpA. In the presence of glucose, CcpA binds to catabolite responsive elements (*cre*) located within the *hut* promoter and the *hutP* coding sequence, exerting repression of the *hut* operon (70-72). Transcription of *hut* genes are also strongly repressed when grown in the presence of amino acids by CodY binding to an operator site (*hutO_A*) just downstream from the *hut* promotor (69). To date, there is little knowledge of histidine utilization in *S. aureus* or the regulation thereof.

**Oxaloacetate generating amino acids:**

Oxaloacetate is a TCA cycle intermediate that provides a link between gluconeogenesis/glycolysis, the TCA cycle, and amino acid metabolism. Oxaloacetate can be utilized in several reactions that direct the carbon flux within central metabolism to ensure efficient use of available carbon sources. The synthesis of citrate, which is catalyzed by citrate synthesis (*GltA*) via the substrates oxaloacetate and acetyl-CoA, is essential for full TCA cycle activity and generation of energy via respiration. However, in the absence of glycolytic sugars oxaloacetate can fuel gluconeogenesis through phosphoenolpyruvate carboxykinase (PckA) activity, which catalyzes the GTP-dependent formation of
phosphoenolpyruvate (PEP) from oxaloacetate (73-75). In *B. subtilis*, mutants lacking PckA cannot grow on non-glycolytic carbon sources, as they are unable to synthesize glycolytic intermediates necessary for growth and replication, such as precursors for peptidoglycan synthesis (75).

Oxaloacetate occupies a key position in central metabolism in which it can be utilized as a substrate in several different pathways depending on nutrient availability. Therefore, it is of no surprise that multiple pathways also exist to fuel its synthesis. Oxaloacetate, the beginning metabolite of gluconeogenesis, can be synthesized from pyruvate via pyruvate carboxylase (Pyc), a reaction utilized to replenish TCA cycle intermediates. Pyc is constitutively expressed and subject to strong allosteric activation by acetyl-CoA (75). Asparagine and aspartate are also able to fuel oxaloacetate synthesis via the hydrolysis of asparagine to aspartate by the enzyme, asparaginase (AnsA). Aspartate can then be converted to oxaloacetate by aspartate aminotransferase (AspB), which catalyzes a transamination reaction via the conversion of aspartate and 2-oxoglutarate to oxaloacetate and glutamate. It is of importance to note that the aspartate provides the carbon backbone for oxaloacetate in this reaction. This reaction is also reversible, allowing for oxaloacetate to fuel aspartate and asparagine synthesis if needed (76). When carbon availability is scarce, *B. subtilis* is able to fuel oxaloacetate synthesis and subsequent gluconeogenic reactions via utilization of aspartate/asparagine, a process that is hypothesized to be involved in sporulation (74, 75). *S. aureus* also has the ability to convert both asparagine and aspartate to oxaloacetate via the asparaginase (AnsA) and
aspartate aminotransferase (AspA) enzymes, however, the contribution of these amino acids to central metabolism remains unknown.

Pyruvate generating amino acids:

Pyruvate is a versatile metabolic intermediate, generated from the breakdown of glucose through glycolysis. Pyruvate can be utilized to generate acetyl-CoA, which either enters the TCA cycle or is converted to acetate, both pathways generating ATP (77). It can also be converted to acetoin during carbon overflow metabolism to avoid over-production of potentially harmful weak-acids (78). During anaerobic fermentation, pyruvate is converted to lactate for the regeneration of NAD$^+$ and redox balance. As previously discussed, pyruvate can also be used as an acceptor of carbon dioxide to replenish oxaloacetate levels and the TCA cycle via Pyc (75, 77). The synthesis of several metabolites, including amino acids, also require pyruvate as a precursor (76).

When carbon availability is limited, several amino acids can fuel pyruvate synthesis. Both *B. subtilis* and *S. aureus* have the ability to directly convert serine and alanine to pyruvate via serine dehydratase (SdaAB) and alanine dehydrogenase (Ald), respectively. Furthermore, threonine and glycine can be converted to serine and subsequently pyruvate via SdaAB, while cysteine can be converted to alanine and then pyruvate via Ald. While little is known about the function of serine utilization in *B. subtilis* metabolism, it has been demonstrated alanine can be utilized as a sole carbon or nitrogen source through its conversion to pyruvate and ammonia via Ald. It has also been demonstrated that Ald is necessary for normal sporulation in *B. subtilis* (79, 80). It is unclear, however,
how these amino acids contribute to central metabolism in *S. aureus* when carbon sources are limited.

The work described in this dissertation is designed to gain a better understanding of the pathways involved in amino acid catabolism in *S. aureus*, as utilization of these pathways may be critical during host colonization and infection. Knowledge of these metabolic networks may help gain insight into improved therapeutic strategies for the treatment of *S. aureus* infections.
Chapter 2

Methods and Materials
Bacterial strains and culture conditions.

The *S. aureus* strains used in this study are all derived from JE2, which is the parent strain of the Nebraska Transposon Mutant Library (NTML) (81). Defined *bursa aurealis* transposon mutants were obtained from the NTML (81). JE2 *ccpA* and JE2 *ackA* have been previously described (41, 82, 83). *S. aureus* strains were grown in either tryptic soy broth (TSB; Becton Dickinson, Franklin Lakes, NJ), complete defined medium (CDM) as previously described (84) except no glucose was added, or CDM with 0.25% glucose (CDMG). Cultures were grown aerobically in a 1:10 volume to flask ratio, shaking at 250 rpm at 37°C. *S. aureus* cultures were inoculated to 0.05 optical density at 600 nm (OD$_{600}$) from overnight cultures grown in either TSB, CDM or CDMG. Bacterial growth was assessed by measuring the optical density at 600 nm. Anaerobic growth was measured in an anaerobic chamber (Coy Laboratory Products, Inc., Grass Lakes, MI) using a 10:1 flask-to-volume ratio in CDM supplemented with 1.0 g/liter L-cysteine hydrochloride (Sigma) with or without the addition of L-lactate (0.5%) and/or sodium nitrate (20 mM). Growth was assessed in the anaerobic chamber with shaking at 37°C at 250 rpm to ensure that the cells did not settle in the flask. For time-kill curves, 100 µM of *N,N*-dicyclohexylcarbodiimide (DCCD) in 95% ethanol was added to cultures during early exponential growth (OD$_{600}$=0.5 for CDM and 1.0 for TSB and CDMG).

Construction of JE2 ΔahrC mutant
All primers (TABLE) used for construction and confirmation of the ahrC deletion were generated based on the sequence of S. aureus USA300_FPR3757 (NC_007793.1). pNF293, the JE2 ΔahrC allelic replacement construct, was first created by insertion of an 861-bp ahrC upstream PCR product (using primers 1999 and 2000; TABLE) into the EcoR1 and BAMH1 sites of pUC19 (85). Second, a 755-bp ahrC downstream PCR product was amplified (using primers 2211 and 2212; TABLE) and ligated into the BamH1 and Pst1 sites of the pUC19 polylinker. The temperature-sensitive pE194 derivative pROJ6448 was ligated into the PstI site of the plasmid (86). The completed construct, pNF293, was electroporated into the RN4220 restriction-negative S. aureus strain (87). Modified plasmid DNA was isolated using Wizard Plus MidiPrep kits (Promega, Madison, WI) and transduced into S. aureus JE2 using phage 80α as previously described (88). Allelic replacement was performed using previously described methods (89). Genetic sequencing (Eurofins Genomics) using primers 2944 and 2945 confirmed in-frame deletion of ahrC in JE2 ΔahrC.

**Construction of JE2 arcA1::kan arcA2::NΣ and JE2 rocF::tetL arcA1::kan arcA2::NΣ mutants**

The pKan plasmid (89) was transduced (88) into transposon mutant JE2 arcA1::NΣ and allelic replacement was performed using previously described methods (89) to construct JE2 arcA1::kan and confirmed by PCR and southern blot analyses. JE2 arcA1::kan was transduced (88) into JE2 arcA2::NΣ to construct JE2 arcA1::kan arcA2::NΣ. To construct JE2 rocF::tetL arcA1::kan arcA2::NΣ mutants, pTet plasmid (89) was transduced (88) into the transposon
mutant JE2 rocF::NS and allelic replacement was performed using previously described methods (89) to construct JE2 rocF::tet. JE2 rocF::tet was transduced (88) into JE2 arcA1::kan arcA2::NS to construct JE2 rocF::tet arcA1::kan arcA2::NS.

**Measurement of bacterial metabolites.** Bacterial cultures were collected (1 ml aliquots) and centrifuged at 14,000 rpm for 3 min. The supernatants were removed and stored at -80°C until use. Acetic acid, glucose, ammonia, and D- and L-lactic acid concentrations were determined using kits purchased from R-Biopharm, according to manufacturer instructions.

**Measurement of TCA cycle and respiration activity.** For all assays, JE2 was grown overnight aerobically in 25 ml of appropriate media. Cultures were inoculated to an OD<sub>600</sub> of 0.05 in TSB, CDM, or CDMG and were grown to early exponential phase (OD<sub>600</sub>=0.5 for CDM and 1.0 for TSB and CDMG). These data were analyzed using an unpaired t-test. A P value of ≤0.05 was considered statistically significant.

**ATP concentration:** Intracellular ATP concentrations were determined using the BacTiter-Glo kit (Promega) according to the manufacturer’s protocol and normalized to total optical units.

**NAD<sup>+</sup>/NADH concentration:** Aliquots of bacterial cultures (24 ml) were harvested by centrifugation at 4°C for 10 min at 4,000 rpm. Intracellular NAD<sup>+</sup> and NADH concentrations were determined using Fluoro NAD<sup>+</sup>/NADH kit (Cell Technology) as previously described (82) and normalized to total cellular protein.
concentrations.

**Oxygen consumption**: Samples were collected and diluted in air-saturated TSB, CDM, or CDMG to an OD$_{600}$ of 0.3. Relative oxygen consumption rates were determined for up to 120 min at 37°C by using a MitoXpress oxygen-sensitive probe (Luxcel Biosciences) according to the manufacturer’s instructions and normalized to total optical units.

**Citrate concentrations**: Aliquots of bacterial cultures (24 ml) were harvested by centrifugation at 4°C for 10 min at 4,000 rpm. The bacterial pellets were washed twice with 1 ml of phosphate-buffered saline, pH 7.4 (PBS) and subsequently resuspended in 1 ml PBS. The cells were lysed using lysing matrix B tubes in a FastPrep FP120 instrument (Qbiogene). The lysates were centrifuged for 3 min at 14,000 rpm and deproteinized using perchloric acid/KOH. Citrate concentrations in the lysates were determined by using a Citrate fluorometric assay kit (BioVision) according to the manufactures protocol and normalized to total cellular protein concentrations.

**Amino acid analysis.** JE2 was grown overnight in 50 ml (500 ml flask) of TSB or CDMG. Cultures were inoculated to an OD$_{600}$ of 0.05 in TSB (from TSB overnight), CDM, CDMG, and CDMP (all from CDMG overnight) and grown for 12 hours. 1 ml of media was collected every 4 hours and centrifuged for 3 min at 14,000 rpm. Supernatant was collected and filterd through 3,000 MWCO Amicon Ultra centrifugal filters (Millipore) according to the manufacturer’s protocol.
Amino acid analysis was performed by the Protein Structure Core Facility, UNMC, using a Hitachi L-8800.

**NMR sample preparation.** Three independent 50-ml cultures of *S. aureus* JE2 were grown to exponential phase (4hr-6hr) and stationary phase (8hr-10hr) in CDM, CDMG, and CDMP containing either $^{13}\text{C}_5$-glutamate, $^{13}\text{C}_5$-proline, $^{13}\text{C}_5$-arginine, $^{13}\text{C}_5$-glucose, or $^{13}\text{C}_5$-pyrurate (Sigma-Aldrich). Cultures were normalized to an OD$_{600}$ of 40 and pelleted by centrifugation (6000 rpm at 4°C for 5 min). Pelleted cells were subsequently washed two times with 10 ml of cold sterile water and resuspended in 1 ml cold sterile water and transferred to a lysing matrix B tube (MP Biomedicals). The cells were lysed using an FP120 FastPrep cell disrupter (MP Biomedicals) and centrifuged at 13,200 rpm for 15 min at 4°C. The pelleted debris was resuspended in 1 ml of cold sterile water centrifuged again as above. Supernatants from the two centrifugation steps were combined and stored at -80°C for NMR analysis.

**NMR data collection and analysis.** The collected supernatants were lyophilized and reconstructed in NMR buffer (KH$_2$PO$_4$/K$_2$HPO$_4$ buffer in D$_2$O, pH 7.4, with 500 uM TMSP as an internal standard). 2D 1H-13C HSQC spectra were collected on a Bruker DRX Avance 500-MHz spectrometer equipped with a 5-mm triple-resonance cryoprobe (1H, 13C, and 15N) with a z-axis gradient, a BACS-120 sample changer, Bruker ICON-NMR, and an automatic tuning and matching (ATM) unit. NMRPipe (28) and NMRViewJ (29) were used to process and analyze collected spectra. After being referenced in chemical shift and normalized in intensity to the internal standard TMSP, peaks were annotated by
comparing their chemical shifts to the metabolite reference data from the Platform for RIKEN Metabolomics (PRIMe, http://prime.psc.riken.jp/) (30), Human Metabolome Database (HMDB, http://www.hmdb.ca/) (31), Madison Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/) (32), Metabominer (http://wishart.biology.ualberta.ca/metabominer/) (33), and BiomagResBank (BMRB, http://www.bmrb.wisc.edu/) (34) with an error tolerance of 0.08 ppm and 0.25 ppm for $^1$H and $^{13}$C chemical shifts, respectively. The intensity of each metabolite was calculated by averaging the intensities of peaks, which were unambiguously assigned to the metabolite.

**RNA isolation and northern blot analysis.** Cultures of *S. aureus* JE2 and JE2 ccpA were grown overnight in TSB, CDM and CDMG and inoculated to an OD$_{600}$ of 0.05 into TSB (TSB overnight), CDM, CDMG, respectively. Cultures were grown at 37°C in a 1:10 volume to flask ratio at 250 rpm. 15 ml of bacterial culture were collected at mid-exponential phase (3hr-6hr) and post-exponential phase (7hr-10hr) of growth and centrifuged at 5,000 rpm for 10 min at 4°C. Cell pellets were resuspended in RLT buffer with 1% β-mercaptoethanol and transferred to lysing matrix B tubes (MP Biomedicals). They were then processed in an FP120 FastPrep cell disrupter (MP Biomedicals) for 24 s at a setting of 6.0. Cells were then pelleted at 13,000 rpm for 15 min at 4°C. The top-phase was combined with 500 µL of ethanol and the samples were then processed using an RNeasy mini kit, according to manufacturer’s instructions (Qiagen, Inc.) Primers listed in Table S2 were used to make DNA probes that were subsequently labeled with digoxigenin-labeled dUTP (Roche). 1 µg of RNA
was used for northern analysis that was performed using DIG buffers and washes (Roche). Anti-Digoxigenin-AP Fab fragments (Roche) was used with ECF substrate (GE Healthcare) for detection. Blots were visualized using the Typhoon FLS 7000 imaging system (GE Healthcare).
Table 2.1 Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial Strain or Plasmid</th>
<th>Relevant Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pUC19</td>
<td>Gram-negative origin of replication, Amp⁺</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pROJ6448</td>
<td>pE194 containing pC221 nick site functioning in conjugative mobilization, temp sensitive gram-positive origin of replication, Erm⁺</td>
<td>(86)</td>
</tr>
<tr>
<td>pNF293</td>
<td>ahrC allelic replacement vector for makerless, in-frame ahrC mutant; Amp⁺, Erm⁺</td>
<td>This study</td>
</tr>
<tr>
<td>E. coli DH5α</td>
<td>Plasmid cloning host and Gram-negative replicon</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>S. aureus RN4220</td>
<td>Restriction deficient NCTC8325-4</td>
<td>(90)</td>
</tr>
<tr>
<td>S. aureus JE2</td>
<td>LAC cured of all 3 native plasmids</td>
<td>(91)</td>
</tr>
<tr>
<td>S. aureus JE2 ald::ΝΣ</td>
<td>bursa aurealis mutation in alanine dehydrogenase</td>
<td>This study</td>
</tr>
<tr>
<td>S. aureus JE2 sdaAA::ΝΣ</td>
<td>bursa aurealis mutation in serine dehydratase, alpha subunit</td>
<td>This study</td>
</tr>
<tr>
<td>S. aureus JE2 aspA::NΣ</td>
<td><em>bursa aurealis</em> mutation in aspartate amino transferase</td>
<td>This study</td>
</tr>
<tr>
<td>------------------------</td>
<td>--------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>S. aureus JE2 ansA::NΣ</td>
<td><em>bursa aurealis</em> mutation in asparaginase</td>
<td>This study</td>
</tr>
<tr>
<td>S. aureus JE2 rocF::NΣ</td>
<td><em>bursa aurealis</em> mutation in arginase</td>
<td>This study</td>
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<tr>
<td>S. aureus JE2 putA::NΣ</td>
<td><em>bursa aurealis</em> mutation in proline dehydrogenase</td>
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<tr>
<td>S. aureus JE2 gudB::NΣ</td>
<td><em>bursa aurealis</em> mutation in glutamate dehydrogenase</td>
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<tr>
<td>S. aureus JE2 hutU::NΣ</td>
<td><em>bursa aurealis</em> mutation in urocanate hydratase</td>
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<td>S. aureus JE2 sucA::NΣ</td>
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<td>This study</td>
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<tr>
<td>S. aureus JE2 succC::NΣ</td>
<td><em>bursa aurealis</em> mutation in succinyl-CoA synthetase, beta subunit</td>
<td>This study</td>
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<td>S. aureus JE2 sdhA::NΣ</td>
<td><em>bursa aurealis</em> mutation in succinate dehydrogenase, flavoprotein subunit</td>
<td>This study</td>
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<td>S. aureus JE2 fumC::NΣ</td>
<td><em>bursa aurealis</em> mutation in fumarate hydratase</td>
<td>This study</td>
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<td>S. aureus JE2 mgo1::NΣ</td>
<td><em>bursa aurealis</em> mutation in malate quinone oxidoreductase</td>
<td>This study</td>
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<td>S. aureus JE2 pckA::NΣ</td>
<td><em>bursa aurealis</em> mutation in phosphoenol pyruvate carboxykinase</td>
<td>This study</td>
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<tr>
<td>S. aureus JE2 ΔackA::ermB</td>
<td>Allelic exchange mutant in ackA; Erm’</td>
<td>(82)</td>
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<tr>
<td>S. aureus JE2  pyc::NΣ</td>
<td>bursa aurealis mutation in pyruvate carboxylase</td>
<td>This study</td>
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<tr>
<td>-----------------------</td>
<td>------------------------------------------------</td>
<td>-----------</td>
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<tr>
<td>S. aureus JE2  arcA1::kan arcA2::NΣ</td>
<td>bursa aurealis mutations in arcA1 and arcA2; TetR, ErmR, KanR</td>
<td>This study</td>
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<td>S. aureus JE2  rocF::tet arcA1::kan arcA2::NΣ</td>
<td>bursa aurealis mutations in rocF, arcA1, and arcA2; ErmR, KanR</td>
<td>This study</td>
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<td>S. aureus JE2  gltA::NΣ</td>
<td>bursa aurealis mutation in citrate synthase</td>
<td>This study</td>
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<td>S. aureus JE2  acnA::NΣ</td>
<td>bursa aurealis mutation in aconitase</td>
<td>This study</td>
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<td>S. aureus JE2  icd::NΣ</td>
<td>bursa aurealis mutation in isocitrate dehydrogenase</td>
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<td>S. aureus JE2  gltB::NΣ</td>
<td>bursa aurealis mutation in glutamate synthase, large subunit</td>
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<td>bursa aurealis mutation in ornithine amino transferase</td>
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<td>S. aureus JE2  rocA::NΣ</td>
<td>bursa aurealis mutation in Δ¹-pyrroline-5-carboxylate dehydrogenase</td>
<td>This study</td>
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<tr>
<td></td>
<td>S. aureus JE2 ΔahrC</td>
<td>Markerless deletion in SAUS200_1469, arginine repressor</td>
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</tr>
<tr>
<td>pKAN</td>
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<td>pTnT with <em>aphA</em>-3</td>
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<tr>
<td>pTET</td>
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<td>pTnT with <em>tet</em>(M)</td>
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Table 2.2 Oligonucleotides used in this study

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<td>2838</td>
<td>GCTTCATCAACATGCGGTAA</td>
<td>Forward primer to amplify putA</td>
</tr>
<tr>
<td>2839</td>
<td>ATGATGCGATGGTCATGTGT</td>
<td>Reverse primer to amplify putA</td>
</tr>
<tr>
<td>2840</td>
<td>TTCCACCCAGATGGTGGATGA</td>
<td>Forward primer to amplify gudB</td>
</tr>
<tr>
<td>2841</td>
<td>GGCATTACCAGAAACCTTGAA</td>
<td>Reverse primer to amplify gudB</td>
</tr>
<tr>
<td>2932</td>
<td>GAAGCAGTGGGTGATGCAGC</td>
<td>Forward primer to amplify rocA</td>
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*Restriction site denoted in italics*
Chapter 3

Amino acid catabolism in *Staphylococcus aureus*
Introduction

*S. aureus* has the ability to establish infections in a wide variety of metabolic niches within the human host including, among others, the heart, bone, kidney, blood, and soft tissue. Significant advances have been made in the study of bacterial virulence factors and their functions in human disease and infection of these various organ systems. However, we have only begun to study how particular carbon and nitrogen sources that are available to *S. aureus* in specific host niches affect virulence factor expression and subsequent invasion, metastasis or quiescence. As these metabolic pathways are required for bacterial proliferation within the host, knowledge of these pathways may reveal novel avenues for drug development.

A critical component of the innate immune response is the synthesis of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) within activated phagocytes (92). Nitric oxide has wide ranging effects on bacterial cells including the disruption of lipid biosynthesis, central metabolism, respiration and DNA replication (93). Data from an evolving model demonstrates that, in contrast to other staphylococcal species, *S. aureus* has the ability to grow under NO stress by fermenting glucose via lactate dehydrogenase generating lactate and facilitating NAD\(^+\) regeneration to maintain glycolytic flux and redox balance (3, 10, 11, 94, 95). Under NO stress conditions, glycolysis is an essential process as a mutation in the glycolytic enzyme pyruvate kinase (*pyk*) severely inhibits initiation of abscess formation in a skin and soft tissue murine model (95). Indeed, inhibitors of pyruvate kinase have proved promising against *S. aureus* and other gram-positive pathogens (96-99).

Following invasion of host tissue, *S. aureus* replicates in abscess communities that are encapsulated within fibrin deposits and infiltrating immune cells (100). Much
recent work has led to an overarching view of abscess formation including the definition of essential virulence factors such as coagulase, Von Willebrand factor, ClfA, FnbpA, and FnbpB (101). As the abscess matures within the fibrin wall, the center includes a large cluster of viable cells called the staphylococcal abscess community (100, 102). It is hypothesized that these niches have low concentrations of NO allowing for respiratory activity (3, 103); however, it is predicted that S. aureus must grow on non-preferred carbon sources as glucose is not abundant (10, 104). Indeed, S. aureus lacks the ability to utilize phospholipids, triglycerides, and potentially other short chain fatty acids as carbon sources due to the lack of both β-oxidation and glyoxylate shunt pathways. Therefore, other carbon sources that are available include lactate, excreted from S. aureus during fermentative growth, as well as peptides and free amino acids. Recent work from Spahich and colleagues found that the addition of lactate plus peptides supports growth of S. aureus under low NO stress (10). Growth under these conditions was dependent upon lactate quinone oxidoreductase (lqo), which assimilates lactate in an NAD-independent manner and requires an electron acceptor such as oxygen or nitrate (11). Importantly, growth under these conditions also required peptides to serve as a carbon source to facilitate gluconeogenesis.

To date, little is known about amino acid catabolism in S. aureus; however, bioinformatics analysis suggests that pathways are present to catabolize multiple amino acids including those that generate pyruvate, 2-oxoglutarate, and oxaloacetate (Table 1.1). Although it is unclear in which microniches amino acid/peptide catabolism is important, it has been presumed that S. aureus utilizes peptides/amine acids as carbon sources as it encodes multiple oligopeptide permeases and free amino acid transporters (6-8). In addition, S. aureus encodes metallo, serine and papain-like cysteine proteases that have the ability to degrade host proteins (9). We have recently demonstrated that at
least some of these amino acid catabolic pathways, such as those linking arginine and proline, are repressed by carbon catabolite repression (via CcpA) and are only induced when S. aureus is growing on non-preferred carbon sources (5, 37, 41). Thus, a S. aureus ccpA mutant interconverts arginine and proline via an intact urea cycle (5, 37, 41).

In this study, we utilized genetic and NMR metabolomics approaches to investigate amino acid catabolism in S. aureus USA300 JE2. We found that glutamate catabolism via GudB, which functions to replenish the TCA cycle via 2-oxoglutarate, is required for growth in a glucose-free medium. In addition, ATP generated from the Pta/AckA pathway is also required for growth, presumably due to a significantly reduced respiration rate. These data provide a model suggesting that certain amino acid catabolic pathways are essential for growth in niches where glucose is limiting.

**Results**

**Growth of S. aureus in Complete Defined Medium lacking glucose (CDM).**

Staphylococcal growth and central metabolism have been well studied in tryptic soy broth (TSB), which contains 14 mM glucose and an ill-defined concentration of peptides and free amino acids (1). Therefore, to fully assess amino acid catabolism, a complete defined medium was prepared as described by Hussain and colleagues except that no glucose was added (84). This medium contains 18 amino acids but excludes glutamine and asparagine. Aerobic growth of JE2 in CDM reached an OD\textsubscript{600} maximum of 3.0 following 8 hours (Figure 3.1). 2.3 mM acetate was produced by 6 hours of growth (Figure 3.1) indicating catabolism of the glucogenic amino acids yielding pyruvate and the generation of acetate via Pta/AckA (82, 105). Acetate was subsequently consumed from the medium as a secondary carbon source and presumably oxidized via the TCA
Figure 3.1. Growth of *S. aureus* JE2 in CDM. Aerobic growth of JE2 in CDM displaying acetate and ammonia production. Data are representative of experiments performed three times.
cycle (Figure 3.1) (106). Amino acid analysis of the spent medium demonstrated that alanine, serine, glycine, threonine, arginine, proline, glutamate, and aspartate were rapidly consumed by 8 hours of growth, whereas histidine was not consumed until 12 hours of growth (Figure 3.2). Consistent with these data was the generation of ammonia, which reached a maximum concentration of 4.3 mM at 6 hours of growth (Figure 3.1) and is indicative of amino acid catabolism (57). There was a gradual consumption of other amino acids (cysteine, lysine, phenylalanine, tyrosine, leucine, isoleucine, valine, and methionine) indicating probable use for protein synthesis but not for catabolic purposes (Figure 3.2). Based on bioinformatics analyses, S. aureus JE2 encodes genes for enzymes that function to catabolize amino acids to the metabolic intermediates pyruvate (from alanine, serine, glycine, threonine, and cysteine), 2-oxoglutarate (from glutamate, glutamine, histidine, arginine, and proline), and oxaloacetate (from aspartate and asparagine) (Table 1.1). Complete catabolic pathways could not be identified for tryptophan, isoleucine, leucine, lysine, methionine, phenylalanine, tyrosine, and valine. Outside of cysteine, which was not catabolized by JE2 in CDM, these analyses agreed with our experimental observations as detailed in Figure 3.2. To determine which catabolic pathways are required for growth in CDM, mutants from each predicted pathway (Table 1.1: ald, alanine; sdaAA, serine, glycine, and threonine; aspA, aspartate; ansA, asparagine; rocF, arginine; putA, proline; gudB, glutamate; and hutU, histidine) were chosen from a sequence defined transposon library (81) and growth analysis was performed (Figure 3.3 and Figure 3.4A-H). These data demonstrated that of these genes mutated, only gudB (Figure 3.4G), which encodes glutamate dehydrogenase and catalyzes the reaction between glutamate and 2-oxoglutarate, was required for growth in CDM. In addition, a mutation within, hutU yielded a markedly increased lag phase (Figure 3.4H) whereas a mutation in putA (Figure 3.4F) resulted in a growth yield defect.
Figure 3.2. Amino acid consumption of JE2 following growth in CDM. JE2 was grown aerobically in CDM and amino acid concentrations (mM) were measured in the supernatant at 0, 4, 8 and 12 hours of growth.
Figure 3.3. Amino acid catabolic pathways in S. aureus. Green arrows indicate those mutations that result in no significant growth defect as compared to wild type JE2 in CDM. Blue arrows indicate those mutations that result in intermediate growth defects as compared to wild type JE2 in CDM. Red arrows indicate mutations that completely inhibit growth of JE2 in CDM. Black arrows indicate that these pathways were not tested. Please see Figure 3.4 for growth analyses.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37° C) of JE2 bursa aurealis mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37° C) of JE2 bursa aurealis mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37° C) of JE2 _bursa aurealis_ mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37° C) of JE2 bursa aurealis mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37° C) of JE2 *bursa aurealis* mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Figure 3.4. Growth of JE2 and transposon mutants in CDM. Aerobic growth (250 rpm, 1:10 volume to flask ratio, 37°C) of JE2 *bursa aurealis* mutants from the Nebraska Transposon Mutant Library in CDM. Data represents means ± SEM of three biological replicates.
Collectively, these data suggest that carbon derived from glutamate and those amino acids that can be converted to glutamate, particularly proline and histidine, serve as the major carbon sources for fueling the TCA cycle and subsequent gluconeogenic reactions via phosphoenolpyruvate carboxykinase (pckA) (Figure 3.3). Based on these observations, we predicted that mutations in the TCA cycle enzymes generating oxaloacetate from 2-oxoglutarate would also be required for growth. Therefore, transposon mutants in sucA (2 oxoglutarate dehydrogenase), sucC (succinyl Co-A synthetase), sdhA (succinate dehydrogenase), fumC (fumarase), and mqo1 (malate dehydrogenase) were grown in CDM (Figure 3.4I-M). As predicted, no growth was observed with the sucA, sdhA, and fumC mutants (Figure 3.3, Figure 3.4I-L). However, growth, albeit delayed, was observed with the mqo1 mutant (Figure 3.3, Figure 3.4M). This suggests that oxaloacetate can also be supplied by aspartate/glutamate transamination via AspA. Indeed, an mqo1 mutant was unable to grow in CDM lacking aspartate (CDM-D; Figure 3.5). Lastly, as predicted, no growth was observed for the pckA mutant, which is a critical enzyme required for gluconeogenesis via the generation of phosphoenolpyruvate from oxaloacetate (Figure 3.3, Figure 3.4N). The putA (proline), rocF (arginine), hutU (histidine), and rocA (arginine and proline) transposon mutants were grown in CDM lacking glutamate (CDM-E) to determine which amino acid substrates that fuel glutamate synthesis are most crucial (Figure 3.6). As a result we found that the putA and rocA mutants had significant growth yield defects whereas mutations in rocF and hutU had only moderate growth defects compared to wildtype JE2 grown in CDM-E. Thus, these data show that proline, arginine and histidine all serve to fuel glutamate synthesis when glutamate is limiting; however, proline via PutA and RocA is the major source of glutamate.
Figure 3.5. Growth of *S. aureus* JE2 in CDM-D. Growth of JE2 and JE2 *mqo1::N* in CDM lacking aspartate (CDM-D). Data represents means ± SEM of three biological replicates.
Figure 3.6. Growth of *S. aureus* JE2 in CDM-E. Growth of JE2 putA::NΣ, JE2 rocF::NΣ, JE2 rocA::NΣ, and JE2 hutU::NΣ in CDM lacking glutamate (CDM-E) in comparison to wildtype JE2. Data represents means ± SEM of three biological replicates.
As shown in Figure 3.1, growth of JE2 in CDM produces acetate suggesting that the glucogenic amino acids generating pyruvate (serine, threonine, glycine, and alanine) are important for ATP synthesis via substrate-level phosphorylation in the Pta/AckA pathway (82). To determine the importance of this pathway, JE2 ΔackA (82) was grown in CDM and, as shown in Figure 3.4O and Figure 3.3, growth was significantly reduced suggesting that generation of ATP via this pathway may be critical to growth using amino acids as carbon sources. Lastly, we found only a minor growth defect with the pyc mutant, suggesting that pyruvate flux is directed primarily towards acetate synthesis, instead of oxaloacetate and the TCA cycle (Figure 3.4P and Figure 3.3).

NMR metabolomic analysis of amino acid catabolism in S. aureus.

The previously described experiments suggested that proline primarily serves to fuel glutamate synthesis and thus may serve as the major carbon source to fuel gluconeogenesis when glutamate is limiting. In addition, it is predicted that the glucogenic amino acids serine, threonine, glycine and alanine are primarily catabolized to acetate to fuel ATP synthesis. To address these hypotheses and to determine the fate of carbon following amino acid catabolism, $^{13}$C labeled glutamate, arginine, proline, aspartate, histidine, serine, threonine, alanine, and glycine were individually added to CDM and assayed via NMR metabolomics in mid- and post-exponential growth.

**Glutamate, proline, and arginine**

Metabolic intermediates originating from $^{13}$C labeled glutamate, proline and arginine were very similar and included gluconeogenic intermediates (glucose-6-P and phosphoglycerate), and intermediates that function in nucleotide biosynthesis (dihydroorotate, ribose, uracil and adenosyl homocysteine) and glycolipid/lipoteichoic acid synthesis (glucose 1-phosphate) (Figure 3.7 and Figure 3.8A-C) (107). In addition,
both aspartate and asparagine were detected from all three $^{13}$C labeled amino acids suggesting these amino acids were synthesized from oxaloacetate and the TCA cycle. It is important to note that glutamate was synthesized from both proline and arginine, presumably via RocA (Figure 3.3, Figure 3.7, and Figure 3.8A-C). However, as previously reported, proline and arginine were not produced from glutamate (41). Importantly, these results suggest proline and arginine can both serve as carbon sources as they both serve as precursors for glutamate, the key intermediate linking carbon and nitrogen metabolism. However, the relative amount of glutamate generated from proline was approximately five-fold greater than the amount generated from arginine (Figure 3.7, Figure 3.8B and 3.8C). And, as previously noted, a mutation in rocF had minimal effect on growth in CDM and CDM-E. It is important to note that JE2 contains two copies of arginine deiminase, which can generate citrulline and ornithine from arginine while bypassing RocF (50, 57). However, an arcA1/arcA2 arginine deiminase mutant also had no significant growth defect in CDM (Figure 3.4Q). Thus, these NMR data are consistent with our genotypic growth analysis and show that proline is rapidly catabolized to generate glutamate and facilitate subsequent gluconeogenic reactions.

Previous work demonstrated that arginine is synthesized via proline and the urea cycle in a ccpA mutant (41) but not through the well-defined glutamate to arginine pathway as described in B. subtilis and other bacteria (38). Although genes for the arg pathway are present in S. aureus, growth conditions where they are induced are not known. In a similar manner, work from others (5, 37) demonstrated that arginine served as a precursor for proline biosynthesis in a ccpA mutant via RocF, RocD, and ProC. Electrophoretic mobility shift assay (EMSA) analysis demonstrated that CcpA bound to the promoters of both rocF and rocD, but not proC (5).
Figure 3.7. Summary of cellular metabolite concentration changes resulting from uniformly labeled amino acids supplemented in CDM. Summary on following page.
Figure 3.7. Summary of cellular metabolite concentration changes resulting from uniformly labeled amino acids supplemented in CDM. The bubble plot summarizes the metabolites derived from [U-\textsuperscript{13}C\textsubscript{4}] aspartate (D), [U-\textsuperscript{13}C\textsubscript{5}] glutamate (E), [U-\textsuperscript{13}C\textsubscript{6}] proline (P), [U-\textsuperscript{13}C\textsubscript{6}] histidine (H), [U-\textsuperscript{13}C\textsubscript{6}] arginine (R), [U-\textsuperscript{13}C\textsubscript{4}] threonine (T), [U-\textsuperscript{13}C\textsubscript{3}] serine (S), [U-\textsuperscript{13}C\textsubscript{3}] alanine (A) or [U-\textsuperscript{13}C\textsubscript{2}] glycine (G) based on an analysis of triplicate 2D \textsuperscript{1}H-\textsuperscript{13}C HSQC experiments. Due to the wide dynamic range of NMR intensities, the original NMR peak intensities were transformed by natural logarithm for the purpose of visualization. The relative size of the bubble indicates the transformed value where a larger diameter indicates a higher NMR peak intensity. The bubbles are colored using a red color-gradient to represent the original NMR peak intensity, where an increase in red color intensity represents a corresponding increase in the original NMR peak intensity. Each level increase in red intensity corresponds to a tenfold increase in the NMR intensity. The bubbles with a purple outline (○) identify metabolites observed during exponential phase. Bubbles with a green outline (●) identify metabolites observed during stationary phase. Metabolite names are colored based on an assigned category: urea cycle (orange), amino acid (green), glycolysis/gluconeogenesis (black), pentose phosphate pathway/nucleosides (red), and others (blue).
Figure 3.8 NMR Peak intensity of CDM supplemented with $^{13}$C labeled amino acids. Metabolites derived from $[U-^{13}C_5]$ glutamate (E) (A), $[U-^{13}C_6]$ proline (P) (B), $[U-^{13}C_6]$ arginine (R) (C), $[U-^{13}C_6]$ histidine (H) (D), $[U-^{13}C_4]$ aspartate (D) (E), $[U-^{13}C_3]$ alanine (A) (F), $[U-^{13}C_3]$ glycine (G) (G), $[U-^{13}C_3]$ threonine (T) (H), $[U-^{13}C_3]$ serine (S) (I), based on an analysis of triplicate 2D $^1$H-$^{13}$C HSQC experiments. Samples were assayed in either early (OD$_{600}$=0.8-1.0) or late exponential (OD$_{600}$=2.0-2.2) phases of growth.
Figure 3.8  NMR Peak intensity of CDM supplemented with $^{13}$C labeled amino acids. Metabolites derived from $[\text{U-}^{13}\text{C}_5]$ glutamate (E) (A), $[\text{U-}^{13}\text{C}_6]$ proline (P) (B), $[\text{U-}^{13}\text{C}_6]$ arginine (R) (C), $[\text{U-}^{13}\text{C}_6]$ histidine (H) (D), $[\text{U-}^{13}\text{C}_4]$ aspartate (D) (E), $[\text{U-}^{13}\text{C}_3]$ alanine (A) (F), $[\text{U-}^{13}\text{C}_2]$ glycine (G) (G), $[\text{U-}^{13}\text{C}_4]$ threonine (T) (H), $[\text{U-}^{13}\text{C}_3]$ serine (S) (I), based on an analysis of triplicate 2D $^1\text{H-}^{13}\text{C}$ HSQC experiments. Samples were assayed in either early (OD$_{600}=0.8-1.0$) or late exponential (OD$_{600}=2.0-2.2$) phases of growth.
Figure 3.8  NMR Peak intensity of CDM supplemented with $^{13}\text{C}$ labeled amino acids. Metabolites derived from [U-$^{13}\text{C}_5$] glutamate (E) (A), [U-$^{13}\text{C}_6$] proline (P) (B), [U-$^{13}\text{C}_6$] arginine (R) (C), [U-$^{13}\text{C}_6$] histidine (H) (D), [U-$^{15}\text{C}_4$] aspartate (D) (E), [U-$^{15}\text{C}_3$] alanine (A) (F), [U-$^{13}\text{C}_2$] glycine (G) (G), [U-$^{13}\text{C}_4$] threonine (T) (H), [U-$^{15}\text{C}_3$] serine (S) (I), based on an analysis of triplicate 2D $^1\text{H}$-$^{13}\text{C}$ HSQC experiments. Samples were assayed in either early (OD$_{600}$=0.8-1.0) or late exponential (OD$_{600}$=2.0-2.2) phases of growth.
Figure 3.8  NMR Peak intensity of CDM supplemented with $^{13}$C labeled amino acids. Metabolites derived from [U-$^{13}$C$_5$] glutamate (E) (A), [U-$^{13}$C$_6$] proline (P) (B), [U-$^{13}$C$_6$] arginine (R) (C), [U-$^{13}$C$_6$] histidine (H) (D), [U-$^{13}$C$_4$] aspartate (D) (E), [U-$^{13}$C$_3$] alanine (A) (F), [U-$^{13}$C$_3$] glycine (G) (G), [U-$^{13}$C$_3$] threonine (T) (H), [U-$^{13}$C$_3$] serine (S) (I), based on an analysis of triplicate 2D $^1$H-$^{13}$C HSQC experiments. Samples were assayed in either early (OD$_{600}$=0.8-1.0) or late exponential (OD$_{600}$=2.0-2.2) phases of growth.
Figure 3.8  NMR Peak intensity of CDM supplemented with $^{13}$C labeled amino acids. Metabolites derived from $[U-^{13}\text{C}_5]$ glutamate (E) (A), $[U-^{13}\text{C}_6]$ proline (P) (B), $[U-^{13}\text{C}_6]$ arginine (R) (C), $[U-^{13}\text{C}_6]$ histidine (H) (D), $[U-^{13}\text{C}_4]$ aspartate (D) (E), $[U-^{13}\text{C}_3]$ alanine (A) (F), $[U-^{13}\text{C}_2]$ glycine (G) (G), $[U-^{13}\text{C}_3]$ threonine (T) (H), $[U-^{13}\text{C}_3]$ serine (S) (I), based on an analysis of triplicate 2D $^1\text{H}$-$^{13}$C HSQC experiments. Samples were assayed in either early (OD$_{600}$=0.8-1.0) or late exponential (OD$_{600}$=2.0-2.2) phases of growth.
Thus, when *S. aureus* is growing on non-preferred carbon sources, an intact urea cycle is critical for the interconversion of arginine and proline. Since CDM does not contain a preferred carbon source, it is predicted that CcpA-repression would be alleviated and pathways to interconvert proline and arginine would be transcriptionally active. As predicted, proline synthesis was detected when $^{13}$C labeled arginine was added to CDM, however, arginine synthesis was not detected from $^{13}$C labeled proline (Fig. 3.7). Instead, ornithine was detected from $^{13}$C labeled proline suggesting, in addition to its use in glutamate synthesis, carbon from proline also enters the urea cycle. To further investigate arginine synthesis in CDM, JE2 and JE2 *ccpA::tet* were grown in media lacking either proline (CDM-P) or arginine (CDM-R). It was found that JE2 *ccpA::tetL* grew in both CDM-P and CDM-R, although growth was delayed in CDM-R (Fig 3.9). In addition, as predicted, JE2 wildtype grew in CDM-P, but a *rocF* mutant could not, suggesting that proline synthesis is fueled by arginine in CDM (Fig 3.9). However, unexpectedly, but consistent with the NMR data, JE2 could not grow in CDM-R suggesting that proline cannot serve as a precursor for arginine synthesis in the absence of a *ccpA* mutation.

**Histidine and aspartate**

Carbon derived from $^{13}$C histidine was primarily identified in glutamate metabolism and cysteine biosynthesis intermediates (Figure 3.7 and Figure 3.8D). Consistent with our amino acid analysis where histidine was catabolized more slowly than glutamate, proline, and arginine (Figure 3.2), significantly more metabolites (especially glutamate) were identified in the post-exponential phase as compared to the exponential phase. This observation suggests histidine is catabolized after proline and arginine are limited or depleted.
Figure 3.9. Growth of *S. aureus* JE2 in CDM-P and CDM-R. Growth of JE2 rocF::NΣ, JE2 ccpA::tetL, and JE2 wildtype in CDM lacking proline (CDM-P) or arginine (CDM-R) (D). Data represents means ± SEM of three biological replicates.
As the carbon backbone of aspartate enters central metabolism via oxaloacetate, it is not surprising that many metabolic intermediates derived from $^{13}$C aspartate were identified, including those that function in gluconeogenesis, the pentose phosphate pathway, nucleotide biosynthesis, amino acid biosynthesis, and glutamate metabolism (Figure 3.8E). Indeed, as noted in Figure 3.4C, an aspA mutant, which is unable to synthesize oxaloacetate from aspartate, has an increased lag phase as compared to wildtype.

*Alanine, glycine, threonine, and serine*

The metabolic intermediates derived from these $^{13}$C glucogenic amino acids were very similar, reflecting their direct catabolism to pyruvate (Figure 3.7 and Figure 3.8F-I). Similar to other amino acids assayed, carbon derived from the glucogenic amino acids was identified in gluconeogenic intermediates, other amino acids, nucleotide biosynthesis, and glutamate. However, significantly more extracellular acetate was derived from alanine, threonine and serine as compared to other amino acids (Figure 3.10A). Therefore, we hypothesized a bifurcation exists during amino acid catabolism where pyruvate generated from the glucogenic amino acids is primarily utilized to generate acetate and ATP via AckA and little carbon enters the TCA cycle via citrate synthase until acetate is consumed from the medium at six hours of growth. In addition, those amino acids that generate glutamate enter the TCA cycle at 2-oxoglutarate via GudB and serve as a carbon source to subsequently facilitate gluconeogenesis (Figure 3.3). Although it is clear from the NMR data that the $^{13}$C glucogenic amino acids synthesize glutamate, this hypothesis predicts that mutations within *gltA* (citrate synthase), *acnA* (aconitase), and *icd* (isocitrate dehydrogenase) would not abrogate growth in contrast to mutations in other TCA cycle genes past the 2-oxoglutarate node.
Figure 3.10. Extracellular acetate production via amino acid catabolism and intracellular citrate concentrations as an indicator of TCA cycle activity. (A) Extracellular acetate detected following aerobic growth in CDM containing $[\text{U}^{\text{13}}\text{C}_4]$ aspartate (D), $[\text{U}^{\text{13}}\text{C}_6]$ glutamate (E), $[\text{U}^{\text{13}}\text{C}_5]$ proline (P), $[\text{U}^{\text{13}}\text{C}_6]$ histidine (H), $[\text{U}^{\text{13}}\text{C}_6]$ arginine (R), $[\text{U}^{\text{13}}\text{C}_4]$ threonine (T), $[\text{U}^{\text{13}}\text{C}_3]$ serine (S), $[\text{U}^{\text{13}}\text{C}_3]$ alanine (A) or $[\text{U}^{\text{13}}\text{C}_2]$ glycine (G). NMR peak intensity was assessed in early exponential (early), mid exponential (middle) and late exponential (late) growth based on an analysis of triplicate 2D $^1\text{H}-^1\text{C}$ HSQC experiments. (B) Intracellular concentration of citrate as measured in early and post-exponential phases of growth in CDM (*p<.05).
Indeed, transposon mutants within gltA, acnA, and icd were grown in CDM, and, in contrast to the sucA, sdhA, and fumC TCA cycle mutants, growth was observed albeit with growth rate and yield defects (Figure 3.4R-T and Figure 3.3). In agreement with this premise, intracellular citrate concentrations, as an indicator of TCA cycle activity and entry of carbon into the TCA cycle, increased 3-fold during post-exponential growth in CDM as compared to exponential growth (Figure 3.10B). Collectively, since we detected citrate in early exponential growth in CDM and we observed growth defects with the gltA, icd, and acnA mutants even before acetate is depleted from the medium (6 hours of growth; Figure 3.1), these data suggest that the TCA cycle is active throughout growth in CDM, but carbon flux is reduced through the TCA cycle and is instead routed to acetate when amino acids that generate pyruvate are present in the medium. Once these amino acids are depleted (primarily serine, threonine and alanine), acetate is consumed and enters the TCA cycle to generate 2-oxoglutarate.

**Respiration is reduced, but required for viability, during growth of S. aureus in CDM**

As an ackA mutant was unable to grow in CDM, we hypothesized that the ATP generated by acetate kinase is critical for growth under these conditions. We reasoned that the two NADH generated by 2-oxoglutarate dehydrogenase (SucA) and malate quinone oxidoreductase (Mqo1) are utilized during gluconeogenesis to regenerate NAD\(^+\) and maintain redox balance. Thus, the net NADH may be reduced during growth in CDM below levels needed to drive oxidative phosphorylation from the staphylococcal NADH:quinone oxidoreductase. Therefore, experiments were designed to determine if respiration is reduced during growth in CDM as compared to TSB or CDM containing 14 mM glucose (CDMG). The relative rate of oxygen consumption during growth in CDM was approximately 50% of the rate in TSB; and the rate was significantly lower than that
detected in CDMG (Figure 3.11A). Consistent with these observations, the relative intracellular ATP concentration during growth in CDM was 12% of the concentration in TSB (Figure 3.11B). Collectively, these data suggest that growth in CDM yields significantly decreased respiratory activity and coincident ATP synthesis.

*S. aureus* contains a branched respiratory chain and has at least two terminal menaquinol oxidases, QoxABCD (cytochrome *aa₃* oxidase) (4, 108) and CydAB (cytochrome *bd* oxidase) (109). To date, the presence of a third terminal oxidase, a cytochrome *bo* oxidase, is unclear (110). Respiratory complexes that feed the menaquinone pool include succinate dehydrogenase (Sdh) (111), lactate quinone oxidoreductase (Lqo) (10, 11, 112), and malate quinone oxidoreductase (Mqo) (10, 11). Furthermore, *S. aureus* does not contain a complex I NADH oxidoreductase, but instead encodes a type 2 non-electrogenic NADH:quinone oxidoreductase (Ndh2) that may be linked to an NuoL-like protein MpsA that functions to translocate cations, but has no NADH binding capability (113). The lack of growth demonstrated by a succinate dehydrogenase mutant (Figure 3.3 and Figure 3.4K) and the decreased ATP concentration and oxygen consumption noted during growth in CDM led us to address the importance of the electron transport chain during growth in CDM. Transposon mutants deficient in NADH:quinone oxidoreductase (*nrd*; SAUSA300_0844), and quinol oxidase (*qox*) were cultured in CDM, however, both the *nrd* and *qox* mutants were unable to grow aerobically in CDM (Figure 3.12A). Furthermore, growth of *S. aureus* was halted in CDM
Figure 3.11. Metabolic indicators of JE2 growth in CDM, TSB or CDMG. Relative rate of oxygen consumption (A) and ATP concentration (B) following growth of JE2 in CDM, CDMG or TSB. Rates of JE2 grown in TSB were set at 100%. Samples for (A) and (B) were collected in mid-exponential phase (***p<.001; ****p<.0001).
when the ATP synthase inhibitor DCCD was added to the medium in early exponential phase (Figure 3.12B). Conversely, DCCD had little effect on growth in CDMG or TSB (Figure 3.12C and 3.12D). Collectively, these experiments suggest that ATP generated via both substrate level phosphorylation and oxidative phosphorylation is required for growth in CDM and the importance of the PTA-AckA pathway under these conditions is most likely linked to the reduced rate of respiration.

**Growth of *gudB* and *ackA* mutants in CDM is impaired under anaerobic growth.**

The infection model proposed by Spahich and colleagues suggests that *S. aureus* growing under mild NO stress within an anoxic, glucose-depleted abscess requires both lactate and peptides. Thus, we assessed whether *gudB* and *ackA* were also required for growth in CDM under anaerobic growth conditions. JE2 was grown in CDM with or without lactate and/or nitrate, which was used as a terminal electron acceptor. These experiments demonstrated that enhanced growth was only observed in CDM containing lactate and nitrate as previously described (10, 11) (Figure 3.13A). Thus, JE2 *gudB::N*Σ and JE2 *ackA* were grown in CDM containing lactate and nitrate. As predicted, no growth was observed for either of these mutants (Figure 3.13B).

**S. aureus does not utilize proline or arginine during growth in CDMG.**

During our initial studies with growth in CDM, we found that several differences were noted if 14 mM glucose was added to the medium. First, aerobic growth of *S. aureus* JE2 in CDMG reached an OD$_{600}$ maximum of approximately 5.5 following 6 hours of growth at which time glucose was exhausted from the medium (Figure 3.14A). In contrast to growth in TSB (106) and CDM (Figure 3.1), acetate was not completely consumed from CDMG suggesting that growth arrest and subsequent acetate oxidation
Figure 3.12. Importance of aerobic respiration during growth in CDM. Aerobic growth of JE2, JE2 qoxB::NΣ, and JE2 nrd2::NΣ in CDM (A). Growth of JE2 in CDM following the addition of the ATP synthase inhibitor DCCD in early exponential phase (B). Growth of JE2 in CDMG following the addition of the ATP synthase inhibitor DCCD in early exponential phase (C). Growth of JE2 in TSB following the addition of the ATP synthase inhibitor DCCD in early exponential phase (D). Data represents means ± SEM of three biological replicates.
Figure 3.13. Importance of anaerobic respiration during growth in CDM. Anaerobic growth (24 hour) of JE2 in CDM containing either 3 mM sodium nitrate or 0.5% lactate (CDML) (A). Anaerobic growth (24 hour) of JE2, JE2 _gudB::NΣ_, and JE2 _ackA::NΣ_ grown in CDM containing sodium nitrate (3 mM) and lactate (0.5%) (B). Data represents means ± SEM of three biological replicates.
via the TCA cycle was halted due to depletion of critical amino acids or other C4/C5 compounds as previously discussed, although the exact mechanisms are not clear (Figure 3.14A) (111). Amino acid analysis of spent medium demonstrated that aspartate, glutamate, lysine and the glucogenic amino acids alanine, serine, glycine and threonine were rapidly depleted from the medium (Figure 3.15). However, the remaining amino acids, including arginine, histidine and proline that are known to generate glutamate during growth in CDM, were incompletely utilized following 12 hours of growth. In fact, a mutation in gudB had little effect on growth in CDMG in contrast to that observed with growth in CDM (Figure 3.14B). As discussed previously, investigations have shown that arginine and proline catabolism is repressed via CcpA and carbon catabolite repression (5, 41). However, following glucose consumption at 6 hours of growth, rapid arginine and/or proline depletion was not observed. Furthermore, no growth was observed in CDMG when proline (CDMG-P) or arginine (CDMG-R) was subtracted from the medium (Figure 3.14C). In contrast, the lack of glutamate in CDMG (CDMG-E) did not result in a significant growth defect (Figure 3.14C). NMR analysis using $^{13}$C$_6$-glucose confirmed that glucose was metabolized to synthesize glutamate, thus facilitating growth in CDMG-E (Figure 3.16). However, almost undetectable levels of $^{13}$C labeled arginine or proline were detected during growth with CDMG containing $^{13}$C$_6$-glucose confirming previous data demonstrating that arginine and proline need to be acquired from exogenous sources when S. aureus grows on preferred carbon sources such as glucose (5, 41). Collectively, these data suggest that growth of S. aureus in CDMG results in rapid utilization of glucose serine, threonine, alanine, glycine, glutamate, lysine and aspartate. However, proline and arginine, key amino acids that fuel glutamate synthesis when
Figure 3.14. Growth of *S. aureus* JE2 in CDMG. Aerobic growth of JE2 in CDMG displaying glucose consumption and acetate, ammonia, and lactate production (A). Growth of JE2 and JE2 *gudB::N* in CDMG (B) and JE2 in CDMG, CDMG lacking arginine (CDMG-R), CDMG lacking glutamate (CDMG-E) and CDMG lacking proline (CDMG-P) (C). Data represents means ± SEM of three biological replicates.
Figure 3.15. Amino acid consumption of JE2 following growth in CDMG. JE2 was grown aerobically in CDMG and amino acid concentrations (mM) were measured from the supernatant at 0, 4, 8 and 12 hours of growth.
Figure 3.16. NMR Peak intensity of CDMG supplemented with $^{13}$C labeled glucose. Metabolites derived from [U-$^{13}$C$_6$] glucose following aerobic growth in CDMG based on an analysis of triplicate 2D $^1$H-$^{13}$C HSQC experiments. Metabolites were assayed in early, mid or late exponential growth.
grown in CDM, are not extracted from the medium. Furthermore, it is unclear how lysine is rapidly utilized during growth in CDMG as pathways that function to catabolize lysine were not identified in *S. aureus* FPR3757 (Table 1.1).

**Discussion**

Our knowledge of how *S. aureus* proliferates within multiple niches of the host is limited. Nevertheless, recent studies have found that *S. aureus* can adapt to changing carbon sources and oxygen availability found within specific organ systems. For example, Hammer and colleagues determined that *S. aureus* defective in aerobic respiration were unable to mediate infection in the heart or liver, but colonization and abscess formation in the kidney was unaffected (4). Complementing these findings, *S. aureus* defective in anaerobic fermentation were less virulent than wildtype in a mouse model of kidney infection (3). Additionally, mutants defective in CcpA are less effective at infecting the liver where glucose is abundant, but infection burden in the kidney is not affected (5). Thus, taken together, these data suggest that *S. aureus* uses distinct central metabolic pathways to adapt to different carbon or oxygen sources that are available within the organ system or site of infection.

An elegant model has emerged demonstrating how *S. aureus* survives within a staphylococcal abscess (10). The antimicrobial action of NO occurs at multiple nodes of central metabolism including the TCA cycle and respiration. However, the NO concentration at the center of staphylococcal abscesses is thought to be below 1 µM allowing respiration and other metabolic processes to be functional (103, 114). Furthermore, glucose is depleted due to phagocytic cell activity whereas lactate concentration is abundant due to excretion during staphylococcal growth (10, 104). Thus, the major carbon sources for *S. aureus* at the center of abscesses are lactate and
peptides. Lactate is utilized via LQO that generates pyruvate and subsequently acetate via Pta/AckA whereas, peptides provide a carbon source for gluconeogenesis. The goal of this manuscript was to assess amino acid catabolism and to determine the importance of particular amino acid catabolic pathways during growth without glucose or other preferred carbon sources.

Bioinformatic analyses of the USA300 LAC (50) and other staphylococcal genomes in public databases found that S. aureus has the potential ability to catabolize 11 amino acids to either pyruvate, oxaloacetate, or 2-oxoglutarate (Table 1.1). Growth in CDM and subsequent amino acid analysis verified these predictions for 10 of the 11 amino acids. The lone amino acid not catabolized, but predicted based on bioinformatics analyses, was cysteine. This may be because CDM contains MgSO$_4$ as a sulfur source. It is well established that S. aureus cannot utilize sulfate, sulfite, or sulfonates as the source of sulfur in cysteine biosynthesis whereas it can use thiosulfate, sulfide, or glutathione (115). Thus, we predict that a feedback mechanism exists for repressing cysteine catabolism if an appropriate sulfur source for cysteine synthesis is not present. In addition, we observed that lysine was catabolized when S. aureus was grown in CDMG, but not CDM. Some species are known to ferment lysine to acetate (116), but metabolic pathways to perform this function are not immediately recognized in the S. aureus genome.

Glutamate is the major amino donor for most anabolic enzymatic reactions and yet also functions as a key intermediate in carbon metabolism (12, 25). Our results suggest that glutamate, and those amino acids that can be converted into glutamate, serve as central carbon sources to facilitate growth in media lacking a preferred carbon source such as glucose. First, glutamate dehydrogenase (GudB), an enzyme that catalyzes the conversion of glutamate to 2-oxoglutarate, is required for growth in CDM.
In addition, all enzymes (excluding Mqo1, which is rescued by anaplerotic reactions generating oxaloacetate) that function in the TCA cycle downstream of 2-oxoglutarate are required including phosphoenolpyruvate carboxykinase (PckA) that generates PEP from oxaloacetate. Thus, glutamate fuels the TCA cycle allowing subsequent gluconeogenic reactions and presumably serves as the major amino group donor to cellular reactions.

Second, although NMR analysis found that $^{13}$C carbon from all of the amino acids tested eventually was incorporated into glutamate, only mutations in those genes that code for enzymes that function to generate glutamate from proline or histidine resulted in a significant growth defect. Outside of arginine, all other amino acids must generate glutamate via the TCA cycle and glutamate synthase (GOGAT). However, a mutation in gltB (encoding large subunit of GOGAT; Figure 3.4U) conferred no growth defect in CDM suggesting the importance of glutamate synthesis from other amino acids besides proline and histidine is minimal. As indicated by NMR, arginine can also directly be converted to glutamate by the arginase pathway (RocF-RocD-RocA) (Figure 3.3). Alternatively, independent of RocF, arginine deiminase (57) can synthesize ornithine from arginine, which can then be catabolized to glutamate via RocD and RocA. However, individual rocF (Figure 3.4E) and arcA1/arcA2 (Figure 3.4Q) mutants did not have a growth defect in CDM whereas a rocF/arcA1/arcA2 (Figure 3.4V) mutant only had a post-exponential growth defect suggesting that arginine catabolism does not significantly contribute to exponential growth. However, it was surprising to note that a rocD mutant had a similar growth phenotype in CDM as the rocA mutant (Figure 3.4W-X); RocD converts ornithine, which can be synthesized from arginine via RocF, to P5C (Figure 3.3). Since the rocF (Figure 3.4E) mutant did not have a significant growth defect, one would predict that a rocD mutant would phenocopy a rocF mutant. S. aureus
also encodes a nitric oxide synthase (nos), which catalyzes the formation of citrulline from arginine generating NO (62). Thus, it is possible that arginine is primarily catabolized via NOS and not arginase or arginine deiminase during growth in CDM, but this is currently unknown. Nevertheless, complementing these genetic findings, comparison of NMR peak intensities suggested that glutamate synthesis via proline was approximately five-times and six-times that synthesized from arginine in exponential and post-exponential phase, respectively, and approximately five-times and 2.5-times that synthesized from histidine in exponential and post-exponential phase, respectively (Figure 3.8A-D). Thus, as our NMR and amino acid utilization data demonstrate, histidine is utilized after proline is limiting. Collectively, our data suggest that proline is the primary substrate for glutamate synthesis during exponential phase and histidine is utilized once proline is limiting. Furthermore, arginine is a minor source of glutamate synthesis in both exponential and post-exponential phases of growth.
Chapter 4

Regulation of Glutamate Biosynthesis in *Staphylococcus aureus*
Introduction

Bacterial survival depends on the ability of the organism to sense and respond to environmental stresses such as temperature and pH fluctuations, as well as alterations in nutritional availability and quality. As carbon and energy sources within specific host niches vary, bacteria must be able to efficiently utilize available nutrients to survive. Pathogenic bacteria exhibit a wide range of central metabolic capabilities, allowing the bacteria to persist within the human host even when stressful environments are encountered. Global regulators such as CcpA and CodY are responsible for modulating the metabolic response to changing environmental conditions, assuring that nutrient and energy requirements are fulfilled (117, 118).

While commonly thought of as the building blocks of proteins, amino acids can be utilized as substrates for a variety of bacterial cell processes depending on the nutrient availability of the surrounding environments (43). It has been established in several gram-positive bacteria that arginine catabolism via ADI generates ammonia for pH homeostasis and ATP to fuel cell processes (15, 16, 57). *B. subtilis* has the ability to use amino acids, such as arginine and proline, as sole carbon or nitrogen sources in nutrient-limiting conditions (32, 44-46, 48). Indeed, in the previous chapter it was demonstrated that *S. aureus* utilizes proline, histidine, and arginine as substrates for glutamate synthesis in the absence of glucose, therefore providing carbon for TCA cycle activity and gluconeogenesis.

CcpA Regulation

Because of the diverse capabilities of amino acids, their catabolic pathways are under the control of multiple layers of regulation. Regulation of amino acid catabolism ensures the transcription of only those genes necessary for the efficient utilization of
available nutrient sources, which avoids wasting energy by synthesizing unnecessary macromolecules. Most bacteria can use various compounds as carbon sources, including amino acids. Carbon catabolite regulation ensures that secondary carbon sources are not utilized when preferred carbon sources, such as glucose, are in abundance, thus, preventing energy expenditure as a result of the expression and activity of unnecessary catabolic systems (1, 119, 120). In pathogenic bacteria, carbon catabolite repression has also been linked to virulence factor expression as the proteins encoded by virulence genes are often involved in nutrient acquisition/utilization (120).

The catabolite regulator, CcpA, is highly conserved among low-G+C, gram-positive bacteria including *B. subtilis* and *S. aureus* (121). When preferred carbon sources such as glucose are transported into the cell, the accumulation of ATP and the glycolytic intermediates fructose-1,6-bisphosphate (FBP) and glucose-6-phosphate (Glu-6-P) leads to the phosphorylation of the HPrK kinase/phosphorylase at Ser-46 (122-124). Phosphorylation at this position results in an interaction with CcpA, which in turn binds to catabolite responsive elements (cre) within promoter/operator regions, thereby enhancing or repressing expression of the target gene (125-127). In *B. subtilis*, more than 100 genes that function in carbon acquisition and metabolism are CcpA regulated (12). Similarly, the CcpA regulon in *S. aureus* spans hundreds of genes involved in carbon metabolism. Several genes regulated by CcpA are involved in amino acid metabolism, including *argGH*, *rocD*, *rocF*, *rocA*, and *ald* (5, 37, 41, 128). Furthermore, CcpA can also directly or indirectly regulate virulence gene expression in *S. aureus* (83, 128).
CodY Regulation

CodY is also a highly conserved global regulator among low-G+C gram-positive bacteria, responding to nutritional availability (129). CodY is a DNA-binding protein responding to intracellular concentrations of two classes of metabolites: the branched-chain amino acids isoleucine, leucine, and valine (BCAAs) and GTP (130, 131). BCAAs bind to the GAF domain of CodY and induce a conformational change within CodY, affecting the C-terminal DNA binding domain and increasing the affinity of CodY for its DNA-binding sequence, thereby repressing or activating target genes (132). When nutrients become limiting, decreasing intracellular BCAA pools signal a transition from exponential- to stationary-phase growth and result in a decrease in affinity with the CodY GAF domain, which in turn decreases the affinity of CodY-DNA binding. Similarly, CodY senses the energy status of the cell via interaction with GTP, sensing intracellular GTP concentrations. However, the location of the GTP binding site of the S. aureus CodY has not been identified (133).

In S. aureus, CodY directly or indirectly controls the expression of over 200 genes, including those involved in metabolism and virulence, thus linking nutrient availability to virulence. CodY directly regulates genes involved in central and amino acid metabolism during nutrient limitation, prioritizing nutrient uptake and biosynthesis to ensure survival. Indeed, genes involved in carbon overflow metabolism and the TCA cycle, such as pyruvate carboxylase and 2-oxoglutarate dehydrogenase, were repressed during nutrient-rich, exponential-phase growth. Similarly, CodY repressed several amino acid biosynthetic pathways, including glutamate synthase, in nutrient-rich conditions (134). Regulation via CodY allows the organism to scavenge available nutrients for energy and biomass production, before diverting carbon and nitrogen towards biosynthesis of essential building blocks such as amino acids (135). In this chapter, we
utilized genetic and transcriptional studies to investigate the regulation of glutamate biosynthesis via the substrates proline, arginine, and histidine in *S. aureus* USA300 JE2.

**Results**

*putA, rocD, hutU, rocA, gudB,* and *rocF* are regulated via carbon catabolite repression and CcpA.

The studies described in chapter 3 suggest that proline and arginine catabolism is dependent upon carbon catabolite repression. Indeed, previous studies demonstrated that the interconversion of proline and arginine is repressed via CcpA at *rocF, rocD,* and *putA* in the presence of glucose (5, 41) indicating those amino acids that can be converted to glutamate can be used as alternative carbon sources. Therefore, it was hypothesized that the conversion of histidine to glutamate in addition to the conversion of arginine and proline to glutamate via pyrroline-5 carboxylate and RocA and the subsequent conversion of glutamate to 2-oxoglutarate via GudB would also be CcpA repressed as these reactions would only be necessary if there were no other preferred carbon sources available (Figure 3.3). Therefore, northern blots were utilized to assess transcription of *putA, rocD, hutU, rocA, gudB,* and *rocF* in both JE2 and JE2 *ccpA::tetL* following growth in CDMG and CDM. These experiments revealed that *putA, rocD, rocA,* *gudB,* and *rocF* transcription was induced after glucose was exhausted from CDMG (post-exponential growth; Figure 4.1A). In addition, as expected, transcription of *putA, rocD, rocA, gudB,* and *rocF* in exponential growth was *ccpA*-dependent as transcription was detected in JE2 *ccpA::tetL* following exponential growth in CDMG (Figure 4.1A). Furthermore, transcription of *putA, rocD, rocA, gudB,* and *rocF* was detected during exponential growth in CDM, which was expected as CDM contains only amino acids as carbon sources (Figure 4.1B). Therefore, repression by CcpA should be alleviated.
Figure 4.1. Transcriptional analysis of putA, rocD, rocA, gudB, rocF, and hutU following growth in CDMG and CDM. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth (both CDMG (A) and CDM (B)) from JE2 and JE2 ccpA::tetL. C. Northern analysis of hutU transcription in JE2 from 8 hr to 10 hr of growth. JE2 ccpA::tetL late RNA was used as a positive control. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
Transcription of *hutU*, however, was only detected in JE2 *ccpA::tetL* in exponential phase but not in exponential phase of wildtype JE2. Based on the NMR and amino acid utilization data (Figure 3.7 and 3.2), we predicted that *hutU* transcript would be detected in post-exponential phase following proline and arginine depletion in CDM. Indeed, *hutU* transcription was detected by 12 hours of growth in wildtype JE2 (Figure 4.1C). In addition, *hutU* transcripts were not detected in CDMG, even in the *ccpA* mutant. These data may suggest that growth arrest occurs in CDMG before potentially multiple regulatory networks induce *hutU* expression. Indeed, histidine catabolism in *B. subtilis* is regulated via both CcpA and CodY (70, 136). Collectively, these data demonstrate that pathways that function to fuel glutamate catabolism, and *gudB*, which fuels the TCA cycle from glutamate, are all regulated by CcpA and are induced only when glucose is not present in the medium.

**CodY regulates proline, arginine, and histidine utilization pathways**

Previous studies of CodY regulation in *S. aureus* have determined that over 200 genes are directly or indirectly regulated by CodY, including many of those involved in amino acid metabolism and transport (134). Recent studies from Waters and colleagues demonstrated that regulation via CodY in *S. aureus* allows for prioritization of nutrient uptake and biosynthesis during growth with limited or varying carbon and nitrogen sources. They constructed *codY* allelic exchange mutants with varying degrees CodY activity due to point mutations at conserved arginine and glycine residues within the ILV-binding pocket. These studies demonstrated that amino acid uptake genes were generally more sensitive to changes in CodY activity than their cognate biosynthesis genes, allowing the pathogen to scavenge the surrounding environment for nutrients before utilizing carbon, nitrogen, and energy for their biosynthesis. Furthermore, these studies demonstrated that not all biosynthesis pathways are regulated at the same level.
of CodY activity. For example, although lysine, methionine, and threonine are all synthesized from aspartate, lysine and methionine biosynthetic genes were expressed with only minor reductions in CodY activity compared to threonine biosynthetic genes (135). Lysine can be utilized as a substrate for peptidoglycan synthesis (137, 138); therefore it is possible that minor changes in CodY activity affects lysine biosynthesis to support both protein production and peptidoglycan synthesis (135). The sensitivity of methionine biosynthesis to CodY activity could reflect adaptation of *S. aureus* to its host, as methionine is limited in physiological fluids (135, 139, 140). Collectively, these studies demonstrate a spectrum of CodY activity regulates *S. aureus* metabolism, prioritizing nutrient uptake and biosynthesis in nutrient depleted environments.

The studies described in the previous chapter suggest that when grown in the absence of glucose, *S. aureus* utilizes glutamate, and those amino acids that can be utilized as substrates for glutamate synthesis (proline, arginine, and histidine), as carbon sources to fuel TCA cycle activity and gluconeogenesis. Growth and NMR studies demonstrated that proline, arginine, and histidine all serve as substrates for glutamate synthesis in glucose limiting conditions (Figure 3.6 and 3.7). Proline utilization via PutA and RocA appeared to be the major source of glutamate, as the *putA* and *rocA* transposon mutants had significant growth yield defects when grown in CDM lacking glutamate (CDM-E) compared to wild type JE2; whereas mutations in the pathways utilizing arginine (*rocF*) and histidine (*hutU*) had only moderate growth defects compared to wild type JE2 grown in CDM-E. Furthermore, the relative amount of glutamate generated from proline was approximately five-fold greater than the amount generated from arginine (Figure 3.8). As recent studies have suggested that CodY regulates preferential uptake and utilization of nutrients (135), we hypothesized that CodY would function to regulate proline, arginine, and histidine utilization as substrates for glutamate synthesis when grown in
carbon limiting conditions. Indeed, the codY transposon mutant had significant growth defects when grown in CDM and CDM-E, suggesting that CodY positively affects the transcription of glutamate synthesis pathways (Figure 4.2). Therefore, northern blots were utilized to assess the transcription of rocA, putA, rocD, hutU, rocF, and gudB in both JE2 and JE2 codY::NΣ following growth in CDM. These experiments revealed a decrease in induction of putA and rocA in the codY mutant when compared to wild type JE2 during exponential-growth in CDM (Figure 4.3A). Furthermore, northern blot assessment of the transcription of these genes during growth in CDMG demonstrated a complete loss of not only putA and rocA transcription, but also a loss of rocD and gudB transcription, following glucose exhaustion in the codY mutant when compared to wild type JE2 (Figure 4.3B). These data suggest that in the absence of CcpA repression, CodY activity positively regulates transcription of genes involved in the synthesis of glutamate via proline and arginine catabolism.

As previously described, hutU transcription was predicted to occur in JE2 during stationary phase following proline and arginine depletion in CDM, however hutU transcription was only detected during exponential growth in a ccpA mutant. We hypothesized CodY would also be functioning to regulate hut transcription during growth in CDM, as both CcpA and CodY function to repress histidine catabolism in B. subtilis. Indeed, hutU transcripts were detected during post-exponential growth in JE2 codY::NΣ, suggesting that both CodY and CcpA function to repress histidine catabolism in S. aureus (Figure 4.3A). Collectively, these data suggest that when preferred carbon sources like glucose are limiting and CcpA repression is alleviated, CodY activity regulates glutamate synthesis via induction of proline and arginine catabolic genes, while repressing histidine catabolism.

Regulation via the arginine transcriptional regulators ArgR1, ArgR2, and AhrC
Figure 4.2. Growth in CDM-E. (A) Aerobic growth of JE2 and JE2 codY::NΣ in CDM-E. (B) Growth of JE2 and JE2 argR1::NΣ CDM-E. Data represents means ± SEM of three biological replicates.
Figure 4.3. Transcriptional analysis of *putA*, *rocD*, *rocA*, *gudB*, *rocF*, and *hutU* in *S. aureus* JE2 and JE2 *codY::NΣ*. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM (A) and CDMG (B) from JE2 and JE2 *codY::NΣ*. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
In *B. subtilis*, the expression of both arginine and proline catabolic genes are controlled by multiple transcriptional regulators, sensing several environmental cues. In addition to regulation via CcpA sensing the availability of glucose and CodY sensing the nutritional status of the cell through assessments of BCAA levels, as well as the energy status of the cell via intracellular GTP concentrations, *B. subtilis* encodes regulators that sense the availability of specific amino acids. Proline can serve as the sole carbon or nitrogen source in *B. subtilis* through utilization of the *putBCP* operon. The transcriptional activator, PutR, induces the expression of this operon in the presence of proline. However, CodY activity will repress *putBCP* expression by displacing PutR (46).

*B. subtilis* can also utilize arginine as a sole carbon or nitrogen source via the arginase pathway (*rocABC* and *rocDEF* operons), which is induced via the positive transcriptional regulator RocR when in the presence of arginine, ornithine, citrulline, or proline (32, 48). A second regulatory protein, AhrC, also acts a positive regulator of these operons in the presence of arginine, while repressing the arginine biosynthetic *arg* operon (141).

As previously discussed, the *S. aureus* genome does not encode orthologous *putR* or *rocR* genes, however, it does encode the arginine transcriptional regulator, *ahrC*. Arginine transcriptional regulation was first described in *Esherichia coli*, in which the transcriptional regulator, ArgR, repressed the transcription of the arginine biosynthetic *arg* operon. In the presence of arginine, ArgR binds to one or more 18-bp palindromic sequences referred to as ARG boxes, repressing transcription of *arg* genes (142). In *B. subtilis*, the ArgR-type regulator, AhrC, was shown to not only repress transcription of arginine biosynthesis but also activate arginine catabolism via the arginase pathway in the presence of arginine (143). In *Lactococcus lactis*, another gram-positive bacterium, both ArgR and AhrC have been shown to regulate arginine metabolism. Here, in the absence of arginine ArgR has a higher affinity for the promoter
of the arginine catabolic arc operon than it does for the biosynthetic arg operon, possibly due to additional ARG box binding sites within the arc promoter. This prevents arginine degradation via the ADI pathway, while allowing for de novo arginine synthesis via the arg operon. The addition of arginine results in the formation of an AhrC-ArgR protein complex, with high affinity for the ARG box binding sites within the promoter of the arg operon. ArgR repression of the arc promoter is alleviated as it complexes with AhrC, repressing the expression of arginine biosynthetic genes and allowing for arginine catabolism via the ADI pathway (54).

*S. aureus* USA300 JE2 has three ArgR-type transcriptional regulators, AhrC, ArgR1, and ArgR2. The transcriptional regulator AhrC, which shares 61% amino acid sequence homology to the *B. subtilis* AhrC protein, is encoded by ahrC on the chromosome in an operon with the DNA recombination gene, recN. ArgR1 and ArgR2 are encoded by the genes, *argR1* and *argR2*, which are monocistronic units of the two ADI operons found within the *S. aureus* USA300 JE2 genome. The native ADI operon contains the gene *argR1*, encoding the native arginine transcriptional regulator, ArgR1. While the acquired arginine catabolic mobile element (ACME) encodes a second ADI operon, and therefore a second arginine transcriptional regulator, ArgR2 (Figure 4.4) (50). As AhrC and ArgR function to regulate utilization of arginine as alternative carbon and nitrogen sources in other gram-positive bacteria, we hypothesized they may have similar functions in *S. aureus* when grown in CDM and utilizing amino acids as carbon sources. Northern blots were utilized to assess transcription of *putA*, *rocD*, *hutU*, *rocA*, *gudB*, and *rocF* in JE2, the transposon mutants JE2 *argR1::N* and JE2 *argR2::N*, and JE2 ΔahrC following growth in CDM (Figure 4.5, 4.7, and 4.8). An in-frame, markerless deletion was constructed in JE2 ΔahrC to avoid any polar mutations with recN. These experiments revealed that in *S. aureus*, the arginine repressor ArgR1 functions to
Figure 4.4 Comparison of native (argR1 – arcR1) and acquired (argR2 – arcC2) ADI operons and arginine transcriptional regulators (argR1, argR2, and ahrC). Percentage of amino acid identity between genes of the native and acquired ADI operon and between all three arginine transcriptional regulators are shown.
Figure 4.5. Transcriptional analysis of *putA*, *rocD*, *rocA*, *gudB*, *rocF*, and *hutU* in *S. aureus* JE2 and JE2 *argR1::NΣ*. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM (A) and CDMG (B) from JE2 and JE2 *argR1::NΣ*. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
regulate proline catabolism via \textit{putA} transcription as JE2 \textit{argR1::N} had decreased expression of \textit{putA} during early-exponential growth when compared to wild type JE2 (Figure 4.5A). Indeed, a mutation in \textit{argR1} results in a significant growth yield defect, similar to a \textit{codY} mutant, compared to wild type JE2 when grown in CDM-E (Figure 4.6). Furthermore, \textit{putA} transcription was also decreased in this mutant during post-exponential growth in CDMG (Figure 4.5B). Suggesting that in the absence of carbon catabolite repression, ArgR1 can function to activate proline catabolism.

As \textit{argR2} is an acquired arginine repressor, not native to the chromosome, we expected to see minimal effects on growth and gene expression in the \textit{argR2} transposon mutant when grown in CDM. The \textit{argR2} mutant had a similar growth phenotype as JE2 when grown in CDM, however, a moderate growth yield defect was observed compared to JE2 when grown in CDM-E, suggesting regulation of glutamate synthesis via ArgR2. Northern blot analysis revealed increased \textit{rocF} transcription during early-exponential growth and delayed induction of \textit{rocA} transcription in the \textit{argR2} mutant compared to wild type JE2 when grown in CDM (Figure 4.7). While in the \textit{ahrC} mutant, \textit{rocA} and \textit{rocD} had increased expression during early-exponential growth when compared to wild type JE2, suggesting ArgR2 and AhrC are both functioning to regulate arginine catabolic pathways (Figure 4.8). It is also worth noting that in an \textit{ahrC} mutant, \textit{hutU} transcription was observed during late-exponential growth, similar to what was seen in the \textit{codY} mutant, indicating AhrC activity also regulates histidine catabolism (Figure 4.3A).

**Discussion**

Bacteria are able to adapt efficiently to a wide range of nutritional environments through the use of regulatory systems linking nutritional availability to gene expression. \textit{B. subtilis} integrates the activities of several global regulators, including CcpA and CodY,
Figure 4.6. Growth of *S. aureus* JE2 an arginine transcriptional regulators in CDM and CDM-E. Aerobic growth of JE2 argR1::NΣ, JE2 argR2::NΣ, and JE2 ΔahrC in CDM (A) and CDM-E (B) in comparison to wild type JE2. Data represents means ± SEM of three biological replicates.
Figure 4.7. Transcriptional analysis of *putA*, *rocD*, *rocA*, *gudB*, *rocF*, and *hutU* in *S. aureus* JE2 and JE2 *argR2::NΣ*. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM (A) and CDMG (B) from JE2 and JE2 *argR2::NΣ*. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
Figure 4.8. Transcriptional analysis of putA, rocD, rocA, gudB, rocF, and hutU in *S. aureus* JE2 and JE2 ΔahrC. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM (A) and CDMG (B) from JE2 and JE2 ΔahrC. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
to control gene expression according to what nutrients are available (12). Pathogenic bacteria often contend with its host restricting nutrient availability, thus they have evolved or acquired virulence factors that aid in nutrient acquisition through host tissue destruction. Often, these bacteria utilize the same regulators of metabolism as regulators of virulence, coordinating nutrient availability with virulence. Coordination of regulation in this way reserves expression of destructive virulence factors for nutrient limiting environments (12, 117). In *S. aureus*, virulence and metabolic gene expression have been linked to both CodY and CcpA activity, allowing this pathogen to maximize the limited nutritional resources available within its host. Li *et al.* demonstrated a link between carbon catabolite repression via CcpA and virulence, as a *ccpA* mutant demonstrated attenuated virulence in a liver abscess model of infection compared to wild type, indicating the regulation of carbon source utilization is important during pathogenesis (5). Furthermore, *S. aureus* CodY has been shown to regulate gene expression through a spectrum of CodY activity. As nutrients begin to be depleted from the environment, CodY sequentially activates the expression of virulence genes (typically via loss of repression) encoding proteins that destruct host tissues, as well as nutrient scavenging/metabolism genes allowing the bacterium to acquire and utilize the available nutrients found within the degraded tissue (135).

Understanding the interwoven metabolic and regulatory networks within *S. aureus* in their entirety requires large data sets and a systems biology approach; however, defining individual regulatory nodes provides useful information as to when these metabolic networks may be functional during pathogenesis. We have previously described an emerging model of *S. aureus* abscess formation where it is hypothesized that viable cells within the abscess must utilize lactate, peptides, and free amino acids as carbon and energy sources (10, 11, 104). In our assessment of amino acid
catabolism we determined that S. aureus utilizes glutamate, and those amino acids that can be converted into glutamate, as central carbon sources. Carbon catabolite repression prevents energy expenditure by repressing the expression and activity of unnecessary catabolic systems when preferred carbon sources such as glucose are in abundance (1, 119, 120). It has been well documented that the pathways interconverting proline and arginine are under repression by the catabolite repressor, CcpA, in the presence of glucose as well as their conversion to glutamate via rocA (5, 37, 41, 128). Therefore, it was of no surprise to find induction of putA, rocD, rocF, rocA, and gudB expression following the exhaustion of glucose in CDMG (Figure 4.1A). The increased expression in JE2 ccpA::tetL compared to wild type JE2 during early-exponential growth in CDMG confirms this repression is ccpA dependent (Figure 4.1A). Furthermore, as predicted, transcription of putA, rocD, rocA, gudB, and rocF was detected during exponential growth in CDM (Figure 4.1B), further indicating that glutamate, as well as proline and arginine, can be utilized as carbon sources during nutrient limitation.

Bacterial growth is dependent on the availability of nutrients. Nutritional adaptation allows for the expression of genes involved in the transport and catabolism of the nutrients that are available in the environment. The global regulator, CodY, has been well documented to regulate amino acid metabolism in response to nutrient starvation by responding to the intracellular concentration of both BCAAs and GTP. During the transition from exponential- to stationary-phase growth, decreasing BCAA and GTP concentrations lead to derepression of CodY regulated genes (129-131). Waters and colleagues have developed a model where a spectrum of CodY activity promotes growth in varying BCAA and GTP concentrations. It was determined that under conditions of increasing nutrient depletion, CodY sequentially turns on genes
required for transporting nutrients, before turning on genes involved in the *de novo* synthesis of the compound. Thus, prioritizing nutrient uptake and catabolism, ensures that energy and resources are not wasted on synthesizing nutrients that are available in the environment (135).

In studies performed by Waters *et al.*, transcriptional profiling via RNA-seq experiments indicated that transcripts of the proline transporters, *proP* and *putP*, were among the first to be induced following nutrient depletion (135) suggesting that proline catabolic genes would also be induced by CodY during nutrient limitation. Indeed, northern blot experiments demonstrated induction of glutamate synthesis genes via proline catabolism is CodY-dependent. In the absence of glucose, and therefore when CcpA repression is alleviated, a *codY* mutant demonstrates decreased induction of *putA* and *rocA*, when compared to wild type JE2, suggesting CodY activity facilitates glutamate synthesis via the activation of proline catabolic genes in the absence of a preferred carbon source (Figure 4.1). A *codY* mutant also results in a significant growth yield defect, similar to that seen in a *putA* mutant when grown in CDM-E (Figure 4.2), further suggesting proline catabolism is regulated by CodY activity. Collectively, our assessments of the induction of glutamate synthesis genes via the catabolism of arginine and proline suggest that the presence of glucose represses the transcription of these pathways in a CcpA-dependent manner. In the absence or exhaustion of glucose, CodY activity leads to induction of the proline catabolic genes, *putA* and *rocA*, facilitating the use of proline as a carbon source.

Regulation of both proline and arginine catabolic pathways leading to the synthesis of glutamate have been well characterized in *B. subtilis*. Several transcriptional regulators have been characterized that function to regulate these pathways, including PutR, RocR, AhrC, and the aforementioned CcpA and CodY. While *S. aureus* does not
encode orthologous PutR or RocR proteins, its genome does encode the arginine transcriptional regulator, AhrC. In fact, it has been reported that three arginine transcriptional regulators, ArgR1, ArgR2, and AhrC, are encoded by the *S. aureus* JE2 genome (50). As *S. aureus* arginine and proline synthesis pathways are interconnected and as these ArgR-type regulators have been shown in several gram-positive organisms to regulate both arginine anabolism and catabolism (5, 37, 41), we hypothesized one or more of the ArgR-type regulators encoded by *S. aureus* JE2 functioned to regulate proline and arginine catabolism. Growth studies revealed that when grown in CDM-E a mutation in *argR1* resulted in similar growth defects as seen in *putA* and *codY* mutants, indicating this mutant is unable to utilize proline for glutamate synthesis (Figure 4.6). In conjunction with these findings, and similar to results seen in the *codY* mutant, northern blot analysis demonstrated that after the exhaustion of glucose in CDMG and during exponential growth in CDM, a mutation in *argR1* results in loss of expression of *putA* when compared to wild type JE2 (Figure 4.5). Furthermore, we predicted that a mutation in *argR2* would have little effect on expression of these pathways, as it is an acquired regulator, not native to the chromosome. Much to our surprise, a mutation in *argR2*, as well as a mutation in *ahrC*, resulted in moderate growth defects when grown in CDM-E (Figure 4.6) and decreased *putA* expression following glucose exhaustion when grown in CDMG, albeit less pronounced than the *argR1* mutant (Figure 4.7B and 4.8B). These data suggest that all three arginine regulators, in the absence of glucose and carbon catabolite repression, have the ability to induce *putA* expression, thus facilitating activation of proline catabolism. A mutation in *argR2* also resulted in delayed expression of *rocA* and increased *rocF* transcription during exponential growth in CDM, while an *ahrC* mutant had increased *rocA* expression during exponential growth in CDM when compared to wild type (Figure 4.7A and 4.8A), suggesting these regulators also have a function in arginine utilization and catabolism.
While these studies indicate that in the absence of glucose, ArgR1, ArgR2, and AhrC function in regulating the pathways involved in glutamate synthesis via the catabolism of proline and arginine, the mechanisms of regulation remain unclear. The similar lack of growth in CDM-E (Figure 4.2 and 4.6) and loss of expression of putA during growth in both CDMG and CDM in the argR1 and codY mutants (Figure 4.3 and 4.5) suggest both of these regulators are involved in activation of proline catabolism. As nutrients become limiting within the environment, CodY typically induces gene expression through loss of repression due to conformational changes induced by decreasing BCAA and GTP concentrations. If loss of CodY repression during nutrient depletion functions to induce transcription of putA, we would expect to see increased expression of putA in a codY mutant. However, as we observe complete loss of transcription of putA in the codY mutant we hypothesize induction of an unknown activator, such as ArgR1, by CodY could result in putA transcription.

We observed similar growth in the argR2 mutant compared to wild type JE2 in CDM (Figure 4.9); however, moderate growth defects are seen when grown in CDM-E (Figure 4.6), indicating ArgR2 regulates glutamate synthesis under these conditions. Indeed, when grown in CDM the argR2 mutant demonstrated increased expression of rocF during exponential growth, indicating it represses arginase-mediated arginine catabolism (Figure 4.7A). Delayed expression of rocA in JE2 argr2::N& when compared to wild type JE2 during growth in CDM indicates this mutant is not able to utilize proline or arginine during exponential growth (Figure 4.7A); however, growth defects are not observed when grown in CDM (Figure 4.9). These data suggest the argR2 mutant is able to utilize other sources of carbon for growth during early exponential growth, while arginine and proline may be utilized during post-exponential growth. Indeed, NMR analysis to determine the fate of carbon after amino acid uptake and catabolism in this
mutant would provide useful information as to what pathways are being utilized during growth in CDM.

We observed few effects on transcription of glutamate synthesis via proline and arginine catabolism pathways during growth in CDM in the *ahrC* mutant (Figure 4.8A). However, we did observe increased *hutU* transcription during late-exponential growth in CDM in the *ahrC* mutant when compared to wild type JE2 (Figure 4.8A). Amino acid consumption data demonstrated that histidine utilization followed proline and arginine exhaustion during growth in CDM (Figure 3.2). While *hutU* transcription was not observed in wild type during early or late time points in CDM, it was observed in the *ccpA*, *codY*, and *ahrC* mutants (Figure 4.1B, 4.3A, and 4.8A). In *B. subtilis*, repression of *hut* transcription by CcpA and CodY ensures histidine catabolism is only active when absolutely necessary, as histidine synthesis requires an input of 20 high-energy phosphate bonds (66). Increased transcription during early exponential growth in the *ccpA* mutant indicates that even in the absence of a primary carbon source such as glucose, CcpA still mediates repression of some genes. We observed increased transcription of *hutU* in the *codY* mutant and *ahrC* mutants during post-exponential growth, indicating both of these regulators function to repress histidine catabolism, however the mechanism of repression remains unclear.

In conclusion, the experiments described above indicate the regulation of glutamate synthesis via the catabolism of arginine, proline, and histidine involves several transcriptional regulators, including CcpA, CodY, ArgR1, ArgR2, and AhrC, however the exact mechanisms of regulation remain unclear. It has been well documented by our group and others that the pathways leading to glutamate synthesis via proline, arginine, or histidine degradation are CcpA regulated and not induced in the presence of glucose (5, 37, 41), thus, indicating these amino acids are utilized as a carbon source in glucose
deplete environments. These studies also demonstrated that CodY and ArgR1 activity facilitate the induction of proline catabolism via putA expression. While the mechanism of putA induction remains unclear, it is possible that as nutrients are depleted from the environment, CodY activity results in expression of argR1, which in turn induces transcription of putA, however, further investigation is needed to confirm this hypothesis. While we are uncertain of the exact functions of ArgR2 and AhrC in the regulation of these amino acid catabolic pathways, northern blot and growth analyses suggest that they may function to regulate the sequential utilization of proline, arginine, and histidine. Indeed, further NMR analysis and transcriptional profiling of these regulator mutants should provide useful insight into the mechanisms of the regulation of glutamate synthesis via amino acid catabolism.
Chapter 5

Concluding Remarks
Final observations of amino acid catabolism in *S. aureus* and Future Directions

Pathogenic bacteria must readily adapt to changing carbon and nitrogen sources to successfully proliferate within their human hosts. Understanding what metabolic pathways are required for growth within specific niches may reveal novel avenues for drug development. *S. aureus* is capable of establishing an infection in a variety of metabolic niches within the human host, most commonly skin and soft tissues. The work presented in this dissertation contributes to an evolving model of *S. aureus* survival within abscesses. To persist within the human host, *S. aureus* must not only combat host innate defenses, such as NO production, but must also adapt its metabolic processes to overcome nutrient limitations at sites of infection (10, 11, 95). It is proposed that the major carbon sources available at the center of staphylococcal abscesses are lactate, excreted from bacterial glucose fermentation during NO stress, and peptides. *S. aureus* is able to utilize lactate via lactate quinone oxidoreductast (Lqo) generating pyruvate and subsequently acetate via the ATP-producing PTA/AckA pathway; where as peptides are predicted to provide a carbon source for the bacteria to fuel gluconeogenesis and biomass production (10). The goal of this work was to assess amino acid catabolism in *S. aureus* and to determine the importance of particular amino acid catabolic pathways during growth without glucose or other preferred carbon sources.

*S. aureus* encodes pathways to catabolize multiple amino acids, including those that generate pyruvate, 2-oxoglutarate, and oxaloacetate. The data presented here has demonstrated that the enzyme GudB, which generates 2-oxoglutarate from glutamate, and subsequently those amino acids that lead to glutamate synthesis, is essential for growth in media lacking glucose. We observed that glutamate was primarily synthesized through catabolism of proline via PutA and RocA during growth in the presence of amino
acids. These findings contribute to a previously proposed model that predicts that \textit{S. aureus} utilizes both host- and self-derived proteases to liberate free proline or proline-containing peptides from the fibrin wall when encapsulated within an abscess. A major constituent of the fibrotic wall encapsulating the bacterial cells is collagen and the second most abundant amino acid found in collagen is proline \cite{41,144}; thus, suggesting a reservoir of proline is present at the site of staphylococcal infection (Figure 5.6). Moreover, mutants in the high affinity proline permease, PutP, are less virulent in animal models of infection \cite{145,146}. Collectively, these data and observations suggest \textit{S. aureus} has adapted to the microenvironment of the abscess, utilizing substrates provided by the fibrotic wall that encapsulates it for growth and persistence. Indeed, analysis of the proline catabolic \textit{putA} and \textit{rocA} mutants and the \textit{gudB} mutant’s ability to survive within a murine abscess model is required to determine the functions and importance of these pathways \textit{in vivo}. Furthermore, characterization of proteases and transporters involved in the liberation and consumption of proline proposed to contribute to this model of abscess formation and persistence would provide evidence that \textit{S. aureus} has the ability to utilize proline in nutrient limiting conditions.

The results of the characterization of amino acid catabolism in \textit{S. aureus} suggests that growth on amino acids is dependent upon ATP generation from PTA/AckA, as an \textit{ackA} mutant was unable to grow in CDM. In addition, a functional respiratory chain was required for growth. It is not surprising that growth in the presence of amino acids would require ATP generation from both substrate level phosphorylation and the electron transport chain as amino acid transport requires ATP and is a more energy intensive process than carbohydrate transport. However, it is unclear whether this growth phenotype is due to loss of ATP generation or disruption of intracellular acetyl-phosphate pools, as acetyl-phosphate is a key coeffector molecule in the cell.
Growth analysis of a serine dehydratase and alanine dehydrogenase double mutant, abolishing the cells ability to utilize glucogenic amino acids for pyruvate and subsequent acetate generation, would provide further information on the contribution of ATP generation via PTA/AckA to growth on amino acids.

Arginine is proposed to be limiting within abscesses due to utilization by host innate immune responses and bacterial scavenging, however, its function in S. aureus pathogenesis remains unclear. It is hypothesized that ADI activity could be important for pathogenesis, as arginine catabolism via the ADI pathway provides pH homeostasis and energy generation via ammonia and ATP production to a variety of bacteria, including staphylococcal species, contributing to the overall fitness of these organisms during growth in specific niches (16, 55, 57). It is unclear how arginine is utilized in CDM, as the arcA1/arcA2/rocF triple mutant showed only slight growth yield defects. It is possible that arginine catabolism via ADI could be contributing to ATP production or pH homeostasis during growth in the absence of preferred carbon sources as citrulline formation was detected in NMR analysis of arginine utilization (Figure 3.7). Citrulline is an intermediate of all three arginine catabolic pathways (argininase, ADI, and NOS pathways), which can synthesize ATP and ammonia via ArcC. While it is assumed that transposon mutations in arcA1 and arcA2 facilitate polar mutations, disrupting transcription of all genes involved in the arc operon, it is possible that the ArcC enzyme is functional in these mutants, allowing for ATP generation. Growth analysis and murine abscess models of infection utilizing an arcC1/arcC2 mutant could provide more knowledge of the contribution of ADI during growth on alternative carbon sources. Furthermore, growth analysis of a nos mutant could also provide information as to what pathways are utilizing arginine during growth in these conditions.
Final observations on regulation of amino acid catabolism

As previously discussed, *S. aureus* has shown to be a proline and arginine auxotroph (39), with growth in CDMG lacking either proline or arginine (CDMG-P, CDMG-R) being dependent upon alleviation of CcpA repression (5, 37, 41). Our studies, and others, have demonstrated that the multiple pathways fueling glutamate synthesis (via proline, arginine, and histidine), and subsequent conversion to 2-oxoglutarate via GudB, are all under the repression of CcpA and only active during growth on non-preferred carbon sources (5, 37, 41). It was predicated that growth defects in CDM lacking arginine or proline would be observed in JE2 as CcpA repression is alleviated. Although moderate defects were observed, JE2 could grow in the absence of proline in a RocF-dependent manner, suggesting utilization of arginine for both proline and glutamate synthesis (Figure 5.1A). However, we only observed growth in CDM-R when *ccpA* was inactivated (Figure 5.1B). Complementing these findings, NMR analysis demonstrated that proline utilization does not facilitate arginine synthesis during growth in CDM. It is unclear how CcpA is functioning to repress arginine biosynthesis in the absence of arginine. However, the detection of *argG* transcript, which encodes the urea cycle enzyme argininosuccinate lyase and is required for arginine biosynthesis, during post-exponential growth in CDM in both JE2 and the *ccpA* mutant, demonstrates arginine biosynthesis is induced after the exhaustion of arginine from CDM during post-exponential growth (Figure 5.2A), thus, suggesting another regulator functions to induce arginine biosynthesis in the absence of arginine. The arginine transcriptional regulator, AhrC, has been well characterized in *B. subtilis*, functioning to repress arginine biosynthesis in the presence of arginine, while simultaneously activating arginine catabolism via the arginase pathway (143). While we were surprised to determine AhrC had little effect on the activation of arginine catabolism
Figure 5.1. Growth of *S. aureus* JE2 and JE2 *ccpA::tetL* in CDM and CDM lacking arginine (CDM-R). Aerobic growth of JE2 *ccpA::tetL* in CDM (A) and CDM-R (B) in comparison to wild type JE2. Data represents means ± SEM of three biological replicates.
via the arginase pathway during growth in CDM, our data does suggest AhrC is
functioning to repress transcription of arginine biosynthesis under these conditions.
Northern blot analysis of the ahrC mutant during early-exponential growth in CDM
demonstrated increased argG transcription when compared to wild type JE2 (Figure
5.2B). Arginine is still available in the media during early-exponential growth in CDM
(Figure 3.2), suggesting AhrC is repressing arginine biosynthesis in an arginine-
dependent manner. After further analysis of argG transcription, we were surprised to
determine that while ArgR1 had no effect on transcription, ArgR2 is also functioning to
repress argG during growth in CDM (Figure 5.2C and 5.2D). As ArgR2 was acquired
with the addition of the ACME island to the S. aureus USA300 JE2 genome (50), it
would be interesting to compare regulation of argG to strains without the ACME element
or in a JE2 ΔACME mutant.

In most bacteria, arginine is typically synthesized via glutamate catabolism and
the argJBCDFGH pathway. However Nuxoll et al. demonstrated that this pathway in S.
aureus is inactive and that proline serves as a substrate for arginine biosynthesis via
PutA, RocD and the urea cycle, including ArgG, in the absence of CcpA repression (41).
We hypothesized that AhrC could be functioning to repress the argJBCD operon in the
presence of arginine; however, we observed no expression of argJ in the ahrC mutant
(Figure 5.3). Furthermore, we were unable to detect argJ transcript in ArgR1 or ArgR2
during growth in CDM (Figure 5.3). Interestingly, further characterization of the arginine
transcriptional regulators revealed ArgR1, and to a lesser extent ArgR2, function to
activate putA expression after exhaustion of glucose in CDMG (Figure 4.5B and 4.7B).
AhrC, however, repressed putA transcription in an arginine-dependent manner (Figure
4.8), further implicating its function in repression of arginine biosynthesis in the presence
of arginine. Collectively, these results indicate these arginine transcriptional regulators
Figure 5.2. Transcriptional analysis of argG in S. aureus. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM in wild type JE2, JE2 ccpA::tetL (A), JE2 ΔahrC (B), JE2 argR1::NΣ (C), and JE2 argR2::NΣ (D). Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
Figure 5.3. Transcriptional analysis of argJ in S. aureus JE2 and the various regulator mutants. Northern analysis was performed with RNA isolated from exponential (early) and post-exponential (late) phases of growth in CDM from JE2, JE2 argR1::Nσ, JE2 argR2::Nσ, JE2 ΔahrC, JE2 ccpA::tetL, and JE2 codY::Nσ. Genomic DNA from JE2 was used as a positive control to ensure the argJ probe was functional. Equal loading of RNA was ensured via staining of the RNA gel with ethidium bromide and visualization of the 16s and 23s rRNA (data not shown).
have evolved in the regulation of arginine biosynthesis from glutamate catabolic genes to proline catabolic genes. Perhaps, S. aureus has adapted both its metabolic pathway preferences and its regulatory mechanisms to utilize proline instead of glutamate, as proline is potentially in abundance during infection.

The global regulator, CodY, directly or indirectly controls the expression of over 200 genes in S. aureus, including those involved in metabolism and virulence, allowing the organism to efficiently utilize available nutrient sources and avoiding wasteful energy expenditure by synthesizing unnecessary macromolecules. CodY regulation of proline metabolism was recently implicated in studies performed by Waters et al. as proP and putP genes encoding proline transporters were among the first to be expressed following nutrient depletion and derepression of CodY (135). Furthermore, the ability to transport proline has been linked to virulence, as mutants in putP are less virulent in animal models of infection (145, 146). These data suggest that proline catabolic pathways are also regulated via CodY derepression and nutrient limitation. Indeed, our studies implicated CodY involvement in putA transcription as a codY mutant had decreased induction of expression after alleviation of repression via CcpA (Figure 4.3). CodY activation of genes typically induces gene transcription via loss of repression; therefore, it is hypothesized that CodY activation of putA expression in the absence of CcpA repression occurs through derepression of an auxiliary transcriptional regulator, perhaps one that senses the availability of proline.

Amino acid consumption data indicated that histidine was consumed when glucose is limiting. Indeed, genetic and NMR analyses demonstrated that histidine can be utilized as a substrate for glutamate synthesis via the hut genes; thus, it can also serve as a carbon source in the absence of glucose. As both CcpA and CodY repress histidine catabolism in B. subtilis, we predicted they would also be involved in hut
repression in S. aureus. Much to our surprise, hut induction was not detected in northern blot analyses of early- and late-exponential time points during growth of JE2 in CDM (Figure 4.1), even though NMR studies determined that the presence of histidine facilitated glutamate synthesis during late-exponential growth (Figure 3.7). However, we did detect hut transcription in CcpA and CodY mutants, indicating that histidine catabolism is only induced during carbon and nutritional limitation (Figure 4.1B and 4.3A). Histidine is one of the most expensive amino acids for the cell to synthesize, requiring an input of 20 high-energy phosphate bonds (66), suggesting it would only be catabolized during extreme carbon and nutrient limitations. To further suggest histidine utilization is only induced during times of starvation, AhrC also repressed hut transcription in an arginine-dependent manner (Figure 4.8). Figures 5.4 and 5.5 summarize our proposed model of the regulation of the proline, glutamate, and arginine metabolic pathways in the presence and absence of preferred carbon. Figure 5.6 depicts the proposed model of S. aureus survival within an abscess.

**Future Directions**

The work presented in this dissertation provides evidence that proline can be utilized as a carbon source for bacterial growth in carbon limiting environments via its conversion to glutamate, which is further utilized to drive TCA cycle activity and gluconeogenesis, thus, providing the cell with carbon to fuel biomass production. CcpA repression in the presence of glucose further suggests the importance of the utilization of glutamate and those amino acids that synthesize glutamate as carbon sources, as these pathways are only induced during carbon limitation. Further characterization of the mechanisms of regulation involved in the interconnected pathways of glutamate, proline, arginine, and histidine metabolism indicated that CodY and the arginine transcriptional regulators AhrC, ArgR1, and ArgR2 are involved in controlling the
expression of these pathways. It seems likely that AhrC and ArgR2 function to repress arginine biosynthesis in the presence of arginine and that CodY activity regulates proline catabolism during nutrient limitation. Additionally, ArgR1 appears to activate proline catabolism in a CcpA-dependent manner, however, further experimentation is required to confirm this hypothesis. Indeed, NMR analysis of the fates of proline, arginine, histidine, and histidine in all five regulator mutants (CcpA, CodY, AhrC, ArgR1, and ArgR2) when grown in CDM would provide useful information as to what catabolic pathways these regulators function to activate or repress. Moreover, amino acid consumption analyses in these mutants could indicate if these regulators are involved in the regulation of transport of these amino acids. In conclusion, the findings of this dissertation further characterize the metabolic and regulatory nodes involved in carbon metabolism, which possibly contribute to in vivo survival and pathogenesis.
Figure 5.4 Regulation of glutamate synthesis in the presence of a preferred carbon source. In the presence of a preferred carbon source such as glucose, CcpA functions to repress most genes involved in the interconnected pathways of glutamate, arginine, and proline metabolism.
In the absence of a preferred carbon source such as glucose, CcpA alleviation (not including hut repression) allows for expression of glutamate synthesis genes via amino acid catabolism. Hut transcription is repressed in the absence of glucose by CcpA, CodY, and AhrC activity, in CDM this repression is hypothesized to be functioning due to the presence of proline and arginine. Transcription of putA is positively regulated by ArgR1 and CodY. Northern blot data suggests CodY activation is through derepression of an unknown activator, perhaps sensing proline availability. Transcription of ArgG is repressed by CcpA, ArgR2, and AhrC in the absence of glucose. In CDM, this repression is hypothesized to be due to the presence of arginine during early exponential growth.
Figure 5.6 Proposed model of S. aureus abscess survival. It is proposed the staphylococcal abscess is carbon limited due to both immune and bacterial cells utilizing glucose at the onset of infection. Host factors such as iNOS and arginase (1A) utilize arginine for nitric oxide production (iNOS) and collagen production (arginase). Bacterial cells utilize arginine via ADI for ATP and ammonia production. Due to the depletion of glucose, it is hypothesized that CcpA repression is alleviated, allowing for utilization of amino acids such as proline as carbon sources. The fibrin wall is cleaved by bacterial secreted proteases such as ScpA, SspB, and aureolysin (2), as well as host proteases such as MMP-9 (2), to liberate free proline or proline containing peptides (3). The bacterial cells are then able to utilize these free peptides and amino acids through specific transporters as carbon sources for survival and persistence within the abscess (4).
References


81. Fey PD, Endres JL, Yajjala VK, Widhelm TJ, Boissy RJ, Bose JL, Bayles KW. 2013. A genetic resource for rapid and comprehensive phenotype screening of nonessential Staphylococcus aureus genes. MBio 4:e00537-0051.


