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Arginine Biosynthesis in Staphylococcus aureus

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Arginine Biosynthesis in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus causes a wide variety of infections including osteomyelitis, endocarditis, and necrotizing pneumonia. It has been known for decades that *S. aureus* is auxotrophic for many amino acids including arginine. However, we discovered that after extended incubation, *S. aureus* can be trained to grow in a defined media lacking arginine. We demonstrate here that this delayed growth is due to the selection of mutations in *ahrC*, a transcriptional regulator of arginine biosynthesis. A mutation in *ahrC* mediates the growth of *S. aureus* in CDM-R by facilitating the biosynthesis of arginine via proline. Mutations in *putA* and a*rgGH* halt the growth of the JE2 *ahrC* mutant while a mutation in *argDCJB* did not. In addition, Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) analysis showed that $^{13}C_5$-labelled arginine was detected in the JE2 *ahrC* mutant when grown in the presence of $^{13}C_5$-labelled proline and not of$^{13}C_5$-labelled glutamate further confirming that proline is the precursor of arginine. Furthermore, our transcriptional analysis results demonstrate that AhrC in *S. aureus* functions to primarily repress *argGH*

and *arcB1* allowing for arginine biosynthesis via proline. Although the mechanism of arginine biosynthesis regulation was shown to be conserved among species, our data demonstrate that AhrC in *S. aureus* functions to regulate arginine biosynthesis differently, with the biosynthesis being mediated through the urea cycle using proline as a precursor instead of the highly conserved and well-characterized glutamate pathway which was shown to be inactive *in-vitro*

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Chapter 1: Introduction

Staphylococcus aureus is a common cause of community-associated and hospital-acquired infections. Clinical manifestations can range from skin and soft tissue infections to more severe conditions such as bacteremia, osteomyelitis, endocarditis, and necrotizing pneumonia[1]. The ability of *S. aureus* to infect multiple organ systems was initially attributed to the acquisition of multiple of virulence factors. However, it was documented that *S. aureus* has evolved to metabolically adapt to cause invasive infections. Therefore, to thrive in different environments, *S. aureus* must scavenge nutrients and coordinate nitrogen and carbon usage. It has been documented that glycolysis is essential for *S. aureus* virulence and tissue invasion[2]. However, within staphylococcal abscesses, glucose, a preferred carbon source for *S. aureus* is limited. Hence, upon depletion of glucose, *S. aureus* must derive carbon and nitrogen source from the catabolism of amino acids such as glutamate, proline, and arginine[3]. These three are the primary interconvertible carbon source in a glucose depleted environment. Proline, as well as glutamate, are abundant amino acids within the abscess. In contrast, arginine is depleted at the site of infection due to inflammatory response and the upregulation of host iNOS and arginase, both requiring arginine as a substrate. Mutation in the arginine biosynthetic pathway attenuated virulence of *S. aureus in vivo* [4, 5]. Therefore, it is important to investigate arginine biosynthesis and its regulation in *S. aureus.* Understanding how *S. aureus* accommodates for arginine depletion in vivo is important and provides insights on host-pathogen interaction and how the pathogen proliferates despite the hostile environment.

S. aureus harbors the genes encoding the arginine biosynthetic pathway *argJBCDFGH* responsible for synthesizing arginine from glutamate[6]. Biosynthesis of arginine from glutamate

is a highly conserved and well-characterized pathway adopted by multiple bacteria, including *Bacillus subtilis*, *Salmonella* enterica serovar Typhimurium, *and Escherichia coli* [7, 8]. However, studies from Nuxoll et al. documented that in *S. aureus* arginine is synthesized from proline rather than glutamate. This study also documented that the arginine biosynthetic pathway is regulated by carbon catabolite repression (CCR) through the activity of carbon catabolite protein A, CcpA. Indeed, a *ccpA* mutant could grow on a media lacking arginine. However, mutations in *putA*,*argG* and *argH*, but not *argC*, completely abrogated the growth of the *ccpA* mutant implicating that the glutamate pathway is inactive *in-vitro*[4]. In agreement with the previous observations, Halsey et al. showed that *putA* and *argGH* transcription are upregulated in a *ccpA* mutant further confirming that arginine biosynthesis is regulated by CCR and that the production of arginine from proline is responsible for the phenotype of the *ccpA* mutant when grown in a media lacking arginine[3]. Hence, based on these studies, one would expect that the growth of *S. aureus* in media lacking glucose and arginine (CDM-R) will be induced since CCR and arginine transcription repression will be alleviated. Surprisingly, *S. aureus* still exhibits arginine auxotrophy when grown in CDM-R. However, growth can be selected after extended incubation. This delayed growth is due to the selection of mutations in *ahrC* which encodes for a transcriptional regulator of arginine biosynthetic genes. In *Bacillus subtilis*, AhrC exerts regulation through the binding to operator site called ARG box found upstream of arginine biosynthetic genes mediating their repression in presence of arginine[9]. Experimental evidence indicates that this mechanism of regulation is conserved among gram-positive as well as gram-negative bacteria[10, 11]. However, little is known about how AhrC regulates arginine biosynthesis in *S. aureus.* This thesis will discuss the importance of proline as an alternative precursor for arginine biosynthesis and the function of AhrC in regulating arginine metabolism in *S. aureus*.

A historical perspective of arginine biosynthesis: Fundamental change and findings.

Early studies assessing the arginine biosynthesis pathway have brought forth many fundamental changes in the perspective of scientists regarding the regulation of biochemical pathways. Crucial experiments were conducted, which contributed to significant knowledge in the field and brought forward new scientific concepts. In 1953, Henry Vogel observed that arginine is an antagonist for the enzyme N-acetylornithinase [12]. In 1957, at the McCollum Pratt Symposium, Vogel explained these data using the term "**enzyme repression."** The substance mediating the inhibition was called the "**represser**" (spelling now changed to repressor). Since then, the term repressor is an accepted term in the scientific field as the molecule that inhibits enzyme activity [13]. The experiments carried out by Werner K. Maas in 1957 demonstrating the kinetics of ornithine carbamoyltransferase demonstrated that both exogenous and endogenous arginine regulate the rate of enzyme synthesis in the metabolic pathway [14]. To further elucidate the mechanism behind enzymatic repression, in 1959, Werner isolated *E. coli* K12 mutants resistant to canavanine, which is an arginine analog that competes for protein synthesis. These mutants overcame the inhibition caused by canavanine and were then derepressed for multiple arginine anabolic enzymes. Werner had mapped the mutation present in the above mutants and termed the mutated gene as an arginine repressor (*argR*). Werner suggested that *argR* regulates the expression of an "**aporepressor**" with arginine acting as the "**corepressor**" [15, 16]. At the 1961 Cold Spring Harbor Symposium, Francois Jacob and Jacques Monod documented two important concepts, the operon model and mRNA [17]. In the same symposium, Werner and Luigi Gorini demonstrated that the arginine biosynthetic genes are scattered across the chromosome, and yet they are regulated by the same regulatory element [16-18]. Later in 1964, Werner and John Clark stated that the repressor *argR* negatively regulates the arginine biosynthetic genes. Thus, they together coined the term **regulon,** which describes the phenomenon where a single regulatory molecule controls a set of unlinked

genes, as seen in arginine biosynthetic enzymes [19]. These findings have therefore changed the perspective regarding biochemical regulation and have immensely contributed to the establishment of the molecular biology field.

Arginine metabolism and bacterial pathogenesis

S. aureus catabolizes arginine through the highly conserved arginine deiminase (ADI) pathway. Catabolism of arginine through ADI generates ornithine, ammonia, and carbon dioxide and 1 ATP. ADI consists of three enzymes encoded on the *arc* operon: arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB), and carbamate kinase (ArcC). Recent evidence documented that methicillin-resistant community-associated *S. aureus* (CA-MRSA) has acquired a second copy of the *arc* operon found on the arginine catabolic mobile element (ACME) which was shown to contribute to the ability of this particular strain to thrive within acidic environments and cause skin and soft tissue infections (SSTI) [20].

Furthermore, arginine serves as a substrate for the NO synthase enzyme (NOS) in *S. aureus* resulting in nitric oxide (NO) production. It was demonstrated that NOS functions in hydrogen peroxide resistance, biofilm formation, and toxin production. Also, evidence has shown that endogenous nitric oxide mediates *S.aureus* daptomycin resistance, facilitates nasal colonization, and promotes protection from oxidative stress [21].

In conclusion, arginine is a part of an extensive metabolic network. It functions in pH homeostasis, serves as a nitrogen and carbon source and substrate for multiple enzymes all contributing to the virulence of pathogen within a particular host and affecting *S. aureus* physiology and host interactions[22, 23].

Arginine biosynthesis in *Staphylococcus aureus*

The arginine biosynthetic pathway from glutamate is a highly conserved and well-characterized pathway found among a wide array of bacteria, including *Bacillus subtilis*, *Salmonella* enterica serovar Typhimurium, *and Escherichia coli.* Genome sequencing has demonstrated that *S. aureus* harbors the arginine biosynthetic genes *argJBCDFGH*; these genes encode the enzymes responsible for synthesizing arginine from glutamate (**Fig.1.1**). Although *S. aureus* harbors the biosynthetic capability to synthesize arginine, it still exhibits auxotrophy for arginine when grown on a complex laboratory media. Nuxoll et al. demonstrated that *S. aureus* arginine biosynthesis is under carbon catabolite repression and that proline serves as a precursor for arginine biosynthesis instead of glutamate (**Fig.1.2**). The highly conserved glutamate pathway was shown to be inactive under *in-vitro* conditions. It is still unclear when or if *S. aureus* derepresses the glutamate biosynthetic pathway or if there is any niche where this pathway is activated during host invasion. The enzymes' names participating in arginine biosynthesis along with their EC number and gene symbol are illustrated in Table.1.1

Further, *S. aureus* encodes a set of virulence factors that enables it to destroy host tissue [24]. Among these, SspB and SspA, are proteases that degrade collagen, a proline-rich protein found in animals [25-28]. Degradation of collagen results in the release of free proline, which can be used as a substrate for arginine production. Thus, this mechanism adopted by *S. aureus* might be beneficial; since collagen is an abundant protein surrounding the fibrotic wall of a staphylococcal abscess and its use to produce arginine exhibits that *S. aureus* metabolically adapts and evolves to acquire nutrients that are readily available at the site of infection to support its growth.

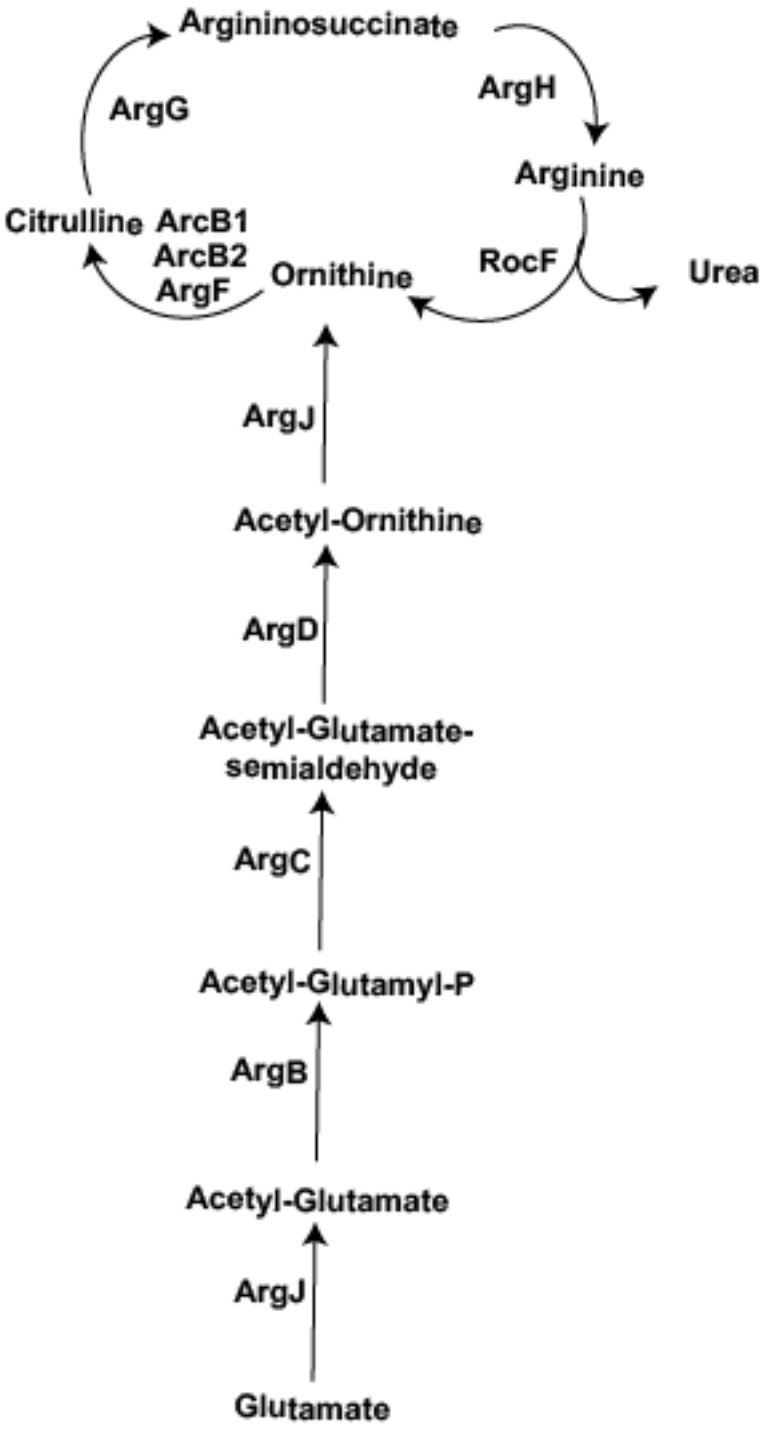


Figure 1.1 Arginine Biosynthesis via Glutamate

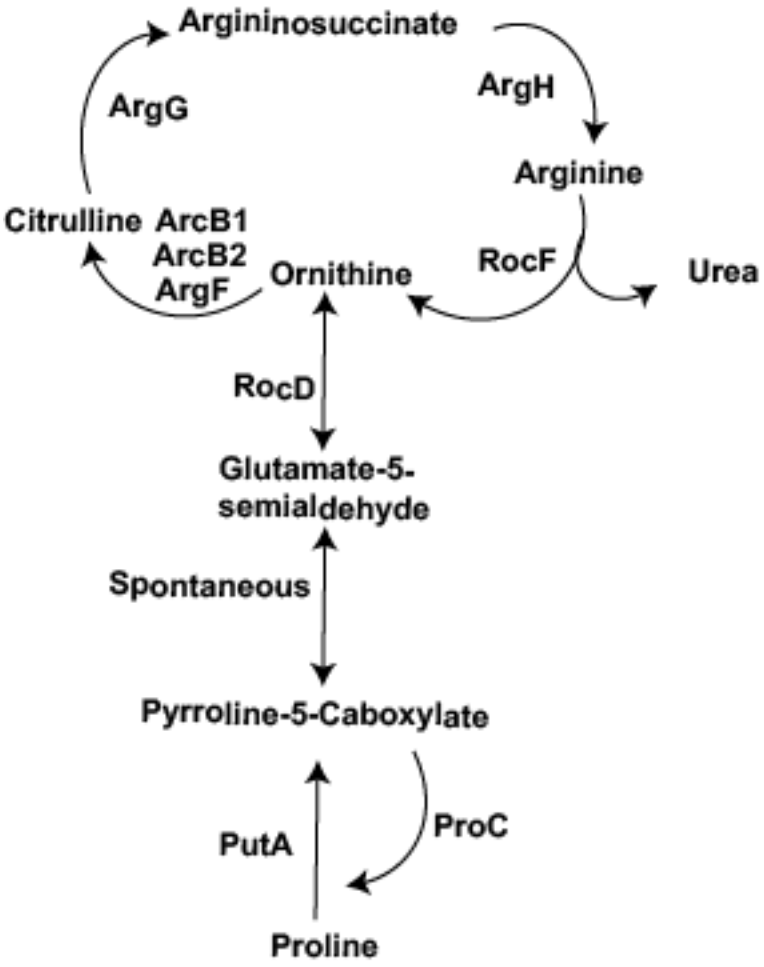


Figure 1.2 Arginine biosynthesis via urea cycle in *S. aureus.*

Table 1.1 Enzyme name with the corresponding EC number and their gene symbol.

Enzyme Name	EC number	Gene symbol
Glutamate N-acetyltransferase/amino-acid acetyltransferase	EC:2.3.1.35 2.3.1.1	*argJ*
N-Acetylglutamate kinase	EC 2.7.2.8	*argB*
N-Acetylglutamylphosphate reductase	EC 1.2.1.38	*argC*
N-Acetylornithine aminotransferase	EC 2.6.1.11	*argD*
Ornithine carbamoyltransferase	EC:2.1.3.3	*arcB1,arcB2,argF*
Argininosuccinate synthetase	EC 6.3.4.5	*argG*
Argininosuccinase	EC 4.3.2.1	*argH*
Arginase	EC:3.5.3.1	*rocF*
Proline dehydrogenase	EC:1.5.5.2	*putA*

Regulation of arginine biosynthesis in *Staphylococcus aureus*.

Transcriptional regulation of arginine biosynthesis was first studied in *Escherichia coli* where it was disclosed that ArgR, an L-arginine dependent repressor, represses transcription of arginine biosynthetic genes by binding to the DNA operator sites found upstream of the *arg* genes, overlapping the promoter sequence. Each operator site, known as an ARG box, consists of a pair of 18 bp palindromic sequences (separated by 2-3 bp) [10, 27]. In *Bacillus subtilis*, AhrC, an ArgR homologue, also represses the arginine biosynthetic pathway in presence of arginine, but in contrast to the mechanism of regulation in *E. coli*, additional transcriptional activation of arginine catabolic genes is mediated by AhrC[9]. AhrC forms a hexamer, a dimer of trimers, through oligomerization of its C-terminal domain. The dimer-trimer interface results in the formation of the arginine binding pocket consisting of Gln104, Asp111, Thr121, Cys123, Asp125, and Asp126. The N-terminal domain has a DNA binding motif, this motif was shown to be part of the winged helix-turn-helix family (wHTH). In the presence of arginine, AhrC binds to the operator sequence, known as ARG box, located within the promoter of *argC* and *argG*, repressing the transcription of the arginine biosynthetic genes[9].

S.aureus encodes three ArgR-type transcriptional regulators, ArgR1 and ArgR2, and AhrC. ArgR1 and ArgR2 are encoded by *argR1* and *argR2* genes, respectively. *argR1* belongs to the native ADI operon while *argR2* is a monocistronic unit of the second ADI operon encoded by the acquired arginine catabolic mobile element (ACME). *ahrC* is co-transcribed with the DNA recombination gene, *recN*, both found in the same operon on the chromosome[28]. Halsey et. al demonstrated that ArgrR1 activates the transcription of *putA* when carbon catabolite repression is alleviated while AhrC represses the transcription of *rocA* and *rocD*. These data together suggest that both ArgR1 and AhrC regulate arginine biosynthesis via proline. However, unlike other bacteria, where the regulation of arginine biosynthesis is extensively studied and well-characterized, little is known how *S. aureus* transcriptionally regulates arginine biosynthesis. As previously mentioned, *S. aureus*

has adopted a novel proline catabolic pathway to synthesize arginine instead of using the glutamate canonical pathway. Also, *S. aureus* harbors three transcriptional regulators instead of one, imposing limitations in studying the regulation of the arginine biosynthetic pathway. Despite the conservation of the mechanism of regulation among different species, *S.aureus* appears to regulate arginine biosynthesis differently, therefore, more studies are needed to investigate how *S.aureus* functions to regulate arginine biosynthesis in the case of arginine starvation.

Chapter 2: Materials and Methods

Bacterial strains and culture Conditions

All strains used in this study are listed in **Table. 2.1**. Defined bursa aurealis transposon mutants were obtained from the Nebraska Transposon Mutant Library and backcrossed to JE2 using Φ11[29]. JE2 *ahrC* markless deletion mutant *ΔahrC* was generated as described in[3].JE2 *ahrC* mutant was isolated after 18-24 hours growth of JE2 in complete defined media lacking arginine (CDM-R). Following the delayed growth on CDM-R, colonies were selected on Tryptic Soy Agar (TSA) and growth was assessed in CDM-R. The colonies that exhibited growth in CDM-R were sent for whole-genome sequencing. Whole-genome sequencing results are illustrated in **Table 2.1**

Bacterial cultures were grown overnight in 5 mL Tryptic Soy Broth at 37°C with shaking at 250 rpm. Overnight cultures were washed with phosphate-buffered saline (PBS) and inoculated to an optical density at 600 nm (OD_{600}) of 0.05. Growth curves were performed in CDM and CDM-R with no glucose added[30] in 96-well plate in the Tecan Device at 37°C with shaking at 250 rpm.

RNA isolation and RT-qPCR analysis

Cultures of *S. aureus* JE2, JE2 *ahrC* and Δ*ahrC* were grown overnight in 3ml CDM at 37°C with shaking at 250 rpm. Overnight cultures were inoculated to an OD_{600} of 0.05 into 25 ml of CDM or CDM-R into 250 mL flask (1:10 volume to flask ratio). Cells were grown to exponential phase and were collected when they reach an OD_{600} = 0.4-0.8. 6 mL of Qiagen RNAprotect Bacteria reagent was added to 3 mL of collected culture. Following, Cells were incubated for 5 minutes at room temperature, pelleted by centrifugation for 10 minutes at full speed. Pellet was resuspended in 185 uL lysis buffer, followed by 15 uL of proteinase K. Next, samples were incubated at rotating platform shaker at room temperature for 10 min then resuspended in 700 uL RLT buffer with 1% β-mercaptoethanol. Suspensions were transferred to lysing matrix B tubes (MP Biomedicals) and

processed in an FP120 FastPrep cell disrupter (MP Biomedicals) for 45 seconds at a setting of 6.0. 760 uL of the supernatant was transferred into a new tube containing 590 uL of 80% ethanol. The samples were then processed using an RNeasy mini kit, according to manufactures instructions (Qiagen, Inc.). cDNA was generated using SuperScript™ IV VILO™ Master Mix with ezDNase™ Enzyme with 1 pg to 2.5 µg total RNA in each 10 uL reaction.

All qPCRs were performed in 20µl reactions (10µl Taq DNA polymerase MasterMix (ThermoFisher), 1µl of 10µM primer sets, 4µl H_2O, 5µl template cDNA). Standards and samples were assayed in duplicate in the LightCycler©480 device. All primer-probe set used are listed in **Table. 2.3** and **2.4**

Liquid Chromatography with tandem mass spectrometry (LC-MS/MS).

LC-MS/MS separation and quantitation were carried out using XBridge Amide 3.5 µm (2.1 x 100 mm) column procured from Waters. Mobile phase A was 10 mM ammonium acetate and 10 mM ammonium hydroxide in water + 5% acetonitrile whereas mobile phase B contains 100% acetonitrile. The flow rate was 0.4mL/min with a gradient mode of mobile phases. The column was maintained at 40 °C. Detection of metabolites was carried out using QTRAP 6500 (Sciex) in multiple reaction mode (MRM). All the labeled metabolites such as canonical and non-canonical amino acid standard mixtures, ^{13}C-arginine, ^{13}C-glutamate and ^{13}C-proline were purchased from Cambridge Isotopes Inc, USA.

Transduction of *putA*:: NΣ, *argH*:: NΣ and *argC*:: NΣ into JE2 *ahrC* mutant and Δ*ahrC* background.

Φ11 transducing lysates were prepared from defined JE2 bursa aurealis mutants with insertions in the *putA*, *argH*, *argC*. These bursa aurealis mutations conferring erythromycin resistance were transduced into JE2 *ahrC* and Δ*ahrC*. Mutants were selected on TSA containing 10 µg/ml erythromycin. Subsequently, Insertions of *putA*,*argH* and *argC* were confirmed by PCR.

Table 2.1 Bacterial Strains used in this study

Bacterial Strains	**Phenotypes**	**Source**
S. aureus JE2	LAC cured of all 3 native plasmids	[29]
S. aureus putA:: NΣ	*bursa aurealis* mutation in proline dehydrogenase	[29]
S. aureus argH:: NΣ	*bursa aurealis* mutation argininosuccinase	[29]
S. aureus argC:: NΣ	*bursa aurealis* mutation acetylglutamylphosphate reductase	[29]
JE2 *ahrC* mutant	Mutant selected after extended growth in CDM-R	This study
ΔahrC	Markerless deletion in SAUS200_1469, arginine repressor	[3]
S. aureus JE2 *argR1::NΣ*	*bursa aurealis* mutation in SAUSA300_2571, arginine repressor	[3]
S. aureus JE2 *argR2:: NΣ*	*bursa aurealis* mutation in SAUSA300_0066, arginine repressor	[3]

Table 2.2 Whole Genome Sequencig results

Isolate	Start	Change	Amino Acid Change	Protein Effect	Product
1	146215	C -> A		Truncation	FIG01107877: hypothetical protein
1	963762	T -> C	L -> S	Substitution	ATP-dependent nuclease, subunit B
1	1655458	C -> A		Truncation	Late competence protein ComGF, access of DNA to ComEA, FIG017774
1	2524007	G -> T	P -> T	Substitution	Nitrite reductase [NAD(P)H] large subunit (EC 1.7.1.4)
1	2784181	A -> G			Arc promoter
2	1358107	A -> C			
2	2784181	A -> G			Arc promoter
4	447901	C -> T	H -> Y	Substitution	Exotoxin 7
4	2784181	A -> G			Arc promoter
8	2784202	A -> T			Arc promoter
8	2784204	-A			Arc promoter
17	1639590	C -> A	C -> F	Substitution	AhrC, repressor of arg regulon
18	804659	A -> T	L -> I	Substitution	Di/tripeptide permease YjdL
18	1564471	C -> T	G -> D	Substitution	Phage lysin, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)
18	2711153	T -> G	I -> L	Substitution	Acyltransferase
18	2784204	A -> T			Arc promoter
21	1550253	C -> A	P -> H	Substitution	L-asparaginase (EC 3.5.1.1)
21	1561036	T -> A			
21	1639949	T -> A	K -> N	Substitution	Arginine pathway regulatory protein AhrC, repressor of arg regulon
22	304912	G -> A	A -> T	Substitution	Glycosyl transferase family protein, putative
22	505342	CGG -> TTT	GG -> GL	Substitution	PTS system, trehalose-specific IIB component (EC2.7.1.69)
22	1034607	G -> T			

22	1721293	C -> A	V -> L	Substitution	peptidase, U32 family small subunit [C1]
22	2784215	A -> T			Arc promoter
24	1427979	A -> G	I -> V	Substitution	Mobile element protein
24	2513697	G -> A	P -> L	Substitution	Two component sensor histidine kinase
24	2784181	A -> G			Arc promoter

Table 2.3 Oligonucleotides used in the RT-qPCR study

Name	Sequence	Comments
oLH1	CCT TAT GGC GAT GAT TGG TTT G	Forward primer for *S. aureus* JE2 *putA* transcriptional analysis by RT-PCR
oLH2	CCA GCA GGT TTC ACA AAT TCT T	Reverse primer for *S. aureus* JE2 *putA* transcriptional analysis by RT-PCR
oLH3	GGT CTT GGT AGA ACT GGG AAA T	Forward primer for *S. aureus* JE2 *rocD* (SAUSA300_0187) transcriptional analysis by RT-PCR
oLH4	CAA GTA CAG CAG ATA CAG GGT ATA A	Reverse primer for *S. aureus* JE2 *rocD* (SAUSA300_0187) transcriptional analysis by RT-PCR
oLH5	CTG TAG AGG ATA CTG CGA AAG T	Forward primer for *S. aureus* JE2 *arcB1* transcriptional analysis by RT-PCR
oLH6	GGA TGC CAA TCG TCT GTT AAT C	Reverse primer for *S. aureus* JE2 *arcB1* transcriptional analysis by RT-PCR
oLH7	CAT TTG AAA CAG CGG CTT ATG A	Forward primer for *S. aureus* JE2 *arcB2* transcriptional analysis by RT-PCR
oLH8	ACA CGA GCA GTA TCT TTG GTA G	Reverse primer for *S. aureus* JE2 *arcB2* transcriptional analysis by RT-PCR
oLH9	AAG AAC GCG ATG TGC ATT TG	Forward primer for *S. aureus* JE2 *argF* transcriptional analysis by RT-PCR
oLH10	AGC ACA CGT GCA GTA TCT TTA	Reverse primer for *S. aureus* JE2 *argF* transcriptional analysis by RT-PCR
oLH11	CAG AGC GAA TGA ATG TGG TAT TT	Forward primer for *S. aureus* JE2 *argGH* transcriptional analysis by RT-PCR
oLH12	TCA GCA GTA TCT GGT GTT TCT T	Reverse primer for *S. aureus* JE2 *argGH* transcriptional analysis by RT-PCR
oLH13	CGG TAG GTT CAG TAT CAG CAA TAA	Forward primer for *S. aureus* JE2 *rocF* transcriptional analysis by RT-PCR
oLH14	TTT CCA CTT GGT GAC TCT TCA G	Reverse primer for *S. aureus* JE2 *rocF* transcriptional analysis by RT-PCR

oLH15	ATG TCT TTA CAC CTG GCT CAC	Forward primer for *S. aureus* JE2 *rocD* (SAUSA300_0860) transcriptional analysis by RT-PCR
oLH16	GGC CTG GTA AAT CCT CAT CAA	Reverse primer for *S. aureus* JE2 *rocD* (SAUSA300_0860) transcriptional analysis by RT-PCR
oLH17	CAA ATG ATC ACA GC**A** TTT GGT ACA G	Forward primer for *S. aureus* JE2 *gyrB* (SAUSA300_0005) transcriptional analysis by RT-PCR. Primer sequence taken from Borgogna 2018 J Infect Dis. Adenine residue in bold was not present in primer sequence listed in the publication.
oLH18	CGG CAT CAG TCA TAA TGA CGA T	Reverse primer for *S. aureus* JE2 *gyrB* (SAUSA300_0005) transcriptional analysis by RT-PCR. Primer sequence taken from Borgogna 2018 J Infect Dis.
oLH19	GAA GAG CGT GAA AGT GGT AAA G	Forward primer for detection of *tpiA* transcript by qPCR
oLH20	GAT TGC CCA GAT TGG TTC ATA AG	Reverse primer for detection of *tpiA* transcript by qPCR

Table 2.4 Probes used in the RT-qPCR study.

Name	Sequence	5' modification	3' modification	Primer pair	Comments
prLH1	TGA GAA GAT TAG CAG AAC GCC CAC A	6-FAM	BHQ-1	oLH1/oLH2	Taqman probe for detection of *putA* transcript by qPCR
prLH2	ATG GAA TGG GAG CAA GTC GTT CCA	HEX	BHQ-1	oLH3/oLH4	Taqman probe for detection of *rocD* (SAUSA300_0187) transcript by qPCR
prLH3	CAC CGG TAC ACC AGA GAA CTT CGC	6-FAM	BHQ-1	oLH5/oLH6	Taqman probe for detection of *arcB1* transcript by qPCR
prLH4	TAC CCA TTT GAG AAC CTG TTG GCC C	HEX	BHQ-1	oLH7/oLH8	Taqman probe for detection of *arcB2* transcript by qPCR
prLH5	AAG TTG CAG CGC ATG ATC AAG GTG	6-FAM	BHQ-1	oLH9/oLH10	Taqman probe for detection of *argF* transcript by qPCR
prLH6	AGA AGA TCC TTA TGC TGC GCC ACC	HEX	BHQ-1	oLH11/oLH12	Taqman probe for detection of *argGH* transcript by qPCR
prLH7	AGG TGT TAT TTG GTA TGA TGC ACA TGG TGA	6-FAM	BHQ-1	oLH13/oLH14	Taqman probe for detection of *rocF* transcript by qPCR

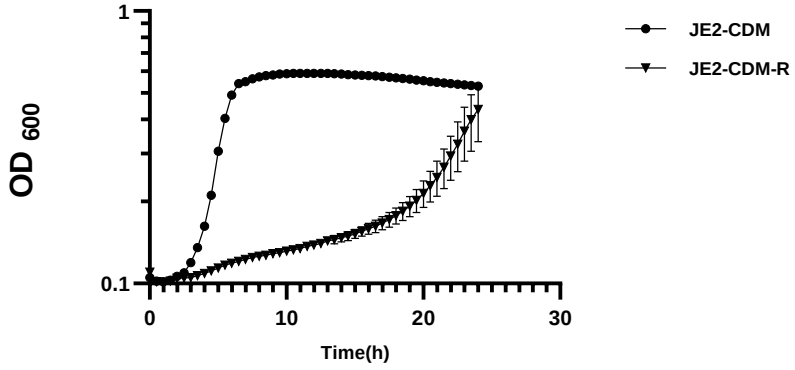
prLH8	TGG TAA TCC ACT TGC TTG TGC TGC	HEX	BHQ-1	oLH15/oLH16	Taqman probe for detection of *rocD* (SAUSA300_0860) transcript by qPCR
prLH9	AAT CGG TGG CGA CTT TGA TCT AGC GAA AG	6-FAM	BHQ-1	oLH17/oLH18	Taqman probe for detection of *gyrB* transcript by qPCR. Probe sequence was taken from Borgogna 2018 J Infect Dis.
prLH10	CCT GCA ACA GCT TTC TTA ACT TGC TCA C	HEX	BHQ-1	oLH19/oLH20	Probe for detection of *tpiA* transcript by qPCR

Chapter 3: Results

Staphylococcus aureus is a functional arginine auxotroph

Based on the study by Nuxoll et al., the growth of *S. aureus* in media lacking arginine can be observed when glucose is not supplemented in the media. Complicating the picture, the growth of *S. aureus* was not observed when grown in a complete defined media lacking arginine without glucose (CDM-R) (**Fig.3.1 A**). However, upon extended incubation, we found that growth is consistently observed after 18-24 hours of growth. To test if this phenomenon is due to the potential induction of a particular metabolic pathway or selection of mutations, we streaked the colonies on Tryptic Soy Agar (TSA) following delayed growth on CDM-R and the growth pattern was tested in CDM-R.These colonies can rapidly grow on CDM-R (**Fig.3.1 B**) suggesting that *S. aureus* is a functional arginine auxotroph unless suppressor mutations facilitate the growth of this strain when exogenous arginine is not supplemented in the media.

A



B

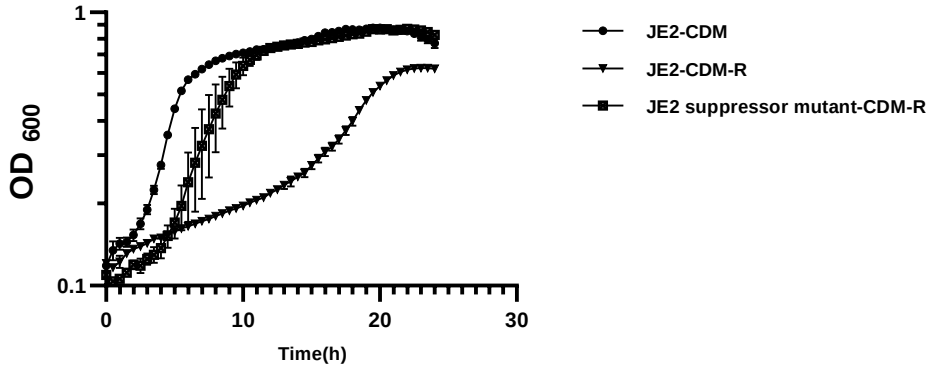


Figure 3.1 Suppressor mutations facilitate the growth of *S. aureus* in CDM-R. Growth analysis of JE2 in (CDM) and (CDM-R). B. Growth analysis of JE2 suppressor mutant in (CDM-R). Data represents means ± SEM of three biological replicates.

S. aureus arcB1 and *ahrC* mutations facilitate growth in CDM-R.

Whole Genome Sequencing (WGS) of suppressors mutants identified two classes of mutations in the isolated colonies. Four isolates have mutations in the promoter region of the *arcA1B1DC*. Interestingly, two isolates have mutations in the Arginine pathway regulatory protein, AhrC. One of the isolates has a substitution mutation changing cysteine (Cys123) into phenylalanine (**Fig.3.2**). This cysteine residue is found at the dimer-trimer interface participating in the formation of an arginine binding pocket. Therefore, since AhrC was reported to be an L-arginine dependent repressor, this mutation will hinder the ability of AhrC to mediate repression of the arginine biosynthetic genes thus enabling the growth of *S. aureus* in complete defined media lacking arginine. The focus of this study will be on the *ahrC* isolate which will be referred to as the JE2 *ahrC* mutant

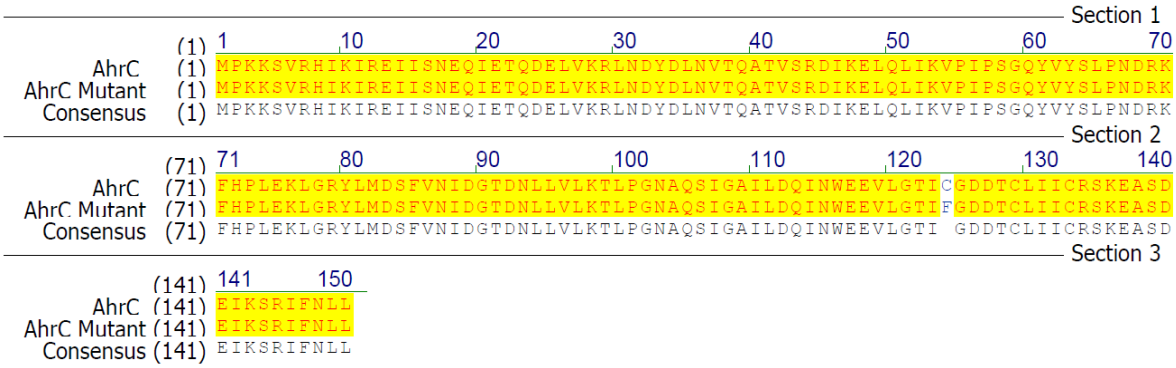


Figure 3.2 JE2 suppressor mutation in the Arginine pathway regulatory protein, AhrC. Alignment of AhrC of the JE2 suppressor mutant against AhrC of wild type JE2.The alignment was done using VectorNTI align-X. Note the change of the cysteine into phenylalanine.

AhrC is the major regulator of arginine biosynthesis in *S. aureus*

To confirm the growth observed in the JE2 *ahrC* mutant, growth analysis of *ahrC* markless deletion JE2 Δ*ahrC* was performed. JE2 Δ*ahrC* exhibits the same phenotype as JE2 *ahrC* mutant when grown in CDM-R confirming that the *ahrC* mutation facilitates the growth of *S.aureus* in the absence of extracellular arginine. Besides, since *S. aureus* encodes three ArgR-type transcriptional regulators, ArgR1 and ArgR2, and AhrC, to eliminate the possibility that ArgR1 and ArgR2 are complementing AhrC function, growth of JE2 *argR1*:: NΣ and JE2 *argR2*:: NΣ was assessed in CDM-R. *argR1*:: NΣ does not grow in CDM-R and *argR2*:: NΣ exhibits a delayed growth reaching OD_{600} after 24 hours (**Fig.3.3**). Taken together, these data suggest that these three transcriptional regulators do not complement each other and that AhrC is the main repressor of arginine biosynthesis.

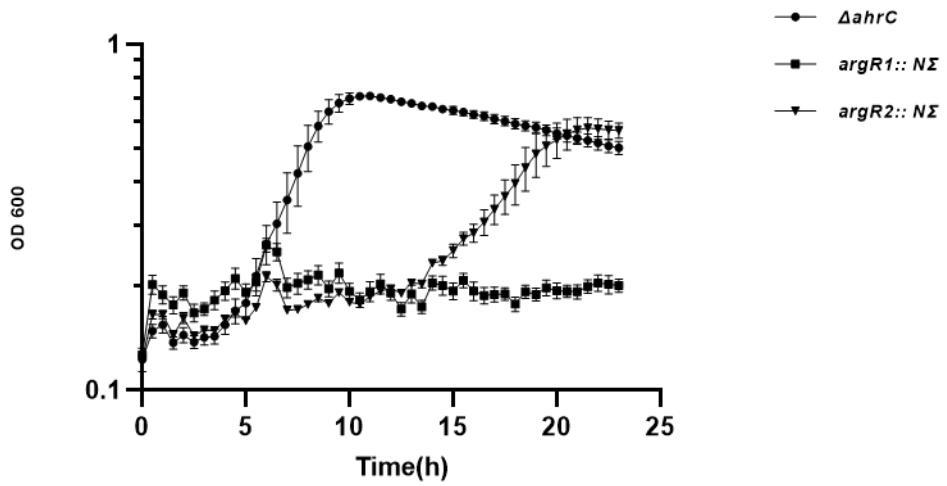


Figure 3.3 Mutation in *argR1* and a*grR2* does not facilitate *S. aureus* growth in CDM-R. Growth curve analysis of *argR1*:: NΣ, *argR2*:: NΣ and Δ*ahrC* in CDM-R. Data represents means ± SEM of three biological replicates.

Arginine biosynthesis is dependent upon proline in *ahrC* mutants

S. aureus harbors the novel proline catabolic pathway as well as the canonical glutamate pathway to synthesize arginine. To investigate which metabolic pathway contributed to the growth of both the JE2 *ahrC* mutant and Δ*ahrC* in CDM-R, transduction of *putA*, *argC* and *argH* was performed. The growth of the resulting mutants was evaluated in CDM-R. Mutation of *putA* and *argH* halted the growth of the JE2 *ahrC* mutant as well as Δ*ahrC*. However, the mutation in *argC* did not affect the growth (**Fig.3.4**) To confirm these results, the JE2 *ahrC* mutant was grown in CDM-R in the presence of either $^{13}C_5$-Pro or $^{13}C_5$-Glu. $^{13}C_5$-labelled arginine was detected in the presence of proline and not glutamate (**Fig.3.5**). These data strongly suggest that *S. aureus* relies on proline as a precursor to synthesize arginine and that unlike AhrC in *Bacillus subtilis*, AhrC in *S. aureus* appears to regulate arginine biosynthesis via proline with no repression being exerted on the *argDCJB* operon.

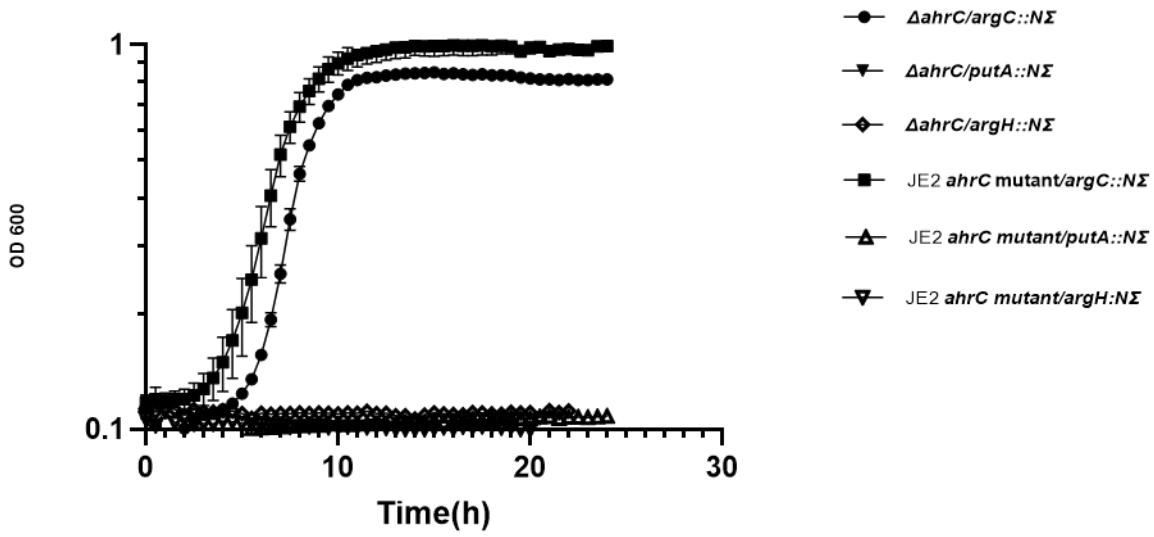


Figure 3.4 Proline catabolism facilitates growth *ahrC* mutants in CDM-R.Growth curves analysis of ΔahrC/putA::NΣ,ΔahrC/argC:: NΣ , ΔahrC/argH:: NΣ, JE2 ahrC mutant/ putA::NΣ,JE2 ahrC mutant/ argC:: NΣ, JE2 ahrC mutant/ argH:: NΣ in CDM-R. Data represents means ± SEM of three biological replicates.

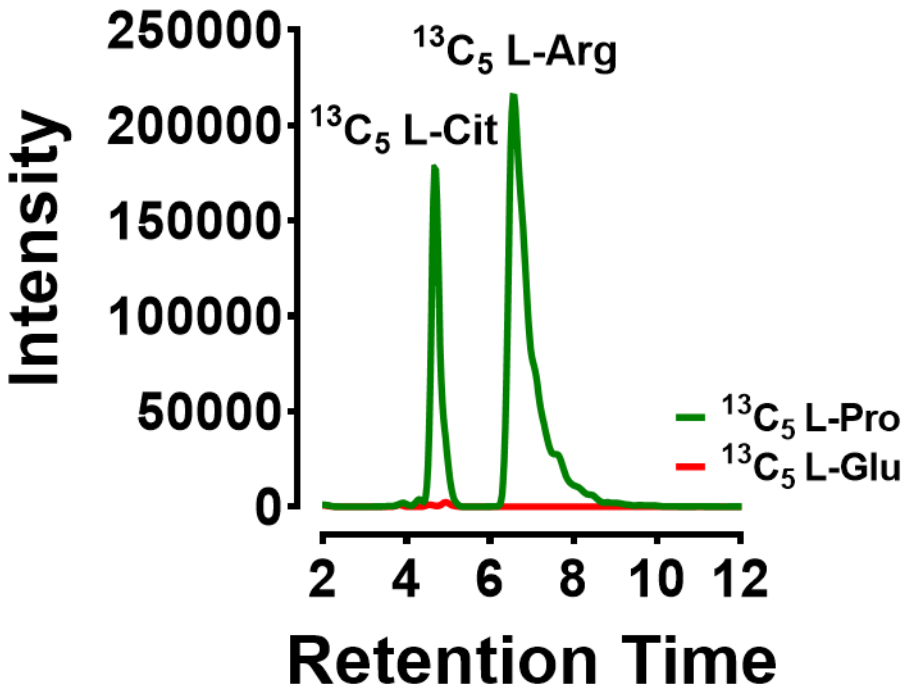


Figure 3.5 Growth JE2 *ahrC* mutant is dependent upon arginine biosynthesis via proline. Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) of JE2 ahrC mutant grown in CDM-R in the presence of either 13C5-Proline or 13C5-Glutamate.Data represent active peaks out of three biological replicates are displayed

AhrC functions to repress *argGH* and *arcB1* in the absence of extracellular arginine.

To investigate how AhrC regulates arginine biosynthesis in *S.aureus,* quantitative reverse transcription (RT-qPCR) was performed to assess the transcription of arginine biosynthetic genes in the JE2 *ahrC* mutant and *ΔahrC* when grown in CDM and CDM-R compared to wild type JE2 in CDM. Transcription of *putA ,argD ,arcB1 ,arcB2 ,arg F,argG ,rocF, rocD* were assessed. A significant change in the gene expression of *argG* and *arcB1* was observed with approximately 3000 and 100 fold change, respectively, in Δ*ahrC* when grown in CDM-R compared to wild type JE2 in CDM (**Fig.3.6**). Similar results were observed in the JE2 *ahrC* mutant with no change of *argDCJB* gene expression in both *ΔahrC* and JE2 *ahrC mutant*. Therefore, the transcription analysis results demonstrate that in *S. aureus*, AhrC functions to repress *argGH* and *arcB1* in absence of arginine allowing for arginine biosynthesis via proline. Indeed, as stated above, growth in CDM-R via an *ahrC* mutation is proline-dependent and glutamate-independent. In addition, our LC-MS/MS demonstrated the accumulation of $^{13}C_5$-ornithine and $^{13}C_5$-citrulline in the presence of ^{13}C5-proline and not glutamate (**Fig. 3.7**). ArcB1 catalyzes the formation of citrulline by using ornithine as a substrate whereas citrulline is subsequently converted into arginine through the activity of ArgG and ArgH, further confirming our transcriptional data as well as that *argGH and arcB1* facilitate the biosynthesis of arginine via proline.

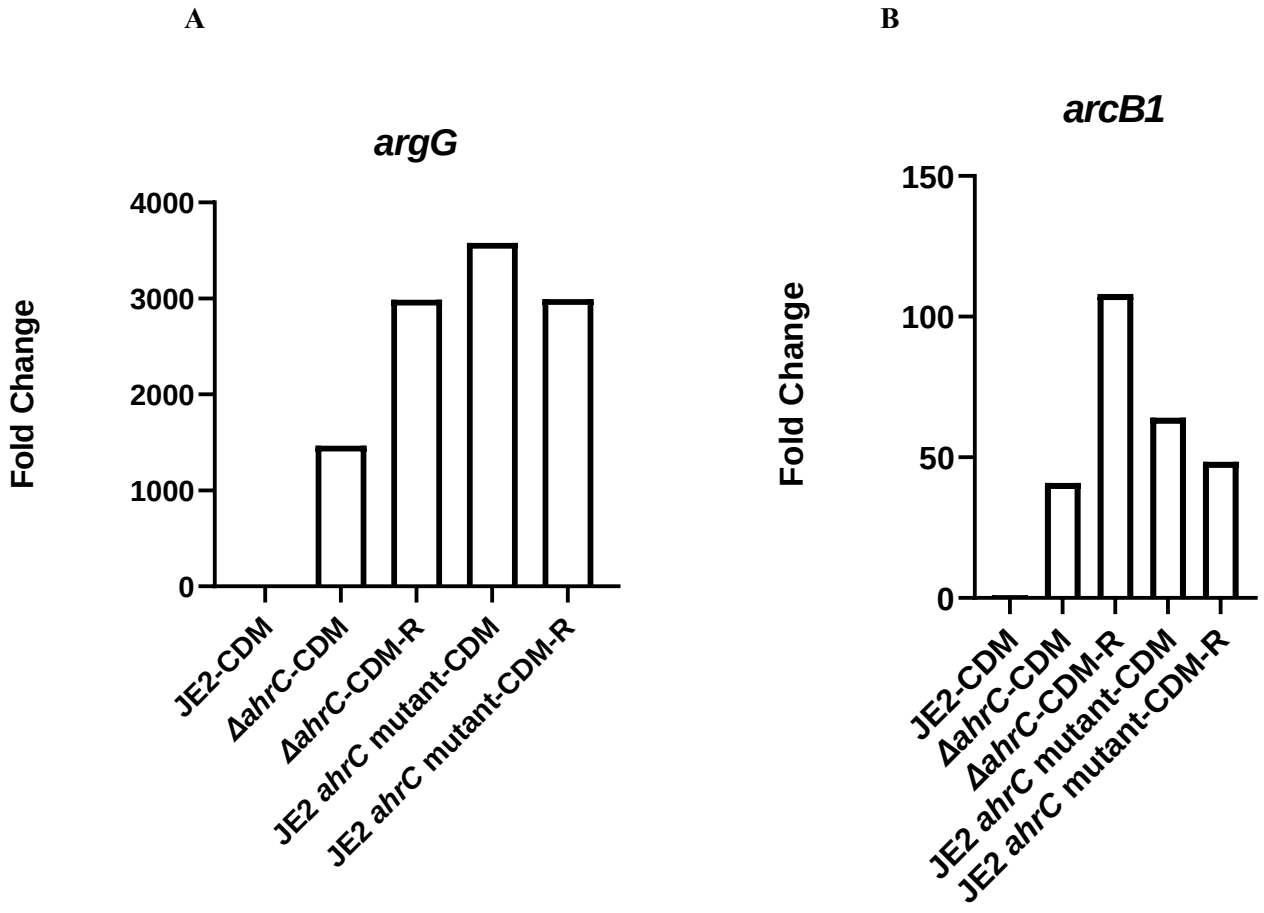
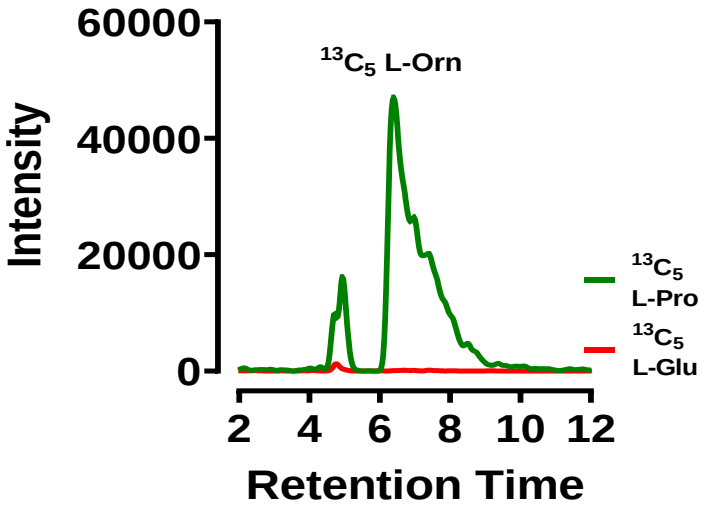


Figure 3.6 *ahrC* mutation augments *argGH and arcB1* transcription allowing for arginine biosynthesis via proline. Quantitative reverse transcription PCR (RT-qPCR) assessing the expression of arginine biosynthesis genes in Δ*ahrC* and JE2 *ahrC* mutant. **A**) Relative expression of *argG, a transcriptional unit representing argGH operon,* in both CDM and CDM-R. **B**) Relative expression of *arcB1, a transcriptional unit representing arcA1B1DC operon,* in both CDM and CDM-R.

A



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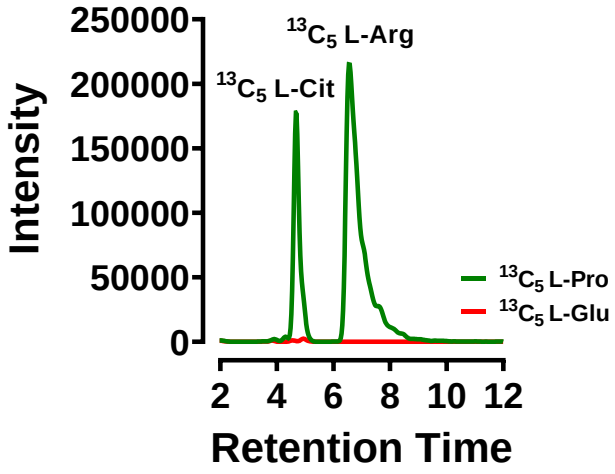

Figure 3.7 ArcB1, ArgG, ArgH catalyzes the formation of arginine from proline. Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) of JE2 *ahrC* mutant grown in CDM-R in the presence of either $^{13}C_5$-Proline or $^{13}C_5$-Glutamate.Accumulation of both A) ornithine and B) citrulline was detected in the presence of ^{13}C5-Proline. Representative date from three biological replicates.

Chapter 4: Discussion

Historically, the extensive studies of the arginine biosynthetic pathway have led to the disclosure of multiple findings, which has set the basic features of metabolic regulation and brought forth numerous scientific notions such as repression, repressor, and regulon [12, 13, 31]. Publications identifying enzymes and genes which are necessary for arginine biosynthesis have brought insights of evolutionary significance [6]. The study on the *arg* regulon has led to multiple discoveries including gene duplication, the mechanism involved in the reactivation of silent genes through the generation of tandem repeats, and the precise function of a transposon as a mobile promoter as well as the origin of divergent promoters [32-35].

Despite harboring the biosynthetic capability to synthesize arginine, *S. aureus* exhibits auxotrophy for arginine when grown on a defined laboratory media lacking arginine [36]. We demonstrated that growth in media lacking arginine can be selected after extended incubation. We have shown that this delayed growth is due to the selection of mutations in *ahrC* loci. AhrC is a transcriptional regulator that represses arginine biosynthesis when arginine is present by binding to an operator site called the **ARG box** found upstream of arginine biosynthetic genes (***argJBCDFGH)***. Experimental evidence indicates that this mechanism of regulation is conserved among gram-positive as well as gram-negative bacteria. our data provided evidence that AhrC in *S. aureus* regulates arginine biosynthesis via proline and not glutamate. Indeed, mutations in *putA* and *argH* have completely abrogated the growth of JE2 *ahrC* mutant as well as Δ*ahrC* in CDM-R while a mutation in *argC* did not affect the growth. Furthermore, our LC-MS/MS demonstrated the accumulation of $^{13}C_5$-arginine when the JE2 *ahrC* mutant was grown in CDM-R in the presence of $^{13}C_5$-proline and not $^{13}C_5$-glutamate further confirming that AhrC functions to regulate arginine biosynthesis via proline and not glutamate. Also, our transcriptional analysis revealed that AhrC represses *argGH* and *arcB1* with no change in gene expression of *argDCJB*. Our LC-MS/MS analysis supports these data with the accumulation of ornithine and citrulline was observed in the

presence of $^{13}C_5$-proline suggesting that proline is being catabolized into arginine by the enzymatic activity of *arcB1*, *argG* and *argH*. These data together suggest that AhrC functions to repress *argGH* transcription allowing for arginine biosynthesis via proline. These data also suggest the presence of an additional transcriptional regulator functioning to repress the production of arginine from glutamate. Further, results from our laboratory demonstrate that a *putA* mutant does not grow in defined media lacking arginine indicating that the glutamate pathway is inactive *in vitro*. However, the selection of *putA* suppressor mutants that can grow in a media lacking arginine has allowed us to identify that the glutamate pathway is induced when **SpoVG**, a DNA binding protein is mutated (data not shown). However, it is still unknown how **SpoVG** regulates arginine biosynthesis and why *S. aureus* has adopted a novel pathway via proline for arginine biosynthesis. Using proline as a precursor suggests that *S. aureus* is maintaining the glutamate pool to be used by other biosynthetic pathways. It is well documented that glutamate is an important precursor to produce siderophores. Siderophores are iron scavenging proteins secreted by *S. aureus* to overcome the iron limitation. *S. aureus* harbors two siderophores, Staphyloferrin A (SA) and Staphyloferrin B (SB). SA production relies on TCA cycle activity, however, interestingly, SB production is independent of TCA cycle activity and all its precursors are generated through the metabolism of glutamate. This independence from the TCA cycle highlights the importance of glutamate in the production of staphyloferrin B, which was shown to be more important *in vivo* than the other siderophores [37]. Iron sequestration from the host contributes to *S. aureus* virulence and its ability to cause severe and invasive infection. Hence, the exploitation of glutamate by the arginine biosynthetic pathway may come at a cost, making *S. aureus* less virulent and attenuated for pathogenesis**.**

Chapter 5: Concluding Remarks and Future Directions.

S. aureus can invade multiple organ systems causing infection in diverse metabolic environments. To survive and proliferate within the host, *S. aureus* has evolved to fine-tune its metabolism to utilize carbon sources unique to each niche. Several studies have been conducted on *S. aureus* metabolism, however, little emphasis has been given on how arginine metabolism facilitates the proliferation of this organism during infection. Investigating important metabolic pathways is crucial and leads to the development of therapeutic that disrupts critical metabolic pathways and limits the growth of this highly adaptable pathogen.

It has been known for decades that *S. aureus* is auxotrophic for many amino acids including arginine. We identified that after extended incubation, *S. aureus* can be trained to grow in a defined media lacking arginine via the selection of mutations. We have also demonstrated that arginine biosynthesis via proline is under repression, with AhrC repressing *argGH* and *arcB1*. Interestingly, the selected mutants still do not synthesize arginine via glutamate. However, using a mutant unable to synthesize arginine via proline, we identified mutants able to synthesize arginine via glutamate. Through the selection of suppressors mutants, we identified SpoVG as a potential transcriptional regulator of glutamate-mediated arginine biosynthesis. In the future study, we will determine how SpoVG regulates arginine biosynthesis and if SpoVG interacts with AhrC to mediate effective and strong regulation. In addition, the biosynthesis of arginine via glutamate is tightly regulated in *S. aureus*. It is unclear when or if *S. aureus* derepresses the glutamate biosynthetic pathway during host invasion. In our future study, we will also determine the impact of the induction of the glutamate pathway on the ability of *S. aureus* to colonize and survive within different niches. The generated data will provide insight into whether the differential regulation selected within *S. aureus* is beneficial or if it negatively impacts other metabolic strategies employed by *S. aureus* to thrive in nutrient-limited environments.

In conclusion, *S. aureus* appears to regulate the arginine biosynthetic pathway differently. How this regulation of biosynthetic pathway impacts *S. aureus* virulence and persistence has not been determined to date. Therefore, further studies are required to understand how metabolic regulation is intricately associated with the ability of *S. aureus* to survive within a hostile environment and how *S.aureus* tailors niche-specific biosynthesis regulation by modulating the function of transcriptional regulators.

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