

ROLE OF BRANCHED-CHAIN AMINO ACID TRANSPORTERS IN
STAPHYLOCOCCUS AUREUS VIRULENCE

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by

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Abstract

Branched-chain amino acids (BCAAs) act as effector molecules that signal a global transcriptional regulator, CodY, to regulate virulence factors in nutrient depleted environments. *Staphylococcus aureus* contains three putative BCAA transporters (BrnQ1, BrnQ2, BrnQ3) whose role in BCAA uptake is unknown. We hypothesize that BrnQ transporters are involved in BCAA uptake and contribute to virulence in *S. aureus* by modulating CodY activity. Results from radioactive uptake assays indicate that BrnQ1 is the predominant BrnQ transporter of isoleucine, valine and leucine. Meanwhile, BrnQ2 is more specific for isoleucine. Furthermore, only the lack of BrnQ1 hinders growth of *S. aureus* in chemically-defined media with limited concentrations of BCAAs; however, BrnQ2 and BrnQ3 show no such growth phenotype. *In vivo*, the *brnQ1* and *brnQ3* mutants are attenuated for virulence in a mouse model of bacteremia, but the *brnQ2* mutant was hyper-virulent, suggesting that the gene plays a more important role within the host.

Keywords

Staphylococci, branched-chain amino acid, transporter, CodY, virulence

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mutant *in vivo*

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List of Abbreviations

ABC	ATP-binding cassette
Amp	Ampicillin
ATP	Adenosine triphosphate
BCAA	Branched-chain amino acid
bp	base pair
CA	Community- acquired
CDM	Chemically-defined media
CFU	Colony forming units
Cm	Chloramphenicol
CoNS	Coagulase negative staphylococci
dNTPs	Deoxynucleotide triphosphates
DNA	Deoxyribonucleic acid
Kb	Kilobase
LB	Luria- Bertani
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
M	Molar
mg	Milligram
MGE	Mobile genetic element
ml	Milliliter
mM	Millimolar
nm	nanometer
OD	Optical density

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pM	Picomolar
PMF	Proton motive force
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
STM	Signature-tagged mutagenesis
Tet	Tetracycline
TSB	Tryptic soy broth
µg	Microgram
µl	Microliter
UV	Ultra-violet
w/v	Weight per volume

Chapter 1- Introduction

1.1. The Staphylococci

1.1.1. The genus *Staphylococcus*

The staphylococci are part of the family of gram-positive cocci, *Staphylococcaceae*. The term “staphylococcus” is a derivative of the Greek word *staphylé*, meaning, “bunch of grapes”, because of their formation of grape-like clusters. The term “coccus,” means grain or berry. Presently, there are 32 species and eight sub-species in the genus *Staphylococcus*, many of which are colonizers of the human body (64), however the two most well-characterized species are *Staphylococcus aureus* and *Staphylococcus epidermidis*.

1.1.2. Characteristics of staphylococci

The staphylococci are non-motile, non-spore forming facultative anaerobes that grow most rapidly under aerobic conditions and in the presence of CO₂. They are also catalase-positive and oxidase-negative, differentiating them from the streptococci, which are catalase-negative (142). Pathogenic staphylococci can be identified by their production of coagulase, which causes plasma to coagulate by converting fibrinogen into fibrin (65). This distinguishes *S. aureus*, a coagulase positive species, from more than 30 species of coagulase negative staphylococci (CoNS), of which *S. epidermidis* is perhaps the most well known.

Staphylococci have a diameter of 0.7–1.2 µm and have a gram-positive cell wall. Their ability to form clusters is due to cell division taking place in more than one plane. *S. aureus* forms medium-sized “golden” colonies on rich medium. *S. aureus* forms β-hemolytic colonies on sheep blood agar, due to the production of several hemolysins such as α-toxin, β-toxin, γ-toxin, and δ-toxin. Some *S. epidermidis* strains can also be β-hemolytic due to the production of δ-toxin (131). Moreover, pigmentation of *S. aureus* is more prominent after 24

hours of growth and when incubated at room temperature (60, 143). In *S. aureus*, the golden pigmentation of the colonies is caused by carotenoids, whose biosynthetic pathway has recently been determined (107). Moreover, the carotenoids have been shown to act as virulence factors, due to their ability to protect the pathogen from oxidants produced by the immune system (79).

Staphylococci have the ability to grow in a wide pH range (4.8–9.4), are resistant to drying, and can persist even at extremely high temperatures. Moreover, *S. aureus* can tolerate high-salt concentrations (7.5–10% NaCl) due to the production of osmoprotectants (142). The ability of *S. aureus* to ferment mannitol permits it to be differentiated from CoNS such as *S. epidermidis* and *S. saprophyticus*. In addition, *S. aureus* can also metabolize various other carbon sources such as glucose, lactose, xylose, sucrose, maltose, and glycerol. Moreover, growing the cells in the presence of novobiocin can further differentiate staphylococci. *S. xylosus* (140) and *Staphylococcus saprophyticus* (136) are intrinsically resistant to novobiocin, however, other CoNS such as *S. epidermidis*, *S. haemolyticus*, and *S. lugdunensis* are susceptible to novobiocin (140).

The bacterial membrane consists of a lipid bilayer composed of layers with asymmetrically placed lipids (97). The lipid composition in the staphylococcal membrane is found to differ during various phases of growth (12, (122, 141), permitting membrane adaptability in response to environmental, host defense-related, and antimicrobial stresses. *S. aureus* membrane is comprised of several phospholipids including, phosphatidic acid, lysyl-phosphatidylglycerol, phosphatidylglycerol, cardiolipin, phosphatidylethanolamine and phosphatidylglucose (97, 122, 141). These phospholipids, along with the carotenoids and the glucolipids, form the chief constituents of the staphylococcal membrane (122).

Because proper cell function depends on the maintenance of optimal membrane

fluidity, staphylococci must adapt to changes within the growth environment by adjusting their membrane fluidity (17). Adjustments in membrane fluidity, that are dependent on changes in lipid composition, must occur in response to environmental changes in pH, pressure, temperature, nutrient availability, xenobiotics, and ion concentrations. In several bacterial species, the disruption of the lipid arrangement can result from the introduction of unsaturated fatty acids into the lipid bilayer. This, in turn, leads to a subsequent increase in membrane fluidity. However, *S. aureus* does not employ this strategy. In contrast, it utilizes anteisobranched of the fatty acids to increase fluidity in response to induced stress, such as exposure to high salt concentrations (73), and increases iso-branched fatty acids and carotenoids to decrease fluidity. Branched-chain fatty acid production is regulated by YycFG: a two-component regulatory system (86).

1.1.3. Clinical Importance of the staphylococci

S. aureus is a commensal and a pathogen. In humans, the major site of *S. aureus* colonization is the anterior nares. About 20–30% of individuals are persistent carriers of *S. aureus* and 30% are intermittent carriers; conversely, 30% of people resist nasal colonization (67, 139).

S. aureus is the major cause for many hospital- and community-acquired infections which can result in severe health consequences (26). Nosocomial *S. aureus* infections affect several parts of the body, including the bloodstream, soft tissues, skin, and lower respiratory tracts. It is also responsible for causing severe diseases such as serious deep-seated infections (i.e osteomyelitis and endocarditis), scalded skin syndrome, toxic shock syndrome, and staphylococcal foodborne diseases (117).

Staphylococcus epidermidis, a well-characterized CoNS, dwells more permanently on

the skin due to its high tolerance to salt, acidic pH, and lipids found on skin (73) compared to *S. aureus*, which is not as persistent on the skin. Numerous staphylococci are also associated with disease in humans and animals. *Staphylococcus saprophyticus* is notorious for causing urinary tract infections (112). Indeed, CoNS frequently bind to tissues via hemagglutinin, fibronectin, teichoic acids, and autolysins on the skin and mucous membranes (41, 100). *Staphylococcus anaerobius* is a pathogen found in sheep and causes skin abscess, but on rare occasion, produces abscesses or sepsis in humans (106).

CoNS also play a role in bacteremia, infections of the central nervous system, endocarditis, urinary tract infection, surgical site infections, and many more. Currently, a major difficulty lies upon differentiating clinically significant, pathogenic and contaminating isolates of CoNS.

1.1.4. Emergence of Antibiotic Resistant Strains of *S. aureus*

β -lactam antibiotics interfere with cell wall synthesis by inactivating the transpeptidases responsible for cross-linking peptidoglycan chains in the cell wall. As a result, the structure of the cell wall is compromised, hampering cell wall-based metabolism and cell division, as well as the function of the cell wall, therefore leading to autolysis (126). Prior to the introduction of antibiotics in the 1940s, mortality was a common consequence of invasive staphylococcal infections (124). In 1941, when penicillin was introduced clinically, mortality due to staphylococcal infections reduced greatly (128). Unexpectedly, in 1942, penicillin resistance in staphylococcal infections was reported (113) and by the late 1940s, penicillin was commonly used in hospitals and penicillin-resistant strains began to appear in the clinical setting, exceeding sensitive strains (12). Penicillin-resistance was due to the production of a serine protease, β -lactamase, which hydrolyzes penicillin's β -lactam ring

(81). Since penicillin was no longer an effective means of treatment for most *S. aureus* infections, semisynthetic β -lactamase-resistant penicillins, such as methicillin and oxacillin, were generated to treat methicillin-sensitive *S. aureus*. However, within a few years of its usage, methicillin-resistant *S. aureus* (MRSA) strains began to surface (103). From 1975 to 1991, hospitals in the United States reported an increase in the proportion of *S. aureus* strains resistant to methicillin. By 1991, 79% of hospitals were reporting MRSA cases (103) and by 2003, approximately 60% of *S. aureus* strains isolated from intensive care units were methicillin resistant (3). As a result, even until now, MRSA is a serious concern for public health.

MRSA is unique due to the presence of the staphylococcal cassette chromosome, *mec* (SCC*mec*); a large mobile genetic element that carries the *mecA* gene. This gene codes for PBP2a, an altered penicillin binding protein, that carries low binding affinity to all β -lactams compared to PBP2 found in methicillin sensitive strains (5, 59). Until recently, vancomycin has remained an effective treatment against most MRSA infections; however, there is growing concern for the potential emergence of vancomycin resistant *S. aureus* strains. These concerns stem from reports of clinical isolates of certain species of coagulase-negative staphylococci that developed resistance to vancomycin (116, 118). Furthermore, vancomycin-resistant enterococci (VRE) had surfaced and spread in many hospital settings in the United States (96).

Although the persistence and high level of antibiotic resistance of strains in the hospital setting have been a serious concern, the rise of community-acquired MRSA (CA-MRSA) has also become a serious public health issue and a financial burden on the health care system. CA-MRSA have been shown to be more virulent and often more lethal compared to traditional hospital-acquired MRSA (HA-MRSA), causing severe and often

fatal infections in otherwise healthy individuals (38). Indeed, community-acquired strains had begun to surface in diverse communities including American Indian and Alaska Natives (2, 10), sports teams (46), prisoners (1), and children (62). By 2002, up to 20% of CA-MRSA isolates were obtained from healthy individuals in the United States who had no established risk factors (40).

A common factor contributing to the pathogenic success of all CA-MRSA is the presence of type IV SCCmec element (24). Relative to other SCCmec elements, the SCCmec type IV is smaller in size. Moreover, it has been suggested that this element does not impose a fitness cost to the bacteria, in contrast to other SCCmec elements, since growth of strains carrying SCCmec type IV is not significantly hampered compared with strains that lack this element (20, 28). This may be due to the absence of resistance genes other than PBP2a, possibly suggesting in part why CA-MRSA disseminates in the community easily. Furthermore, SCCmec type IV was possibly acquired from *S. epidermidis* (144), illustrating the important role of horizontal gene transfer for *S. aureus* evolution (101).

1.1.4.1. CA-MRSA USA300

In the United States, the large-scale outbreak caused by the CA-MRSA USA300 is, one of the most severe in terms of occurrence and severity of infection (38, 88, 94). Indeed, USA300 has become a predominant cause of skin and soft tissue infections in emergency room patients in the United States (94). Moreover, USA300 is also an increasingly common cause of diseases in hospitals (105).

The astonishing pandemic success of USA300 and its dominance over other CA-MRSA clones may, in part, be due to the presence of the type I arginine catabolic mobile element (ACME) that aids in its colonization and transmission. In USA300, ACME is a 31-

kb MGE that is physically linked to SCCmec IV and was likely transferred from *S. epidermidis* (27). This mobile genetic element encodes two main gene clusters: an arginine deiminase (*arc*) and an oligopeptide permease (*opp*) operon. Arginine deiminase activity, which is responsible for ammonia and ATP production, facilitates colonization via energy production and neutralization of the acid environment on the skin (27, 28). In addition, this aids in the prevention of nitric oxide (NO) production, a key molecule utilized by the human innate and adaptive host defenses (92). Oligopeptide permeases are also often key players in peptide and essential nutrient uptake by bacterial cells. They may also play a potential role in quorum sensing, chemotaxis, cell adhesion, and resistance to antimicrobial peptides (27).

1.1.5. *S. aureus* virulence factors

The ability of *S. aureus* to cause a wide array of diseases and persist within the host is primarily due to the extensive arsenal of virulence factors expressed by the bacteria, which include both structural and secreted products participating in pathogenesis (summarized in Table 1).

Table 1. Summary of *Staphylococcus aureus* virulence factors

Virulence Factor	Gene	Function/ Effect
Accessory gene regulator	<i>agr</i>	Regulates expression of cell-surface and secreted proteins
Staphylococcal accessory regulator	<i>sarA</i>	Up-regulates adhesion molecules and down-regulates expression of exo-proteins
Alpha toxin	<i>hla</i>	Cytolytic pore-forming toxin
Beta hemolysin	<i>hlb</i>	Cytolytic pore-forming toxin
Delta hemolysin	<i>hld</i>	Heat stable pore-forming toxin
Gamma hemolysin	<i>hlg</i>	Cytolytic pore-forming toxin
Panton-Valentine Leukocidin	<i>lukPV</i>	Damages leukocyte membranes
Protein A	<i>spa</i>	Bind Fc portion of IgG molecules
Fibronectin binding protein	<i>fnbAB</i>	Adhesion to host tissues
Fibrinogen binding protein	<i>fbp</i>	Adhesion to host tissues
Collagen binding protein	<i>cna</i>	Adhesion to collagenous tissues and cartilage
Coagulase	<i>coa</i>	Activates prothrombin
Clumping factor	<i>clfA</i>	Fibronectin binding protein, adhesion to host tissues
V8 Serine protease	<i>sspA</i>	Cleaves extracellular proteins
Staphopain B cysteine protease	<i>sspB</i>	Cleaves extracellular proteins
Extracellular proteases	<i>splA-F</i>	Secreted proteases
Staphylokinase	<i>sak</i>	Dissolves fibrin clots
Toxin shock syndrome toxin-1	<i>tst</i>	Causes acute systemic illness
Capsule	<i>cap</i>	Polysaccharide capsule production
Exfoliative toxins	<i>etaAB</i>	Cause of staphylococcal scalded skin syndrome
Staphylococcal enterotoxin	<i>ent</i>	Superantigen toxins, common cause of food poisoning, stable in GI-tract

The ability of *S. aureus* to adhere to plasma and extracellular matrix (ECM) proteins deposited on biomaterials contributes to the pathogenesis of device-related infections. *S. aureus* encodes for several specific adhesion molecules that are expressed on the cell surface, many of which interact with a number of host proteins, such as collagen, fibrinogen, vitronectin, fibronectin and laminin (37), and have been designated MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). MSCRAMMs facilitate host tissue attachment, initiation of colonization, and dissemination of infection (44). Once an infection has been established, *S. aureus* is capable of secreting toxins that perturb the membranes of host cells, such as erythrocytes, leucocytes, and platelets. Cytolytic toxins (i.e. α -hemolysin, β -hemolysin, γ -hemolysin, leukocidin, and Panton-Valentine leukocidin (PVL) form β -barrel pores in cytoplasmic membranes and facilitate cell content leakage and lysis (36). Indeed, many of these toxins (i.e. PVL and α -toxin) are highly expressed in CA-MRSA (36).

S. aureus produces a group of heat- and protease-resistant immune-stimulatory proteins. These toxins induce the cross-linking of MHC class II molecules found on antigen-presenting cells with T-cell receptors, thereby causing substantial cytokine production and release, and inducing capillary leakage, epithelial damage and hypotension (11). Moreover, the super-antigens are believed to weaken the host's immune system to allow for the propagation and persistence of infection (70). The staphylococcal enterotoxins are responsible for staphylococcal foodborne diseases (i.e. gastroenteritis), while TSST-1 is responsible for causing toxic shock syndrome (11). Many *S. aureus* virulence factors, and the super-antigen toxins specifically, are usually encoded by mobile genetic elements such as pathogenicity islands or prophages (98).

1.1.6. Regulation of genes involved in virulence

The growth and survival of bacteria is dependent on the cell's ability to adapt to environmental changes. The genes coding for virulence factors are tightly regulated and are synchronized with their biological cycle. The quorum sensing mechanism in *S. aureus* is responsible for controlling the production of a wide array of virulence factors. During early stage of growth in *S. aureus*, there is an upregulation of genes encoding for surface proteins, in contrast to the up-regulation of secreted proteins in late exponential or stationary phase. Prior to invasion, *S. aureus* expresses numerous proteins implicated in adhesion and defense against the host's immune system (protein A, coagulase, fibronectin binding proteins, etc.). Once the cells have established an infection in the host, *S. aureus* produces a series of secreted hemolysins, proteases, cytotoxins, and other degradative enzymes, to propagate invasion.

The regulation of virulence factor expression in *S. aureus* is very complex and falls under the control of two major families of global regulators that have been well-characterized: two-component regulatory systems (TCRS) and the SarA family of proteins. The system encoded at the *agr* (accessory gene regulator) locus encodes a quorum sensing, two-component regulatory system that controls synthesis of a regulatory RNA, RNIII, which due to its extensive secondary structure is able to regulate the expression of some 138 genes at both the transcriptional and translational levels (95, 99). Expression of *agr* peaks during the post-exponential phase of growth and corresponds with an up-regulation of toxin production and simultaneous decrease in expression of cell wall associated proteins (16). Meanwhile, the *sarA* locus is a DNA binding protein that binds as a homodimer to the so-called "Sar box" in the promoter region of its target genes, which include numerous cell-wall proteins, including adhesion molecules, and also acts as an activator of *agr* expression (15).

SarA is most highly expressed during exponential phase when the expression of cell adhesion molecules is at its highest. Indeed, the interplay between these two systems, alongside several other regulators of gene expression, results in *S. aureus* persistence within the host. Although several regulatory systems have been identified as regulating virulence genes (15), there is a limited understanding of the ability of *S. aureus* to sense and respond to the nutritional status of the environment. Indeed, there is another protein that is currently emerging as a link between environmental nutrient availability and regulation of pathogenesis.

1.2. CodY

CodY, a highly conserved regulatory protein, plays a key role in stationary phase adaptation in low-G+C gram-positive bacteria, and is currently emerging as a global regulator of virulence in *S. aureus* (83, 110, 134) as well as in other gram-positive pathogens (13, 30, 53, 57, 84, 110). First identified in two nonpathogenic species, *Bacillus subtilis* and *Lactococcus lactis* (47, 125), CodY is able to sense nutrient availability by directly interacting with metabolite effectors. Functional homologs of CodY, define a unique, winged helix-turn-helix-containing family of transcription factors. By sensing intracellular levels of branched-chain amino acids (BCAAs; isoleucine, leucine, and valine) and/or GTP during growth, CodY is able to respond by repressing genes involved in nitrogen metabolism (oligopeptide transporters, toxins, proteases, genes involved in amino acid biosynthesis and transport) and activating transcription of genes of the carbon overflow pathway (47, 121). As a result, by interacting with BCAAs and GTP, CodY is able to sense both carbon and nitrogen availability, and is also linked with the stringent response. Moreover, the regions of *B. subtilis* CodY predicted to be important for DNA binding (61, 77) and for BCAA

interaction (22, (138) are highly conserved in CodY proteins from many species, including *S. aureus* (77).

For *B. subtilis*, *Clostridium difficile*, *Listeria monocytogenes*, *Bacillus cereus*, and *S. aureus* CodY, the effectors are GTP and the BCAAs (13, 30, 57, 91, 114, 121). GTP and the BCAAs act independently and synergistically to increase CodY affinity for its DNA target sites (49, 138). In contrast, *L. lactis* and *Streptococcus pneumoniae* CodY proteins respond solely to BCAAs (53, 109). In *S. aureus*, abundance of BCAAs and GTP yields high CodY activity in rapidly growing cells. As intracellular BCAA and GTP pools decline, CodY loses its affinity for its DNA target sites, bringing about de-repression of its target genes (129). This allows the cells to transition from growth and division to amino acid metabolism and stress tolerance, highlighting the central role of CodY in gram-positive metabolic regulation and virulence.

1.2.1. Effectors of CodY

BCAAs stimulate conformation change by binding to the N-terminal GAF domain of CodY, and this is thought to increase the affinity of CodY for its DNA target sites (25, 76, 77). In *L. lactis*, a study was conducted to investigate which BCAA(s) is/are needed for CodY activity (48). Indeed, CodY binding was significantly enhanced with the presence of isoleucine, however, both leucine and valine had a negligible effect in DNA binding (48). In *S. aureus*, isoleucine also appeared to be the major cofactor for CodY (110). Moreover, growth inhibition was a direct result of excess isoleucine, due to abnormal activation of CodY. This may be due to blockage of metabolic pathways involved in amino acid synthesis (48). On the other hand, valine was also able to bind to *B. subtilis* CodY, and had a 18-fold higher affinity for DNA (77).

Similar to BCAAs, there is a decline in intracellular GTP levels when bacteria enter stationary phase of growth and are exposed to nutrient limited environments. For example, the intracellular GTP concentration in *B. subtilis* is predicted to range from 2 to 3 mM during the exponential phase of growth, and drops to approximately 300 uM during the early-stationary phase (49, 127). By binding GTP, CodY is able to directly sense the cell's energetic status and may control the expression of several genes responsible for morphological differentiation and development. The binding site of GTP in the CodY protein remains unclear, but it is certainly not located in the GAF domain (49, 76, 77). Lastly, the difference in gene repression by BCAA-CodY or GTP-CodY is not yet elucidated.

A.



B.

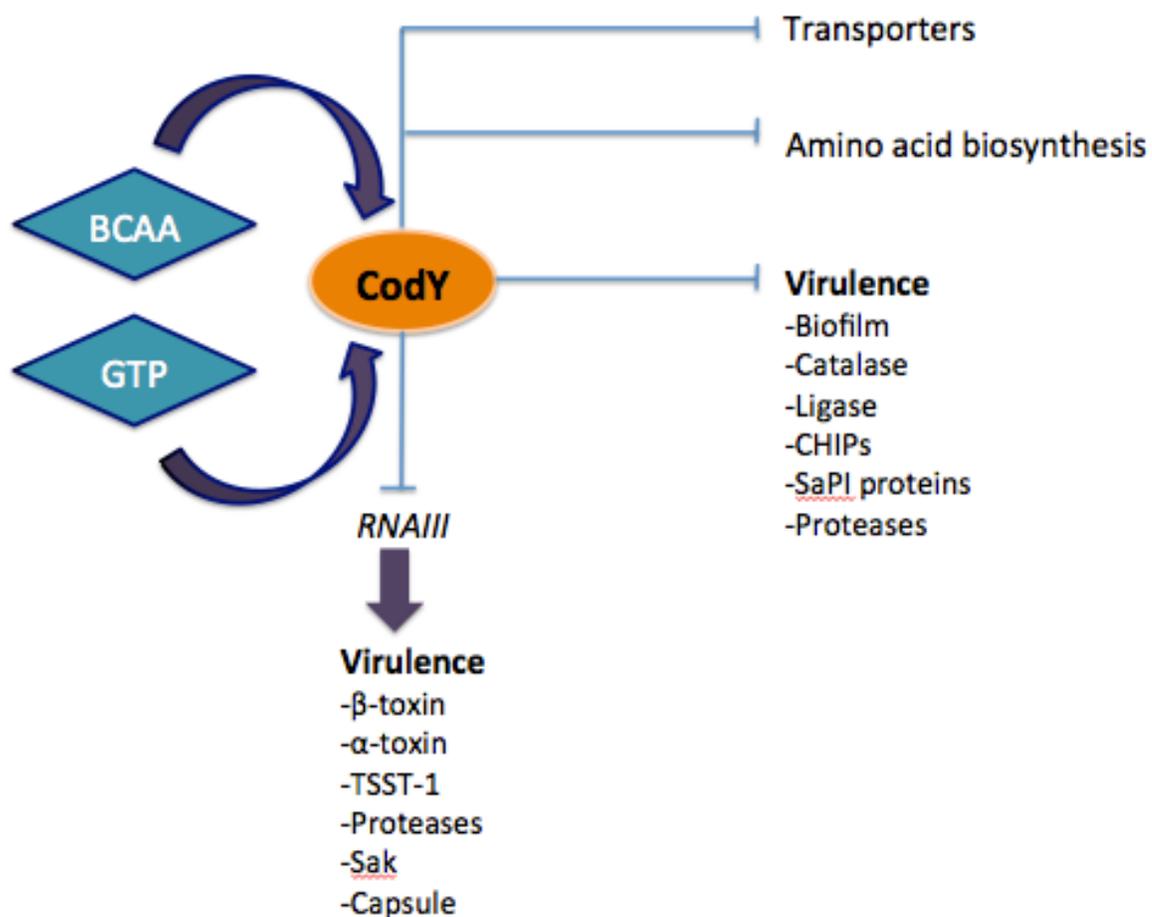


Figure 1. A) Genetic scheme of *codY* operon consisting of genes *hslV* and *hslU* encoding heat shock proteins, and a tyrosine recombinase, *xerC*. B) BCAAs and GTP act as effector molecules and play a role in regulating the function of CodY in *S. aureus*

1.2.2. CodY regulation on global regulators

S. aureus CodY appears to regulate two major global regulatory systems, *agr* and *sarA*, demonstrating that numerous connections and interactions take place between these regulatory proteins (83). CodY directly represses both *sarA* and *agr* systems as demonstrated by various studies (82, 83, 110). Moreover, deletion of *codY* in *S. aureus* strains SA564 and UAMS-1 led to de-repression of mRNA levels for various toxins, such as α -toxin (*hla*) and δ -toxin (*hld*), as well as the accessory gene regulator (*agr*) (RNAII and RNAIII) (83). As a result, it was shown that the culture supernatant of a *S. aureus codY* mutant displayed greater hemolytic activity towards rabbit erythrocytes compared to the wild type strain (83). This suggests that CodY indirectly represses hemolytic activity by repressing the *agr* system during the exponential growth phase. Interestingly, the de-repression of the CodY regulon and the activation of the Agr system simultaneously occur when cells enter the stationary phase. Indeed, in nutrient depleted environments, CodY target genes are de-repressed whereas the Agr system is induced by the high concentration of bacteria (quorum-sensing effect). Under limited isoleucine conditions, the *agr* is prematurely activated due to de-repression of CodY, possibly permitting the bacteria to escape and increasing virulence under nutrient limiting conditions (110). The *agr* system of *S. aureus* does not influence the transcript levels of CodY (110).

1.3. Metabolism

1.3.1. Amino acid requirements for staphylococcal growth

The metabolic regulatory cascades for staphylococci are unconventional and unclear, and there is a limited understanding on the link between nitrogen metabolism and amino acid

availability during infection. However, there is increasing evidence that CodY plays a central role as a regulator that links both metabolism and virulence gene expression in pathogenic bacteria (13, 30, 53, 114).

Most species of staphylococci require a complex mixture of nutrients; however, in general, they require an organic source of nitrogen, supplied by 5 to 12 essential amino acids, e.g. arginine, valine, and B vitamins, including thiamine and nicotinamide (66, 142). Various phenotypic studies that have looked at *S. aureus* nutritional requirements have attempted to determine the amino acids needed for growth (35). It was revealed that *S. aureus* often had numerous amino acid auxotrophies; however, it was also observed that these auxotrophies would revert back to a prototrophic state quite frequently. This suggested that absence, or genetic inactivation of biosynthetic pathways were not responsible for the auxotrophies (35, 42), but that feedback inhibition of the biosynthetic enzymes or repression of synthesis in corresponding pathways may be the likely cause (52). Whole genome sequencing of *S. aureus* was performed to confirm this, and it was determined that, indeed, biosynthetic pathways exist for all amino acids (8, 27, 72). Nevertheless, because a complex mixture of amino acids are required for optimal *S. aureus* growth, it has been suggested that bacteria have evolved the ability to acquire at least some of the amino acids from the host. Genes coding for oligopeptide transporters were also identified in a whole-genome screen, and were shown to be activated and necessary for infection (22, 87). The *S. aureus* genome also consists of genes encoding for proteases, whose activity could play a role in facilitating amino acid or peptide uptake during infection.

1.3.2. Branched-chain amino acids

Branched-chain amino acids provide an essential source of nitrogen for virtually all living organisms. The three branched-chain amino acids (BCAAs), leucine, isoleucine, and valine, are the most hydrophobic of the amino acids and are involved in determining the structures of several globular proteins. Moreover, these amino acids are precursors for the biosynthesis of iso- and anteiso-branched fatty acids, which are the dominant major fatty acids in the membrane (23). The interior of water-soluble globular proteins is primarily composed of hydrophobic amino acids, leucine, isoleucine, valine, phenylalanine, and methionine. This contributes to protein stability during the folding process, and also for the folding pathway that leads to the mature form (29). The hydrophobic residues are involved in the processes of some biologically essential globular proteins, such as the binding of oxygen in myoglobin and hemoglobin, and for substrate binding and catalysis in a variety of enzymes (18). Despite their similarity, the three BCAAs play slightly different roles in proteins. Regardless of this similarity, the differences in the properties of their side-chains, (i.e size, shape, and hydrophobicity), allows them to have unique preferences for various secondary structure motifs. Leucine is more commonly found in α -helices than in β -sheets, whereas both valine and isoleucine have a preference for the opposite (19).

In humans, 35–40% of the dietary essential amino acids in body protein and 14–18% of the total amino acids in muscle proteins are composed of BCAAs (75, 115). The muscle mass of humans is ~40% of the body weight; therefore, a large reservoir of BCAAs can be found in muscle protein. However, in human blood, the total concentration of free BCAAs is approximately ranges from 0.3 and 0.4 mM and is relatively small compared with that found in muscle proteins. As a result, bacteria have evolved various strategies in order to scavenge free BCAAs from the host to obtain these essential nutrients from the host.

1.3.3. Biosynthesis of BCAAs

The pathways for BCAA biosynthesis have mostly been studied in *E. coli* and *S. typhimurium* and to lesser degrees in *Neurospora crassa* and yeast. Many of these studies have experienced complications due to the antagonism that occurs between the three BCAAs. Since the time that it was first reported by Gladstone (43) in 1939 with *Bacillus anthracis*, additional examples have been reported in which antagonism occurs between two or three branched-chain amino acids at various points in metabolism. Such interactions are not surprising, since the three branched-chain amino acids (and their precursors) are analogs of each other. The kinds of antagonism that have been seen include interference with active transport into the cell, end-product control, repression control, and, potentially with incorporation into protein (135). The biosynthesis pathway for each BCAA is outlined in Figure 3.

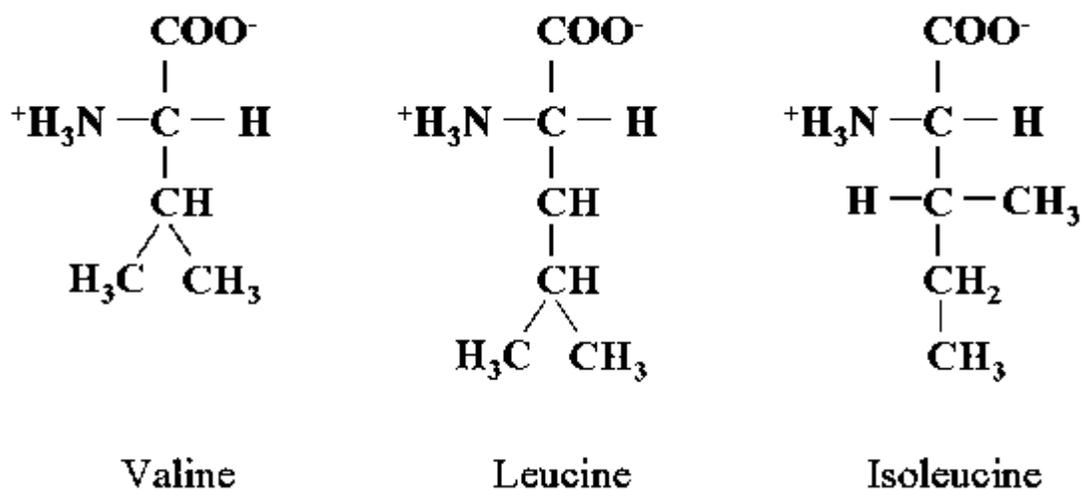


Figure 2. Structure of branched-chain amino acids valine, leucine and isoleucine, each having aliphatic side-chains with a branch (a carbon atom bound to more than two other carbon atoms).

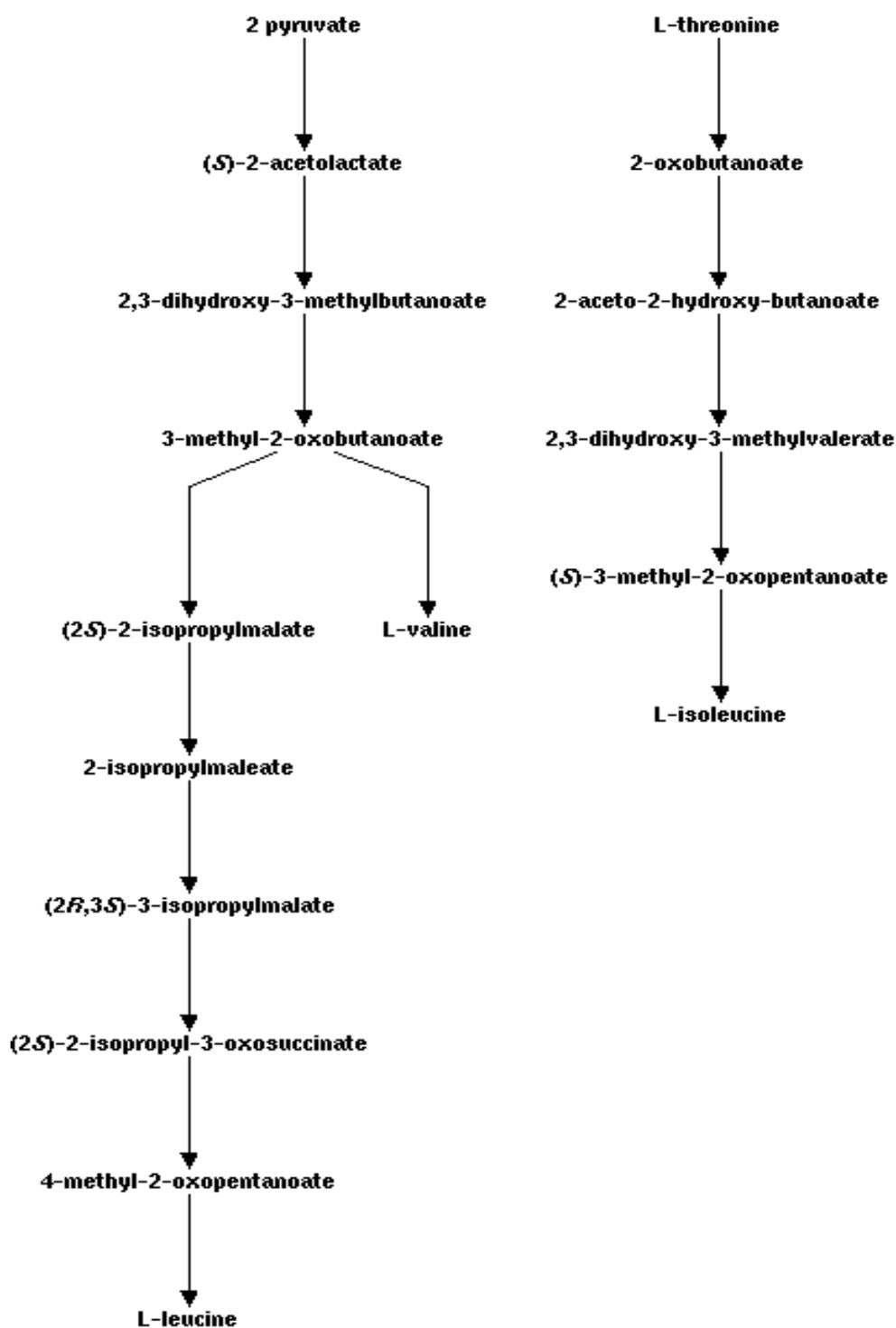


Figure 3. Biosynthesis pathway for branched-chain amino acids in *S. aureus*. Note that isoleucine is synthesized from threonine.

1.4. Preface to bacterial BCAA uptake

Environmental nutrient acquisition by bacteria is essential for survival and persistence. Growth and division stop when cells are exposed to nutrient limitation, causing the population size to remain stationary. A hallmark of this transition is a decline in intracellular BCAA levels (39, 49, 80, 89, 133). Despite the fact that BCAA levels are restricted within the host, pathogenic bacteria still manage to survive, and in some cases, thrive even though the amount of free BCAAs available to them is limited or difficult to obtain. Indeed, bacterial survival is dependent upon the development of specialized BCAA uptake systems that allow them to over-come BCAA withhold strategies of the host to meet their nutritional needs.

1.4.1. ATP-binding cassette (ABC) transporters

In bacteria, ATP-dependent active transport can allow molecules to enter the cell. This mechanism of transport involves the utilization of energy from hydrolysis of ATP to circumvent the electrochemical gradient and facilitate transport of solutes. ATP-dependent translocation occurs via transporters that belong to the ATP-binding cassette (ABC) transporter superfamily. The general structure of ABC transporters includes a membrane channel formed by two proteins containing transmembrane domains, and two cytoplasmic modules that bind and hydrolyze ATP to drive solute transport. In *S. aureus*, ABC transporters have been implicated in the transportation of various solutes including metal ions (78), heme (123), and oligopeptides (54) and are also predicted to transport polyamines, maltose, and amino acids (102).

1.4.2. BCAA uptake systems in bacteria

Many genes involved in transport of BCAAs have been identified by way of full genome sequencing. *E. coli* consists of two different transport systems responsible for BCAA uptake, both of which are high-affinity ABC transporters: LivFGHMJ and LivFGHMK (58). Among them, all three BCAAs bind to LivJ, while only L-leucine binds to LivK (58). The low-affinity BCAA transporter, BrnQ, functions as a Na⁺/BCAA symporter (4). While LivK is specific for L-leucine transport, BrnQ is involved in the uptake of other BCAAs as well as L-leucine. Thus, it was suggested that the complete knockout of *brnQ* and *livK* might cause growth retardation due to L-leucine and L-isoleucine deficiency (104). In *C. glutamicum*, BrnQ also mediates Na⁺-dependent import of all three BCAAs (34). In *L. lactis*, BcaP, the branched-chain amino acid permease, is the major BCAA transporter. Deletion of *bcaP*, along with *brnQ*, a second low-affinity BCAA permease, resulted in reduced viability of the strain due to inability to take up BCAAs (25).

1.5. Research Objectives

The goal of this research was to study the branched-chain amino acid uptake systems in *S. aureus*. We hypothesize that BrnQ transporters are involved in BCAA uptake and contribute to virulence in *S. aureus* by modulating CodY activity. The first objective was to identify putative branched-chain amino acid transporters in *S. aureus* USA300 using BLAST analysis and sequence similarity. The second objective was to generate mutations of each branched-chain amino acid transport system to facilitate study of each transporter. Thirdly, a combination of radioactive uptake assays and growth experiments were used to characterize the role of the branched-chain amino acid transporters in *S. aureus*. The final objective of this research was to determine the role of the branched-chain amino acid transporters on *S. aureus* virulence *in vivo* using a mouse model of bacteremia. The central focus of the research is directed towards understanding the mechanisms and modulators of adaptation and survival of *S. aureus* in nutrient-limited environments.

Chapter 2- Materials and methods

2.1. Bacterial strains and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2. MRSA isolate pulsed-field gel electrophoresis type USA300 LAC that had been cured of the erythromycin resistance plasmid was used in all experiments as the wild type (WT) strain. Unless otherwise indicated, both *E. coli* and *S. aureus* were cultured at 37°C and stored at -80 °C in 15% glycerol. *E. coli* strains were grown in Luria Bertani (LB) medium. *S. aureus* strains were grown either in tryptic soy broth (TSB) or in a chemically-defined medium (CDM). For strains carrying resistance genes, antibiotics were used at the following concentrations: chloramphenicol (10µg/mL) and tetracycline (4µg/mL) for growth of *S. aureus* strains; ampicillin (100µg/mL) for growth of *E. coli* strains. Complete CDM was composed as follows (final concentrations in uM): amino acids from a 10X stock; alanine (672), arginine (287), aspartic acid (684), cysteine (166), glutamic acid (680), glycine (670), histidine (129), isoleucine (228), leucine (684), lysine (342), methionine (20), phenylalanine (240), proline (690), serine (285) threonine (260), tryptophan (50), tyrosine (275), valine (684); vitamins and metal ions from a 1000X stock (final concentrations in uM); thiamine (56), nicotinic acid (10), biotin (0.04), pantothenic acid (2.3), MgCl₂ (1000), CaCl₂ (100); salts from a 10X stock; monopotassium phosphate (40000), dipotassium phosphate (14700), sodium citrate dihydrate (1400), magnesium sulphate (400), ammonium sulphate (7600); carbon source; glucose (27753). The concentrations of individual amino acids were modified in some experiments, as indicated in the Results and Figure legends. The pH of the medium was buffered to 7.0. Solid media were obtained by the addition of 1.5% (w/v) Bacto agar (Difco). Water for preparation of growth media and solutions was obtained by passage through a Milli-Q water filtration system (Millipore Corp.).

Table 2. Bacterial strains used in this study

Strain or plasmid	Description ^a	Source or reference
Strains		
<i>S. aureus</i>		
USA300 LAC	Community-acquired MRSA; WT strain	(88)
USA300	USA300 LAC cured of antibiotic resistance plasmid	(6)
RN4220	r _K ⁻ m _K ⁺ ; capable of accepting foreign DNA	(71)
RN6390	Prophage-cured laboratory strain	(108)
Newman	WT clinical isolate	(33)
H2324	Newman $\Delta brnQ3::Tet$; Tet ^R	This study
H2568	USA300 $\Delta brnQ1$ (SAUSA300_0188)	This study
H2563	USA300 $\Delta brnQ2$ (SAUSA300_0306)	This study
H2578	USA300 $\Delta brnQ3::Tet$; Tet ^R (SAUSA300_1300)	This study
H2586	USA300 $\Delta brnQ1 \Delta brnQ2$	This study
H2580	USA300 $\Delta brnQ1 \Delta brnQ3::Tet$; Tet ^R	This study
H2581	USA300 $\Delta brnQ2 \Delta brnQ3::Tet$; Tet ^R	This study
H2589	USA300 $\Delta brnQ1 \Delta brnQ2 \Delta brnQ3::Tet$; Tet ^R	This study
<i>E. coli</i>		
DH5 α	F ⁻ $\phi 80dlacZ\Delta M15 recA1 endA1 gyrA96 thi-1 hsdR17$ (r _K ⁻ m _K ⁻) <i>supE44 relA1 deoR</i> $\Delta(lacZYA-argF)U169phoA$	Promega

^a Abbreviations: Cm^r, Ap^r, Tet^r designate resistance to chloramphenicol, ampicillin, and tetracycline, respectively

2.2. DNA Methodology

2.2.1. Plasmid isolation from *E. coli*

All plasmids used in this study are listed in Table 3. Plasmid DNA was prepared from *E. coli* using the E.Z.N.A. Plasmid Miniprep Kit (Omega Biotek) according to the manufacturer's index. In summary, approximately 5 mL of stationary phase culture of *E. coli* were pelleted via centrifugation and resuspended in 250 μ L of Solution I/RNase (50mM Tris, pH 8.0, 20 mM EDTA, 100 μ g/mL of RNaseA). Cells were lysed by adding 250 μ L of Solution II (200 mM NaOH, 1% (w/v) SDS) and gently inverting the tubes and incubating the cells at room temperature for 2-5 minutes. To neutralize the solution, 350 μ L of Solution III (guanidine hydrochloride with acetic acid) was added to the lysate and was immediately inverted several times until a precipitate formed. The insoluble material was subsequently centrifuged for 10 minutes at 13000 \times g to form a pellet. The resulting cleared supernatant was aspirated into a HiBind DNA Miniprep Column (I) and was centrifuged at 13000 \times g for 1 minute. 500 μ L of Buffer HB was added to wash the column and ensure that residual protein contaminations are removed. 700 μ L of DNA Wash Buffer diluted with absolute ethanol was added to the column and centrifuged for 1 min at 13000 \times g. The column was subsequently centrifuged for 2 minutes at 13000 \times g to dry the column matrix. Plasmid DNA was then eluted from the column into a fresh microcentrifuge tube by addition of 30 μ L to 100 μ L of dH₂O followed by centrifugation at 13000 \times g for 1 minute.

2.2.2. Plasmid isolation from *S. aureus*

Plasmid DNA isolation from *S. aureus* essentially followed the same protocol as described above for *E. coli* but with slight modification. Bacterial cells that were harvested

were incubated at 37 °C for 30-60 minutes in a 250 μ L mixture of Solution I containing lysostaphin (Sigma) (1 mL Solution I added to 50 μ g of lysostaphin) in lieu of RNase A solution, prior to addition of Solution II.

Table 3. Plasmids used in this study

Plasmid	Description ^a	Source or Reference
pRMC2	Anhydrotetracycline-inducible expression vector; Ap ^r in <i>E. coli</i> ; Cm ^r in <i>S. aureus</i>	(21)
pSO1	pRMC2 containing <i>brnQ1</i> ; Cm ^R	This study
pSO2	pRMC2 containing <i>brnQ2</i> ; Cm ^R	This study
pSO3	pRMC2 containing <i>brnQ3</i> ; Cm ^R	This study

^a Abbreviations: Cm^r and Ap^r designate resistance to chloramphenicol and ampicillin, respectively.

2.2.3. Isolation of chromosomal DNA from *S. aureus*

Chromosomal DNA was obtained from *S. aureus* by pelleting 500 μ l of overnight stationary phase cells grown in TSB culture. 200 μ l of STE (75 mM NaCl, 25 mM EDTA, 20 mM Tris pH 7.5) was added to the cells along with 50 μ g/ml of lysostaphin dissolved in 20 μ L of STE in order to facilitate cell lysis. The cell suspension was incubated at 37 °C for 1 hour. 20 μ l of 10% SDS and 20 μ L of Proteinase K (New England Biolab) were added and incubated overnight at 55 °C. Subsequently, 80 μ l of 5M NaCl was added and mixed by inversion. 320 μ l of a 25:24:1 phenol : chloroform : isoamyl alcohol (Invitrogen) was added and was allowed to sit at room temperature for 30 minutes. The aqueous layer was removed after the mixture was spun at 12000 \times g for 10 minutes. Addition of 300 μ l of 24:1 chloroform : IAA was added and then spun at 12000 \times g for 10 minutes. The aqueous layer was subsequently removed, and 400 μ l of isopropanol was added until the DNA formed a visible mass with gentle inversion. The mixture was allowed to sit for 10 minutes at room temperature, and was then spun at 12000 \times g for 5 minutes. The pellet was washed with 70% ethanol, dried, and resuspended in 100 μ l of dH₂O.

2.2.4. Restriction Enzyme Digests

Restriction enzymes were purchased from Life Technologies, MBI Fermentas, New England Biolabs, or Roche Diagnostics. Reactions were typically carried out in 30-40 μ l volumes over a 1-2 hour incubation at the appropriate temperature (typically 37 °C). Digested DNA was subsequently cleaned using a QIAquick PCR purification kit (QIAGEN) as described by the manufacturer.

2.2.5. DNA ligation

DNA fragments were generally ligated in a 20 µl reaction volume using a 10:1 ratio of insert to vector DNA. Reactions were carried out using the T4 DNA ligase Rapid Ligation Kit (Roche Diagnostics) in accordance with the manufacturer's instructions.

2.2.6. Agarose gel electrophoresis

Agarose gel electrophoresis was used for the separation and analysis of DNA fragments. Agarose gels (0.8% w/v) were prepared using 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA) to which either 1.5 µg/ml of ethidium bromide or 2 µl of SYBR Safe DNA gel stain (Invitrogen) was added. DNA samples to be run were mixed with loading buffer (5% glycerol, 0.04% bromophenol blue, 0.04% xylene cyanol, 10 mM EDTA, pH 7.5) prior to being loaded in the gel. Electrophoresis was typically carried out at 110 V for 20-25 minutes. The 1 kb-Plus ladder (Invitrogen) was used as a standard reference marker for estimation of DNA fragment size. Following electrophoresis, DNA fragments were visualized using a gel doc (Bio-Rad).

2.2.7. Isolation of DNA fragments from agarose gels

DNA fragments were visualized under long wave UV light (365 nm) and excised from agarose gels following electrophoresis. DNA was isolated using the QIAquick Gel Extraction Kit (QIAGEN) using a protocol as described by the manufacturer.

2.2.8. Polymerase chain reaction (PCR)

PCR reactions were carried out in 50 μ l reactions containing: DNA template, 1x PCR buffer, 200 μ M dNTP mix (Roche Diagnostics), 12 pM of forward and reverse primers, and 0.5 units of Taq DNA polymerase. PCR reactions were also carried using KAPA HiFi HotStart PCR Kits (Kapa Biosystems). Briefly, 25 μ l reactions containing template DNA, 1X Kapa HF Buffer (2.0 mM Mg 2+ at 1X), 300 μ M of dNTPs, 300 nM each of forward and reverse primer and 0.5 units of KAPA HiFi HotStart DNA Polymerase. PCRs were performed using the GeneAmp PCR system (Perkin Elmer), DNA engine Gradient Cyclor (Bio-rad) or the MJ Mini Personal Thermal Cyclor (Bio-rad). Oligonucleotide primers were obtained from Integrated DNA technologies.

2.2.9. DNA sequencing

DNA sequencing was performed at the DNA Sequencing Facility of the Robarts Research Institute (London, Ontario, Canada), with sequencing reactions prepared according to their guidelines.

2.2.10. Computer Analyses

DNA sequence analysis, sequence alignments, and oligonucleotides primer design were carried out using Vector NTI Suite 7 software package (Informax, Inc., Bethesda, Maryland). Blast searches were performed using tools available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/>).

2.3. Transformation and transduction methodologies

2.3.1. Preparation of transformation competent *E. coli*

E. coli DH5 α CaCl₂ competent cells were prepared as follows. An overnight, stationary phase culture of DH5 α was diluted 1:100 into 500 ml of fresh LB and grown to an OD₆₀₀ of approximately 0.5 and placed on ice for 30 minutes. The cells were then harvested via centrifugation and resuspended in 100 ml of ice-cold 100 mM CaCl₂ plus 15% glycerol and incubated on ice for 30 minutes. Cells were again collected by centrifugation, resuspended in 4 ml of CaCl₂ plus 15% glycerol, and stored as 100 μ l aliquots at -80 °C.

2.3.2. Transformation of CaCl₂ competent *E. coli*

To transform CaCl₂ competent *E. coli* DH5 α , purified plasmid DNA, PCR product, or ligation mixtures were added to an aliquot of competent cells and kept on ice for 45 minutes, after which cells were subjected to a heat shock treatment at 42 °C for 2 minutes immediately followed by a 2 minute incubation on ice. An 800 μ l aliquot of LB broth was added to the tube, mixed, and the cells were permitted to recover for 1 hour at 37°C before being plated on selective media and incubated overnight.

2.3.3. Preparation of transformation competent *S. aureus*

Strains of *S. aureus* were made competent for transformation via electroporation as follows. An overnight culture of *S. aureus* was diluted 1:100 into 100 mL of fresh TSB and grown to an OD₆₀₀ of approximated 0.3. Cells were then harvested via centrifugation and resuspended in 500 mM sucrose. Cells were subsequently washed three times with ice cold

500 mM sucrose. After the final wash, cells were resuspended in 1 mL of 500 mM sucrose and stored as 80 μ l aliquots at -80°C .

2.3.4. Transformation of electrocompetent *S. aureus*

Electrocompetent *S. aureus* were transformed using purified plasmid DNA (typically 5 μ l from an EZNA miniprep). DNA was added to a tube of competent cells and allowed to incubate on ice for 30 minutes before being transferred to an ice-cold electroporation cuvette (2mm, Bio-Rad) for electroporation. Following incubation, electroporation was performed using a Bio-Rad Gene Pulser II with setting of 2.5 V, 200 mA, and 25 Ω . Ice cold TSB (800 μ l) was immediately added to pulsed cells, which were then recovered for a minimum of 1 hour at 37°C before being plated on appropriate selective media.

2.3.5. Preparation of phage lysate of *S. aureus*

Bacteriophage 80 α was recovered from *S. aureus* RN2564 by UV induction as previously described (119). For routine preparation of infective lysates, 500 μ l of an overnight culture of *S. aureus* was diluted 1:1 in fresh TSB supplemented with 2.5 mM CaCl_2 and incubated at 37°C for 10 minutes. Subsequently, 100 μ l of various dilutions of phage 80 α were added. This mixture was allowed to incubate for an additional 15 minutes at 37°C before being added to 20 ml of TSB with 2.5 mM CaCl_2 and incubated with slow shaking at 37°C until lysis of culture was observed. Following lysis, cell debris was removed via centrifugation at $6000 \times g$ for 10 minutes and the phage lysate was sterilized by passage through a 0.45 μ m filter (Millipore). All phage lysates were maintained at 4°C .

2.3.6. Transduction of *S. aureus*

Transduction of *S. aureus* strains was performed as follows. A 5 ml culture of the recipient strain was grown in TSB with 2.5 mM CaCl₂ to an OD₆₀₀ of 0.9, at which time, 1 ml of culture was harvested by centrifugation and resuspended in 500 µl of fresh TSB. This was further divided into 100 µl aliquots. Various dilutions of phage 80α lysates were added to each aliquot and incubated for 20 minutes at 37°C. This was followed by the addition of 10 µl of 1 M sodium citrate to halt the phage infection. Cells were then exposed to 1/50th the selective concentration of the appropriate antibiotic for 1 hour at 37°C before being plated on appropriate selective media.

2.4. Mutagenesis and DNA cloning methods

2.4.1. Mutagenesis of *brnQ*

Deletions of *brnQ1* and *brnQ2* genes were constructed using the pKOR-1 plasmid as described previously (9). Primer sequences were based on the published USA300 FPR3757 and are displayed in Table 4 (27). Briefly, sequences flanking the *brnQ* locus were PCR amplified with primers for the upstream fragment and primers for the downstream fragment. A PCR amplicon of the joined DNA fragments was recombined into the temperature-sensitive vector pKOR1 using BP clonase reaction (Invitrogen), resulting in the pKOR-1Δ*brnQ1* and pKOR-1Δ*brnQ2* plasmids. The resulting plasmids were first passed through *S. aureus* RN4220, before electroporation into strain USA300. Precise in-frame allelic replacement of the *brnQ* genes was established by a two-step process of temperature shifting and antisense counter-selection (9) and confirmed by PCR and sequence analysis. The *brnQ3::Tet* knockout allele consisted of a tetracycline resistance cassette, excised from

plasmid pDG1513 with restriction enzyme *Pst*I, flanked by DNA sequences homologous to regions upstream and downstream of *brnQ3* – leaving the first 69 bp of the start of the gene and the last 34 bp of the gene. The knockout allele was cloned into the temperature-sensitive *E. coli*/*S. aureus* shuttle vector pMAD, and then passaged through *S. aureus* RN4220 at permissive temperatures prior to being transduced into *S. aureus* RN6390. Recombinant RN6390 was cultured at 30 °C to mid-log phase before the incubation temperature was shifted to 42 °C. The bacteria were further incubated for 16 hours before being plated onto TSA containing tetracycline. Colonies were screened for sensitivity to erythromycin, which indicates loss of the pMAD backbone DNA after integration of the knockout allele into the *S. aureus* chromosome via double homologous recombination. The $\Delta brnQ3::Tet$ deletion was confirmed by PCR and mobilized to *S. aureus* USA300 by transduction using phage 80 α as described above.

2.4.2. Construction of vectors to complement a *brnQ* mutant

Each of the *brnQ* genes, including their endogenous promoters, was cloned into the shuttle vector pRMC2 (21). Primers and restriction enzymes used are outlined in Table 4. As listed in Table 3, the resultant plasmids encoding *brnQ1*, *brnQ2* and *brnQ3* were designated pSO1, pSO2, and pSO3, respectively.

Table 4. Oligonucleotide primers used for mutagenesis and cloning

Oligonucleotides ^a	
<i>brnQ1</i> 5'F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACCT GCAAGAAGGTATTC
<i>brnQ1</i> 5'R	GGGCGTAAGTTAAGAACGA Generation of 5' arm for deletion of <i>S. aureus brnQ1</i>
<i>brnQ1</i> 3'F	/5Phos/AAGGAGGGAGACCGATGAAT
<i>brnQ1</i> 3'R	GGGGACCACCTTGTACAAGAAAGCTGGGTTGGCTAAA GGGTTACCACCA Generation of 3' arm for deletion of <i>S. aureus brnQ1</i>
<i>brnQ2</i> 5'F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCCAT AGACAGGTTTAGC
<i>brnQ2</i> 5'R	TGGAAATACGTTGGAGCGTTG Generation of 5' arm for deletion of <i>S. aureus brnQ2</i>
<i>brnQ2</i> 3'F-	/5Phos/ACCCATCGTTACTAGGCTTT
<i>brnQ2</i> 3'R-	GGGGACCACCTTGTACAAGAAAGCTGGGTTAAAAGC CGCAGCGATATTC Generation of 3' arm for deletion of <i>S. aureus brnQ2</i>
<i>brnQ3</i> 5'F- Sall	<u>TTGTCGACGATTGAACGAGAACAACGAGATGC</u>
<i>brnQ3</i> 5'R- PstI	<u>TTCTGCAGGCGTAAACCCAATGACCCATG</u> Generation of 5' arm for generation of <i>S. aureus brnQ3::Tet</i> allele
<i>brnQ3</i> 3'F- PstI	TTCTGCAGCAACGATATTAGGCTATCTAGTCGGC
<i>brnQ3</i> 3'R- SmaI	<u>TTCCCGGGAGCTCGTTGAGCTGCTATTTCC</u> Generation of 5' arm for generation of <i>S. aureus brnQ3::Tet</i> allele
<i>brnQ1</i> _{SA} 5'F-KpnI	GATCGGTACCGTTCTTAACTTACGCCC
<i>brnQ1</i> _{SA} 3'R-SacI	GATCGAGCTCCAGTTGTTCAATATAAG Cloning of <i>S. aureus brnQ1</i>
<i>brnQ2</i> _{SA} 5'F-KpnI	GATCGGTACCTTTCCAACCTTACTTTCA
<i>brnQ2</i> _{SA} 3'F-SacI	GATCGAGCTCTCAATAGACAGACGGT Cloning of <i>S. aureus brnQ2</i>
<i>brnQ3</i> _{SA} 5'F-KpnI	GATCGGTACCCATGTGTTTTGTTTCTG
<i>brnQ3</i> _{SA} 3'F-SacI	GATCGAGCTCTAATGGCGAGACTCCT Cloning of <i>S. aureus brnQ3</i>

^a Restriction sites in sequences are underlined

2.5. Radioactive transport assays

Cultures were grown overnight at 37°C in complete CDM and subcultured into 5 ml of complete CDM at a starting OD₆₀₀ of 0.1. Bacteria were grown to mid-log phase (OD₆₀₀ of 1.0), cells were washed and resuspended in CDM without amino acids. Cells were harvested by filtration on 0.45- μ m pore-size membrane filters and washed with saline before being resuspended in CDM lacking amino acids. Cells were then shaken at 200 rpm at 37°C for 10 minutes prior to the assay. For the uptake assay, 10 μ L of a mixture containing 15 mM of an unlabeled amino acid of interest and 0.05 μ Ci of amino acid in ¹⁴C-labeled form (Perkin Elmer, MA) were added to 1 mL of culture to initiate transport. 200 μ L were aliquoted at indicated time intervals and rapidly filtered through 0.45- μ m membrane filters. The filters were immediately washed with 20 mL of 0.1 M LiCl₂ at room temperature. Filters were dried and placed in scintillation vials containing 4 mL of scintillation fluid. Radioactivity was measured using the tritium channel of a scintillation system LS 6500 (Beckman). Variations to mixtures and amino acids are as described in the text and figure legends.

2.6. *S. aureus* growth curves

Strains of *S. aureus* to be tested were pre-grown overnight in either TSB or complete CDM. Cells washed and sub-cultured into the same medium or into CDM with decreased concentration of branched-chain amino acids (as outlined in figure legends) to a starting OD₆₀₀ equivalent of 0.0025. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm until stationary phase was reached.

2.7. Murine Systemic Model of *S. aureus* Infection

All protocols were reviewed and approved by the University of Western Ontario's Animal Use Subcommittee, a subcommittee of the University Council on Animal Care. Seven-week-old female immunocompetent BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in microisolator cages. Bacteria were grown to mid-log phase (OD_{600} of approximately 1.0-3.0) in TSB, pelleted by centrifugation, washed twice in PBS, and resuspended in phosphate-buffered saline. Bacterial suspensions in 100- μ L volumes were administered via tail vein ($6-7 \times 10^6$ CFU). Ninety-six hours post-challenge, kidneys, livers, and hearts were excised and placed in a phosphate-buffered solution containing 0.1% (vol/vol) Triton X-100. Organs were homogenized for 10 s, and bacterial loads were calculated following serial dilution of the suspension and drop plating on TSB agar plates. Data are presented as \log_{10} CFU recovered per organ or organ pair in the case of kidneys. Data were analyzed using the Student's unpaired t-test and P values of <0.05 were considered to indicate statistical significance.

Chapter 3- Results

3.1. Identification of putative branched-chain amino acid uptake transporters

A *S. aureus* N315 *braB* mutant was previously identified through signature tagged mutagenesis (STM) as being attenuated in a mouse model of systemic infection (14). BLAST analysis demonstrates that this protein was allelic with USA300 *S. aureus* gene *brnQ3*, encoding a 447-amino-acid branched-chain amino acid carrier protein and 100% amino acid identity to BraB. BLAST searches of the databases show that the USA300 gene product exhibits 34% identity and 56% total similarity to the BrnQ proteins of *Salmonella enterica* sv. Typhimurium and *E. coli*, 34% identity and 54% total similarity to the BrnQ protein of *Corynebacterium glutamicum*, and 35% identity and 55% total similarity to the BraB protein in *Pseudomonas aeruginosa*. BrnQ and BraB proteins are involved in the Na(+)- coupled import of branched chain amino acids (BCAAs) (46, 56, 130). Hydropathy profiles indicate that the proteins are likely to contain 12 membrane-spanning domains. The gene has been annotated in the *S. aureus* COL genome as *brnQ3* since there are two additional *brnQ* paralogs in *S. aureus*. Pairwise alignments demonstrate that the three BrnQ paralogs share approximately 30-35% identity and 50-55% total similarity across the total length of the proteins. The *S. aureus*, *brnQ3* gene was previously identified in screens for salt-sensitive mutants (137), but its function, as well as that of *brnQ1* and *brnQ2* as branched-chain amino acid carrier proteins have not been formally tested. BrnQ1 in USA300 shares 61% identity and 78% total similarity with the BrnQ protein in *L. lactis*, which was shown to contribute to BCAA uptake.

Genes encoding the putative branched-chain amino acid transporters (*brnQ*) were previously identified in a genome-wide screen for CodY targets in *S. aureus* strain UAMS-1 (82). The 1356-nucleotide-containing *brnQ1* gene encodes a protein of 451 amino acid

residues. The *brnQ2* gene constitutes of 1278 nucleotides, encoding a 425 amino acid protein. Finally, the 1344-nucleotide-containing *brnQ3* gene encodes a 447 amino acid protein. The specificities of the BrnQ transporters are largely unknown. As a result, elucidating the role of these BrnQ transporters in BCAA uptake is key in order to determine whether their function plays a role in intracellular BCAA accumulation and, thus, in the regulation of CodY.

3.2. Mutation of the *brnQ* paralogs in *S. aureus* USA300

In order to ascertain a function for each of the *brnQ* paralogs, mutations were introduced in each of the three *brnQ* genes. In-frame markerless *brnQ* deletions were constructed in the *brnQ1* and *brnQ2* genes using pKOR1 as described above. A tetracycline resistance cassette was inserted into the *brnQ3* gene. Finally, a strain was constructed $\Delta brnQ1,2,3$ that contained the mutations in all three of the *brnQ* genes. See Table 4 for details. The mutations were confirmed using PCR amplification across the genes (Fig. 4), and the PCR amplicons were sequenced for confirmation.

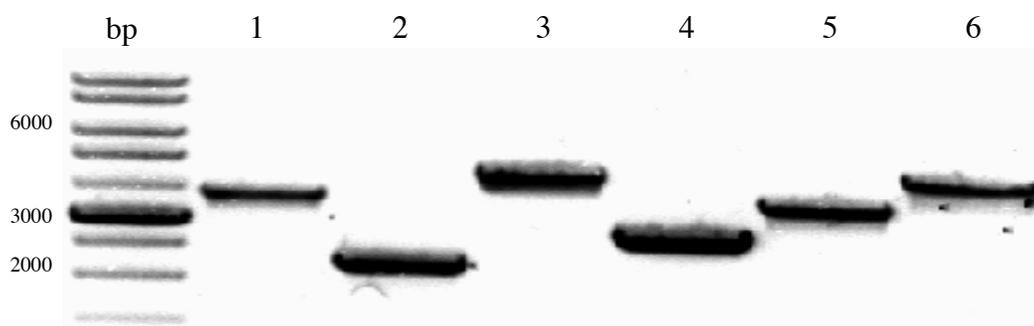


Figure 4. Generation of *brnQ* mutations in *S. aureus* strain USA300. Polymerase chain reaction analysis confirms the presence of *brnQ* genes in wild type USA300 and their absence in the mutant. Lanes 1, 3, and 5 illustrate amplified regions of *brnQ1*, *brnQ2*, and *brnQ3* in WT. Lanes 2, 4, and 6 illustrate amplified regions of *brnQ1*, *brnQ2*, and *brnQ3* in the $\Delta brnQ123$ mutant, where *brnQ3* is replaced with a tetracycline-resistance cassette in the mutant (as shown with amplicon in lane 6 with a higher molecular weight compared to WT).

3.3. Characterization of BrnQ transporters

In order to characterize the role of BrnQ transporters, radioactive uptake assays were performed, as described above, to first determine function and specificity of each transporter. This was accompanied by growth assays, which were conducted to determine if there were any growth deficiencies due to lack of any of the *brnQs*.

3.3.1. Detection of a BCAA uptake deficiency in the USA300 *brnQ123* mutant

Radioactive uptake assays were performed in order to compare uptake of labeled-BCAAs between WT and *brnQ* mutants. As previously described, cells were grown in complete CDM, and resuspended into CDM without amino acids for uptake experiments. Uptake was measured with the addition of either ^{14}C -labeled BCAA alone or ^{14}C -labeled BCAA competing with 1000-fold excess of unlabeled BCAA or non-BCAA to the cells. The initial attempt to determine a role for BrnQ transporters in BCAA uptake involved examining ^{14}C -L-Leucine uptake in wild-type versus the *brnQ123* mutant. Both WT and Δ *brnQ123* displayed comparable ^{14}C -L-Leucine uptake (Fig. 5A), suggesting that either the BrnQ transporters are not involved in BCAA uptake or, alternatively, that there exists an alternate predominant branched-chain amino acid transporter other than the BrnQs whose function may have masked a potential uptake deficiency phenotype. In an attempt to eliminate background with an alternate transporter, a competing BCAA was used in increasing ratios in uptake assays. We measured uptake of labeled leucine in the presence of increasing concentrations of unlabeled isoleucine. We observed that while the levels of leucine uptake did not decrease substantially in the wild type bacteria, the uptake of leucine by the *brnQ123*

mutant decreased to near zero at a 1:1000 ratio of ^{14}C -labeled-leucine to unlabeled isoleucine (Fig. 5, panels B-D). Similar results were seen using ^{14}C -L-Isoleucine and ^{14}C -L-Valine (Figures 6 and 7). This result implicated at least one of the BrnQ transporters in leucine uptake. Since we were able to outcompete the effects of an unidentified transporter(s) using a 1:1000 ratio, all subsequent BCAA uptake assays were conducted in similar fashion to characterize BrnQ-dependent BCAA uptake.

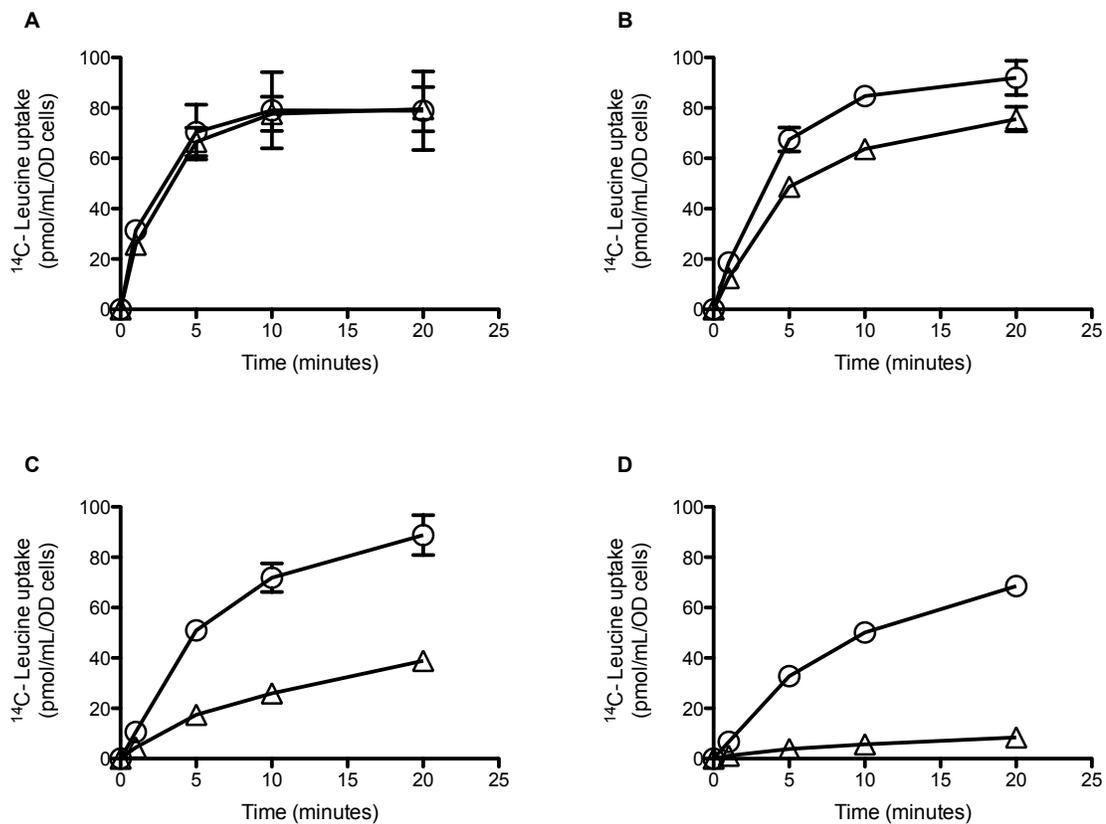


Figure 5. Radioactive uptake assays illustrating the uptake of ^{14}C -L-Leucine in whole cells of *S. aureus* with addition of various ratios of labeled leucine to unlabeled isoleucine. A) ^{14}C -L-Leucine alone; B) 1:10 ; C) 1:100; D) 1:1000. Transport assays using concentrated samples of cells harvested from the exponential phase of growth in complete CDM were performed in the presence of $0.05\ \mu\text{Ci}$ ^{14}C -labeled branched-chain amino acid and various concentrations of unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

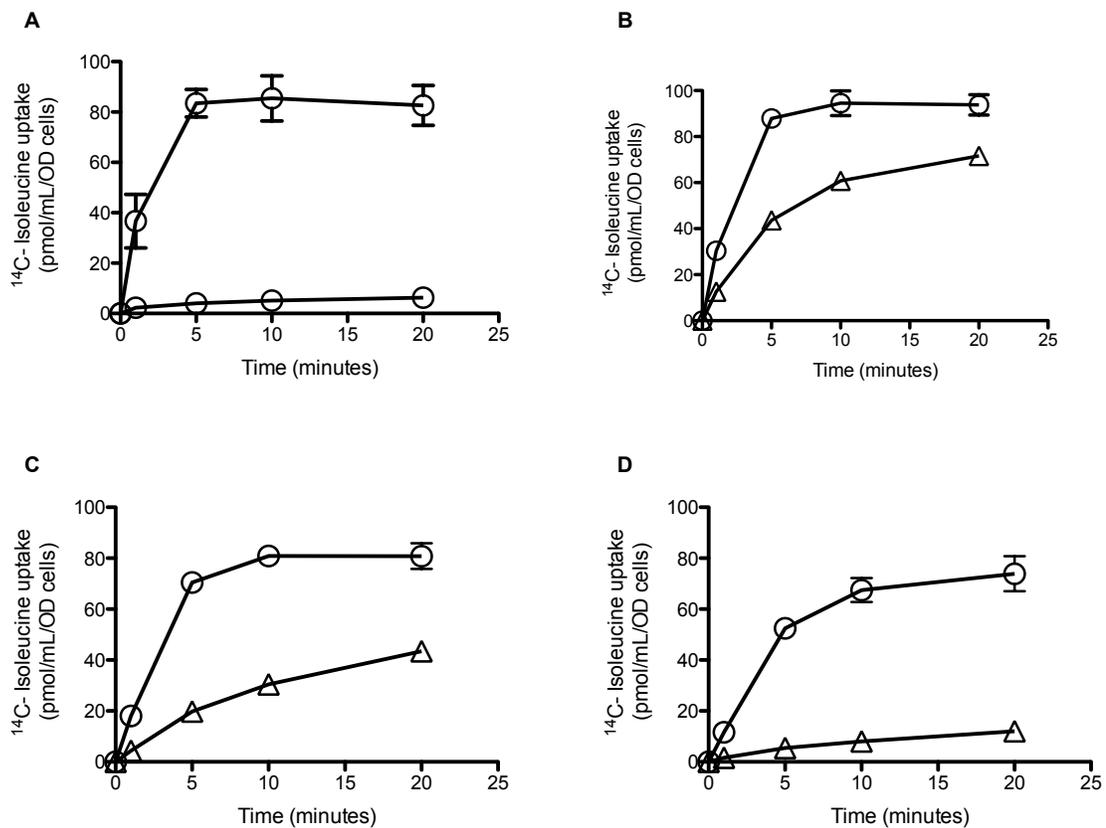


Figure 6. Radioactive uptake assays illustrating the uptake of ^{14}C -L-Isoleucine in whole cells of *S. aureus* with addition of various ratios of labeled isoleucine to unlabeled valine. A) ^{14}C -L-Isoleucine alone; B) 1:10; C) 1:100; D) 1:1000. Transport assays using concentrated samples of cells harvested from the exponential phase of growth in complete CDM were performed in the presence of $0.05\ \mu\text{Ci}$ ^{14}C -labeled branched-chain amino acid and various concentrations of unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

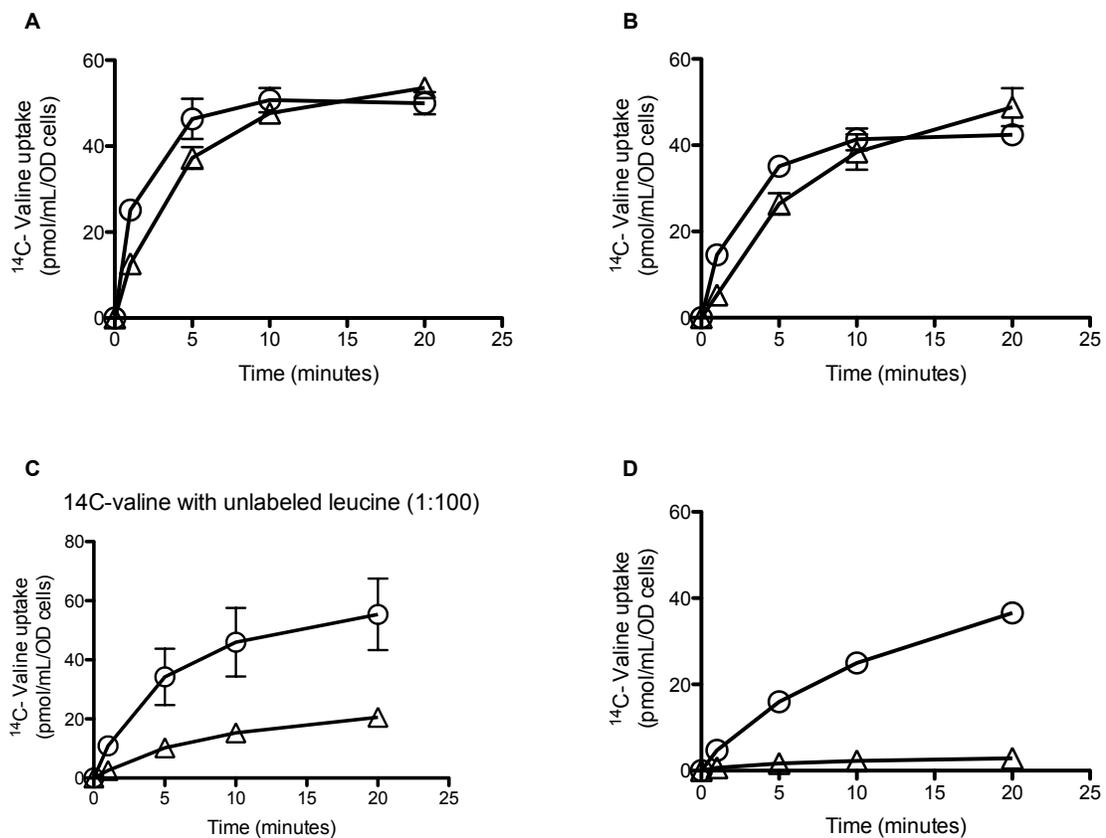


Figure 7. Radioactive uptake assays illustrating the uptake of ^{14}C -L-Valine in whole cells of *S. aureus* with addition of various ratios of labeled valine to unlabeled leucine. A) ^{14}C -L-valine alone; B) 1:10; C) 1:100; D) 1:1000. Transport assays using concentrated samples of cells harvested from the exponential phase of growth in complete CDM were performed in the presence of $0.05 \mu\text{Ci}$ ^{14}C -labeled branched-chain amino acid and various concentrations of unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

3.3.2. BrnQ transporters are not specific for threonine or the non-BCAA proline

Using the same experimental setup described above, we competed labeled BCAAs with unlabeled threonine, a precursor in isoleucine biosynthesis, and proline, an unrelated non-BCAA to see whether a 1000-fold excess of these two amino acids could affect uptake of BCAAs. These two non-BCAAs, when in excess, were unable to outcompete the uptake of BCAAs (Figs. 8 and 9), indicating that the BrnQ transporter(s) involved in the observed uptake is specific for BCAAs.

3.3.3. The BrnQ transporters depend on proton motive force for active transport of BCAAs

A feature of oxidative phosphorylation and membrane transport is the proton gradient across the cytoplasmic membrane. The proton motive force (PMF) thus created is thought to drive ATP synthesis during oxidative phosphorylation, and also, by virtue of associated exchange diffusion systems, the translocation of solutes and ions. Uncouplers like carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) cause a collapse in the proton motive force by making the cell membrane freely permeable to protons (7, 51). Indeed, sensitivity to CCCP can provide a simple test for the dependence of a bacterial transport system on the proton-motive force (50). To determine whether the observed BrnQ-dependent uptake of BCAAs was dependent on PMF, wild type cells were treated with 200 μ M of CCCP. Uptake of labeled L-isoleucine by wild type was abolished by CCCP treatment (Fig. 10), in agreement with the notion that the BrnQ transporters depend on PMF for active transport of BCAAs. The same results were also seen with 14 C-L-Valine and 14 C-L-Leucine (data not shown)

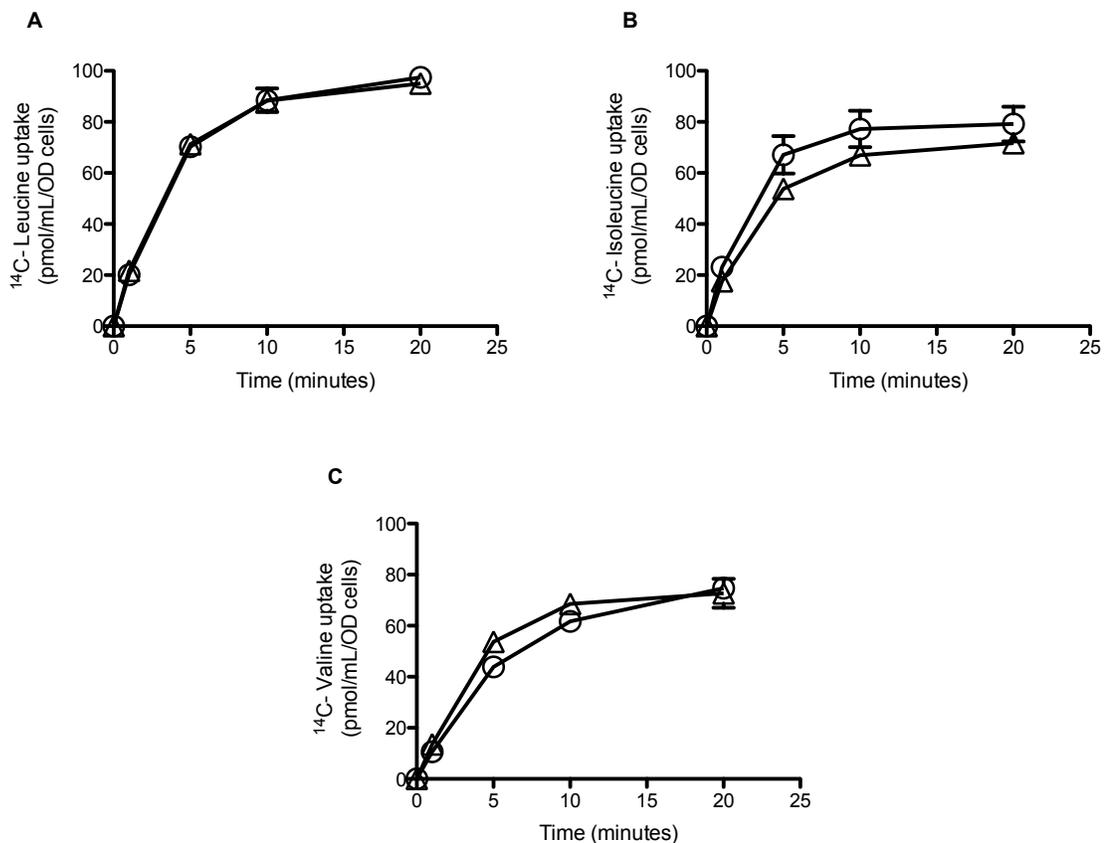


Figure 8. Excess threonine does not inhibit BrnQ-dependent uptake of BCAAs. Uptake assays are shown for WT (open circles) and $\Delta brnQ123$ (open triangles). Transport assays using samples of cells harvested from the exponential phase of growth in complete CDM were performed in the presence of 0.05 μCi ^{14}C -labeled branched-chain amino acid and 150 μM of unlabeled threonine. Results are presented as mean \pm SD of three independent replicates.

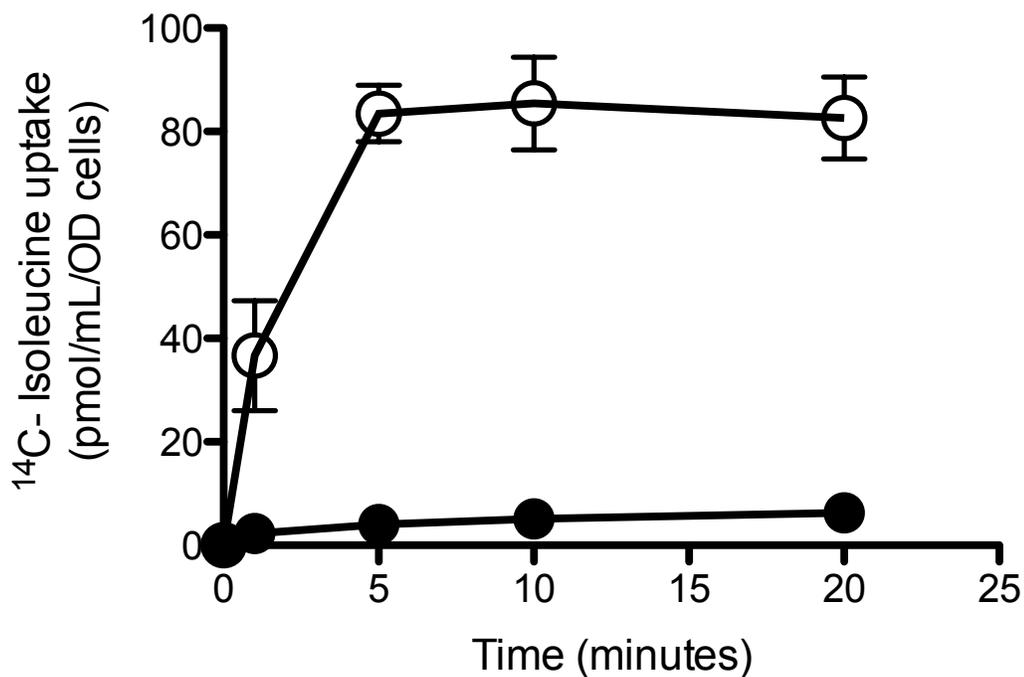


Figure 10. Radioactive BCAA uptake assays illustrating that the uptake of ^{14}C -L-isoleucine is dependent on PMF. Addition of uncoupler carbonyl cyanide m-chlorophenyl hydrazine (CCCP) to WT (closed circles) abolished uptake of labeled BCAA, compared to WT without exposure to CCCP (open circles). Transport assays using cells harvested from the exponential phase of growth in complete CDM were performed in the presence of 0.05 μCi ^{14}C -labeled isoleucine with or without pretreatment with 200 μM of CCCP. Results are presented as mean \pm SD of three independent replicates.

3.3.4. BrnQ1 transports isoleucine, leucine and valine

The preceding experiments set the conditions to test for the uptake of BCAAs using the individual BrnQ mutants in order to determine their specificity and address the question of whether there was a predominant BrnQ transporter. As a result, various conditions were tested with the addition of each labeled-BCAA mixed with a 1000-fold excess of an unlabeled BCAA. The results of a series of uptake assays performed on the *brnQ1* mutant (Fig. 11) clearly demonstrate that the BrnQ1 transporter is capable of transporting all three BCAAs. As illustrated, the *brnQ1* mutant shows an uptake deficiency for leucine and valine that is comparable to that of the *brnQ123* mutant. As previously mentioned, we were able to eliminate the background uptake of an alternate transporter with the addition of unlabeled BCAA, since the transporter is shown to have a preference for the competing unlabeled BCAA due to its excess. As a result, we see an uptake deficiency in both $\Delta brnQ1$ and $\Delta brnQ123$, and the complementation of BrnQ1 function by overproducing BrnQ1 in $\Delta brnQ1$ (via plasmid pSO1) resulted in a strong increase in the uptake rate of ^{14}C -L-Isoleucine, ^{14}C -L-Leucine, and ^{14}C -L-Valine (see closed symbols in panels A,B, D, E, G, H of Fig. 11). Also of note, we observed that the *brnQ1* mutant is still able to take up isoleucine in the presence of excess competing BCAAs, suggesting that another BrnQ transporter plays a pivotal role in isoleucine uptake. When the labeled-BCAA is competing with excess of the same BCAA, but in unlabeled form (Fig. 11 panel C, F, and I), this abolishes uptake of the label in all strains. This is because the BCAA transporters have a preference for the unlabeled form of BCAA only because of its excess. As a result, the unlabeled BCAA in this way is inhibiting uptake of the labeled form, more so emphasizing their role in BCAA transport.

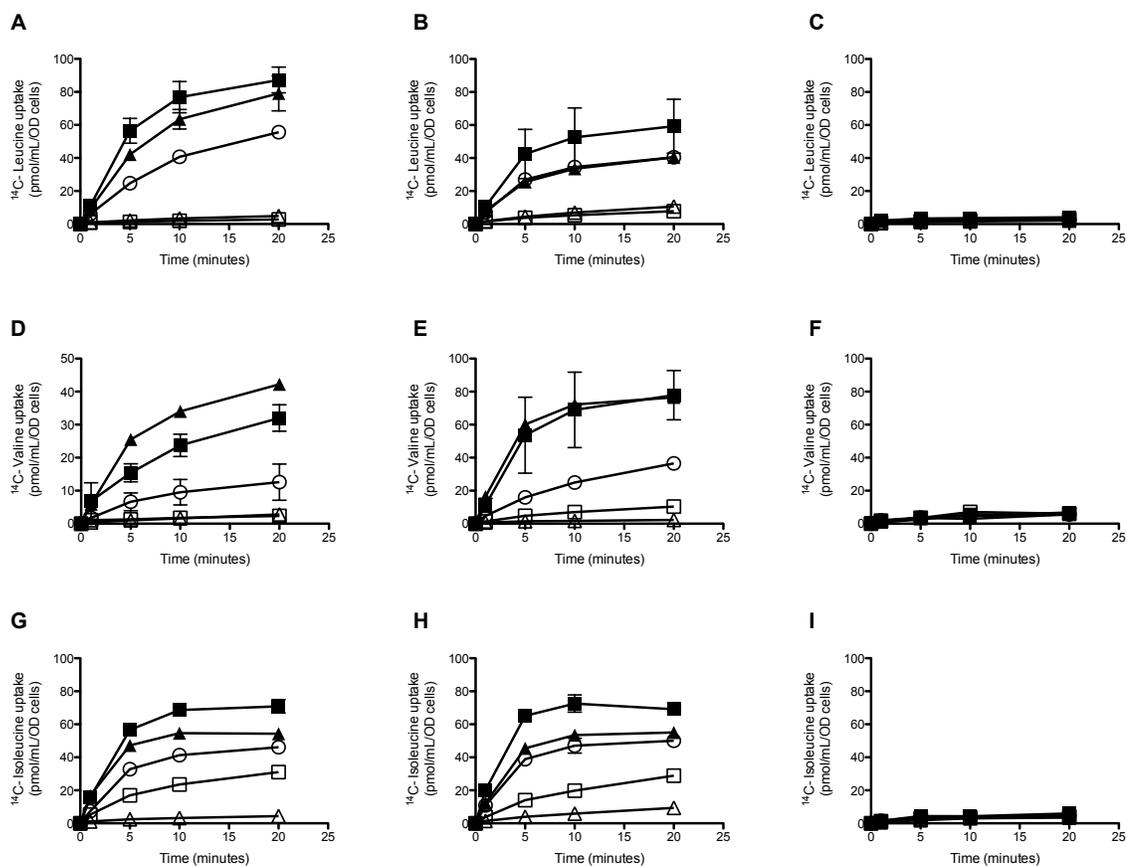


Figure 11. Radioactive BCAA uptake assays illustrating that BrnQ1 can transport all three BCAAs. WT (open circles); $\Delta brnQ1$ (open squares); $\Delta brnQ1 + pSO1$ (closed squares); $\Delta brnQ123$ (open triangles); $\Delta brnQ123 + pSO1$ (closed triangles). A) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Isoleucine B) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Valine; C) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Leucine D) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Isoleucine; E) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Leucine F) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Valine; G) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Leucine; H) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Valine; I) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Isoleucine. Transport assays using concentrated samples of cells harvested from the

exponential phase of growth in complete CDM were performed in the presence of 0.05 μCi ^{14}C -labeled branched-chain amino acid and 150 μM of an unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

3.3.5. BrnQ2 transports isoleucine

The same conditions were tested for the BrnQ2 transported as previously described for the BrnQ1 transporter. However, as opposed to the *brnQ1* mutant, the *brnQ2* mutant did not show an uptake deficiency for any BCAA (Fig. 12). Based upon the data illustrated, we can conclude that BrnQ2 does not play a major role in uptake of either leucine or valine since the uptake of these two amino acids was similar in the $\Delta brnQ2$ and the wild type strains (Fig. 12). Moreover, although $\Delta brnQ2$ did not show significant attenuation of isoleucine uptake, which was presumably due to the presence of *brnQ1*, the isoleucine uptake deficiency in $\Delta brnQ123$ was complemented with *brnQ2* expressed *in trans* (closed diamonds, panels G and H, Fig. 12). This is in agreement with the preceding data shown in Fig. 11 illustrating that the $\Delta brnQ1$ strain was still capable of importing isoleucine. Overexpressed *brnQ2* in the $\Delta brnQ123$ mutant also demonstrated that BrnQ2 has some specificity for valine (Fig. 12, panel E).

3.3.6. BrnQ3 is not a BCAA transporter

The data presented in Fig. 13 illustrate that we could not demonstrate a role for *brnQ3* in BCAA import in *S. aureus*. This conclusion is drawn from the fact that import of all BCAAs was similar between that of the wild type and *brnQ3* mutant strain, and that overproduction of *brnQ3* in $\Delta brnQ123$ did not result in any appreciable increase in any BCAA uptake (Fig. 13, panels A-I). As a result, the role of BrnQ3 is currently unclear. This does not eliminate the transporter from being a non-BCAA transporter, and this remains to be tested.

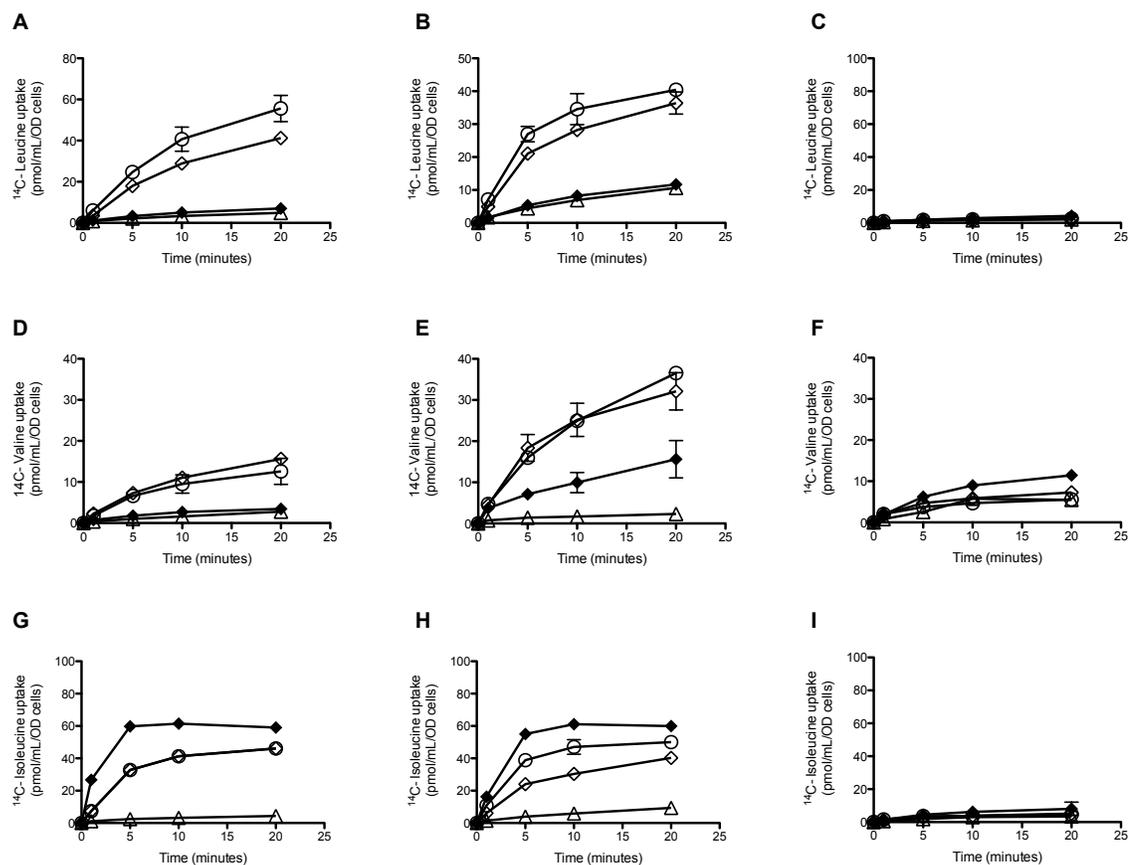


Figure 12 . Radioactive BCAA uptake assays illustrating that BrnQ2 protein transports isoleucine, and valine to a much lesser extent. WT (open circles); $\Delta brnQ2$ (open diamonds); $\Delta brnQ1,2,3$ (open triangles); $\Delta brnQ123 + pSO2$ (closed diamonds). A) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Isoleucine; B) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Valine; C) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Leucine; D) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Isoleucine; E) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Leucine; F) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Valine; G) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Leucine; H) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Valine; I) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-

Isoleucine. Transport assays using concentrated samples of cells harvested from the exponential phase of growth in complete CDM were performed in the presence of 0.05 μCi ^{14}C -labeled branched-chain amino acid and 150 μM of an unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

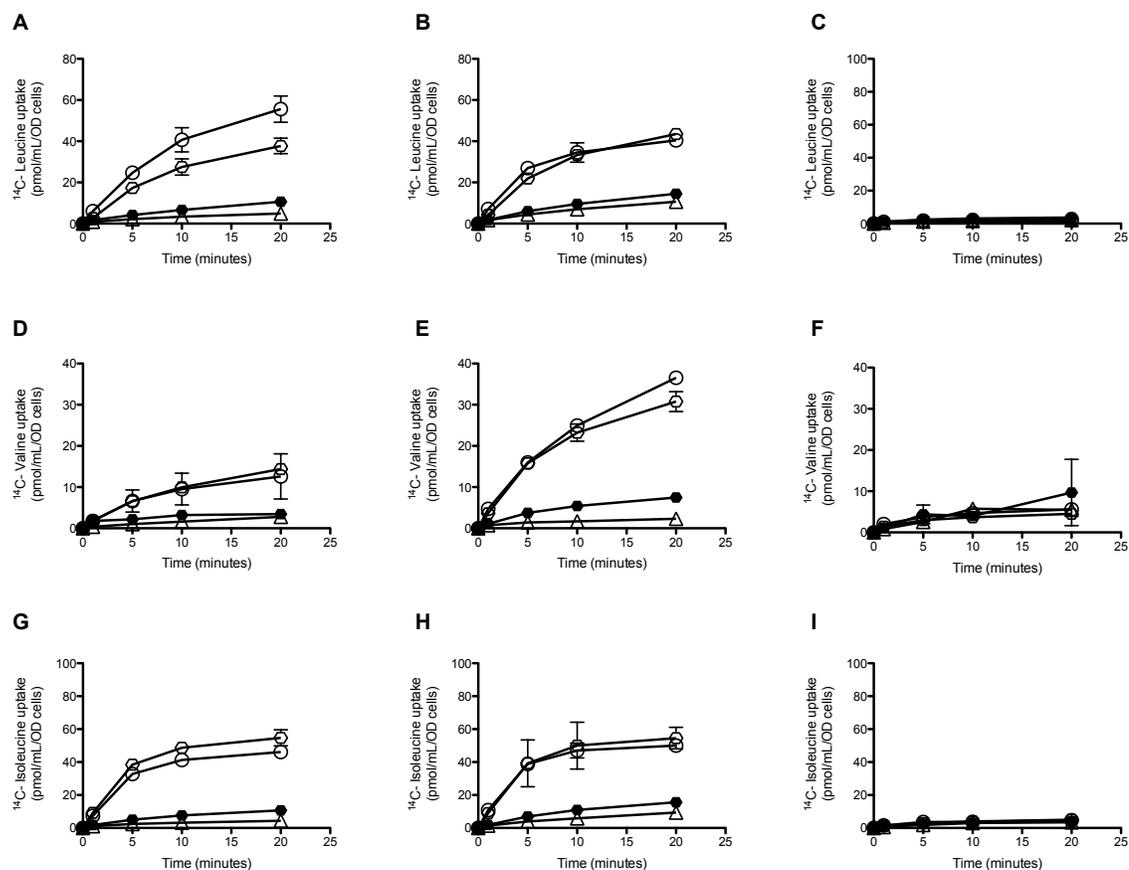


Figure 13. Radioactive BCAA uptake assays illustrating that the BrnQ3 protein does not transport BCAAs. WT (open circles); $\Delta brnQ3$ (open hexagons); $\Delta brnQ123$ (open triangles); $\Delta brnQ123 + pSO3$ (closed hexagons). A) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Isoleucine; B) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Valine; C) Uptake of ^{14}C -L-Leucine when competing with unlabeled L-Leucine; D) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Isoleucine; E) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Leucine; F) Uptake of ^{14}C -L-Valine when competing with unlabeled L-Valine; G) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Leucine; H) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Valine; I) Uptake of ^{14}C -L-Isoleucine when competing with unlabeled L-Isoleucine. Transport assays using concentrated samples of cells harvested from the exponential phase of

growth in complete CDM were performed in the presence of 0.05 μCi ^{14}C -labeled branched-chain amino acid and 150 μM of an unlabeled branched-chain amino acid. Results are presented as mean \pm SD of three independent replicates.

3.3.7. *brnQ* mutants are defective for growth in media containing low concentrations of BCAAs

It was of interest to examine the growth phenotype of the *brnQ* mutants. As shown in Fig. 14, the growth of wild type USA300, the single *brnQ* mutants and the triple *brnQ* mutant were comparable in TSB, a rich medium that contains enzymatic digests of casein and soybean meal, peptides of different lengths and compositions, and glucose as the carbon source. The absence of a growth defect in the mutants in this medium indicates that i) the BrnQ transporters do not serve any apparent physiological function during growth under nitrogen-rich conditions and ii) that the mutants do not contain other major defects that would affect growth in this condition. As a result, to examine a more relevant growth scenario for *brnQ* function, we used a chemically-defined medium containing glucose as a carbon source and all 20 amino acids added individually as the sole source of amino acids, whereby the concentrations of individual amino acids in the growth medium can be modulated. Despite that final biomass for all strains was similar, we observed that $\Delta brnQ1$ had a slightly delayed growth profile in CDM (Fig. 15, panel A). Moreover, growth of $\Delta brnQ123$ was even more affected in this medium (Fig. 15, panel A). When the concentration of BCAAs and threonine (as mentioned previously, threonine is a precursor for isoleucine biosynthesis) was lowered to 0.05% of that in complete CDM (CDM_{0.05}), growth of both $\Delta brnQ1$ and $\Delta brnQ123$ was hampered (Fig. 15, panel B). The growth defect of both $\Delta brnQ1$ and $\Delta brnQ123$ was completely complemented back to a wild type profile with the addition of *brnQ1* in trans (Fig. 15, panel B). This indicates that the presence of the *brnQs*, especially *brnQ1*, is required for optimal growth of *S. aureus* in media containing limiting concentrations of BCAAs.

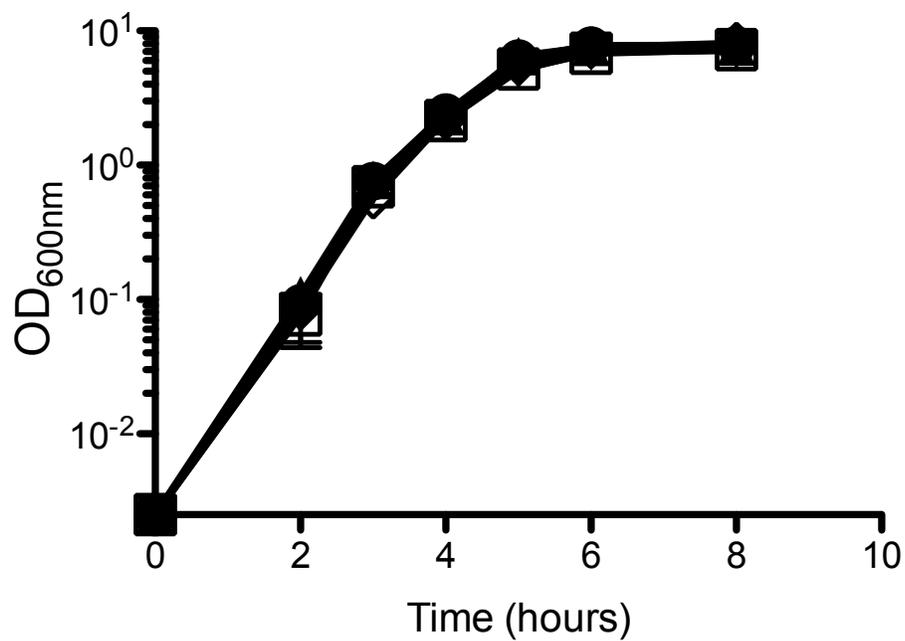


Figure 14. BrnQ transporters do not serve any apparent physiological function during growth under nitrogen-rich conditions. Growth of WT (open circles), $\Delta brnQ1$ (open squares), $\Delta brnQ2$ (open diamonds), $\Delta brnQ3$ (open hexagons), and $\Delta brnQ123$ (open triangles) in tryptic soy broth (TSB).

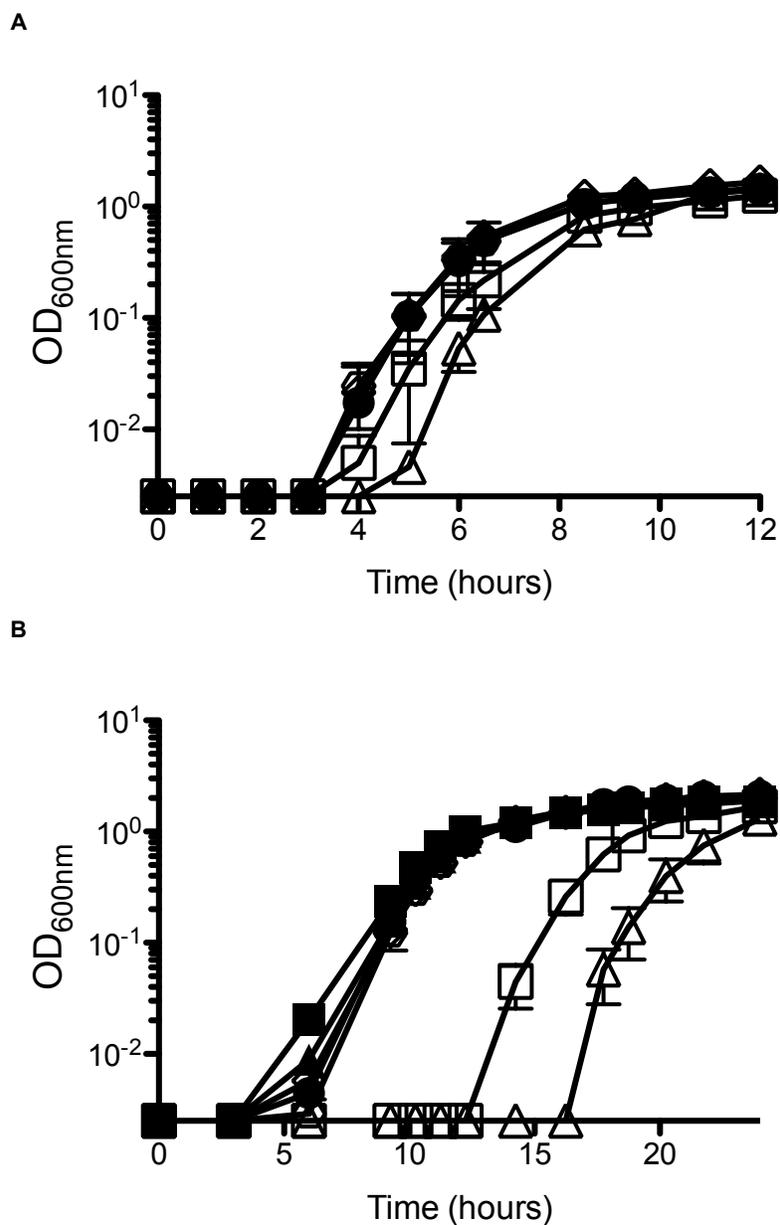


Figure 15. BrnQ transporters are required for optimal growth of *S. aureus* in media containing limiting concentrations of amino acids. Growth of WT (open circles), $\Delta brnQ1$ (open squares), $\Delta brnQ2$ (open diamonds), $\Delta brnQ3$ (open hexagons), and $\Delta brnQ123$ (open triangles), along with complemented strains in panel B only, $\Delta brnQ1$ + pSO1 (closed squares), and $\Delta brnQ123$ + pSO1 (closed triangles), was monitored in complete CDM (panel A) and CDM containing BCAAs and threonine added at 0.05% the concentration of CDM (CDM_{0.05}) (panel B).

3.4. The *brnQ1* mutant and *brnQ3* mutants are attenuated for virulence in a mouse model of bacteremia but the *brnQ2* mutant is hyper-virulent

As previously mentioned, an important growth limitation *in vivo* is very low levels of free BCAAs. Indeed, the total concentration of BCAAs in human blood (0.3–0.4 mM) is relatively low (120). As a result, the ability of *S. aureus* to scavenge these amino acids in environments that contain low concentration of these nutrients would be an important adaptation to growth *in vivo*. Data presented in the preceding section illustrated that the BrnQ transporters play a predominant role for growth in nutrient-limited environments, especially when the concentrations of BCAAs are very low (See Fig. 15).

The virulence potential of the single *brnQ* mutants and the *brnQ* triple mutant compared to the WT strain was assessed in a murine model of bacteremia. 7-week old female balb/c mice were challenged with 6.5×10^6 CFU of each strain via tail-vein injection and the mice were sacrificed 4 days post-challenge. Bacterial burden was then analyzed in kidneys, liver, and hearts from mice that survived the 96-hour experiment. Notably, all mice injected with the $\Delta brnQ2$ mutant strain became extremely sick within 24 hours, showing symptoms such as severe weight loss, and lack of locomotion, eating, drinking, and grooming. As a result, mice injected with $\Delta brnQ2$ either died or required sacrifice before the 96-hour experimental timeline (Fig. 16). Mice challenged with wild type USA300 and $\Delta brnQ1$ mutant all survived the four-day challenge.

Mice that were injected with the triple mutant lost significantly more weight (~6% more) (data not shown), were more stiff, and moved around much less; however, 70% of them survived.

Mice injected with the *brnQ123* mutant demonstrated a higher bacterial burden in both the liver and the kidneys compared to those infected with WT. However, we found that the single *brnQ1* mutant is attenuated in all three organs and the *brnQ3* mutant is attenuated in both the liver and the heart compared to WT.

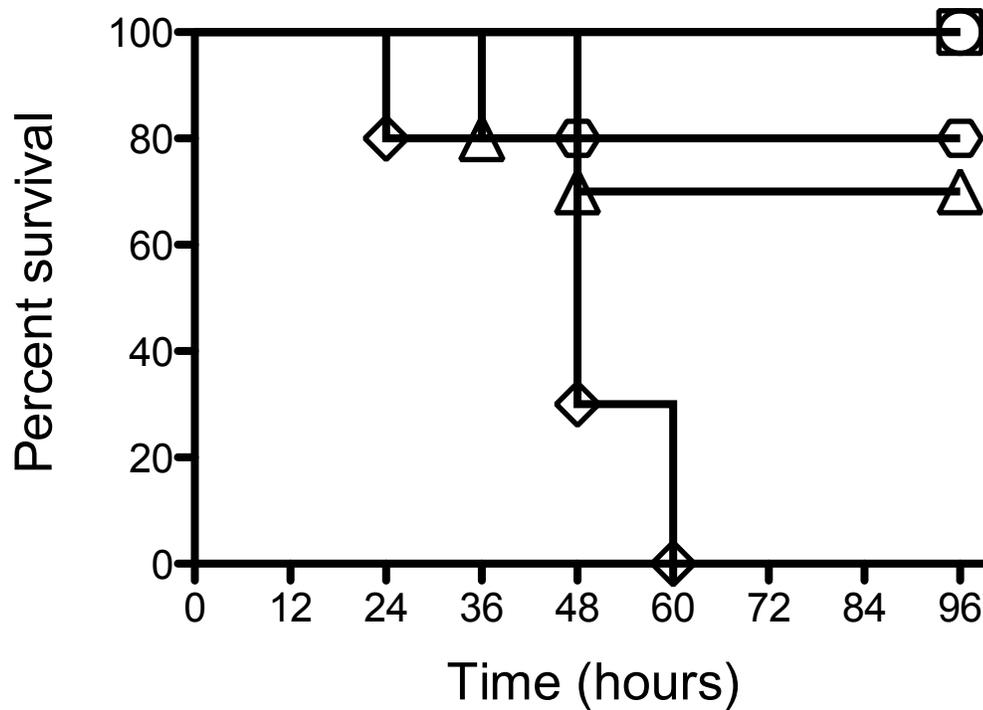


Figure 16. The *brnQ2* mutant is hyper-virulent *in vivo*. Kaplan–Meier survival curve illustrating the percent survival of 10 mice injected with $6-7 \times 10^6$ CFU of WT (open circles), $\Delta brnQ1$ (open squares), $\Delta brnQ2$ (open diamonds), $\Delta brnQ3$ (open hexagons), or $\Delta brnQ123$ (open triangles).

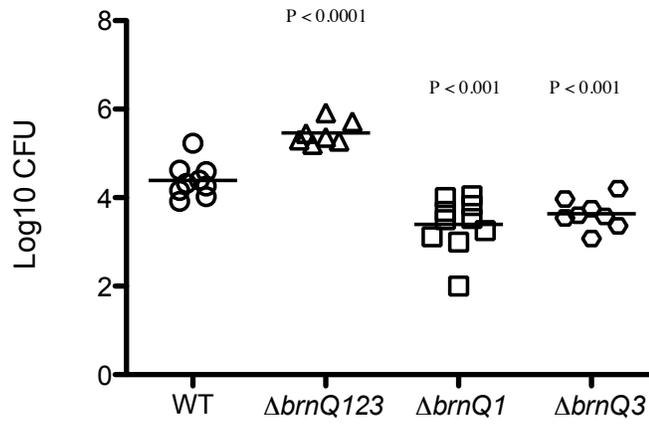
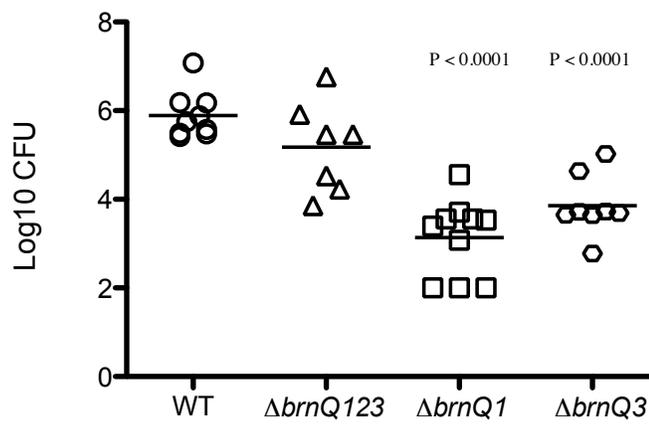
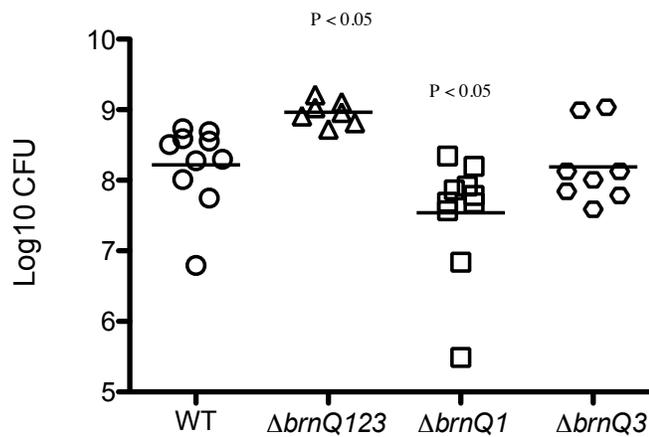
A**B****C**

Figure 17. Virulence potential of WT vs $\Delta brnQ1,2,3$, $\Delta brnQ1$, $\Delta brnQ3$ in a murine model of bacteremia. 7-week old female Balb/c mice were challenged with $\approx 6-7 \times 10^6$ CFU of either WT, $\Delta brnQ1$, $\Delta brnQ3$, or $\Delta brnQ123$. No mice injected with $\Delta brnQ2$ survived so data is not shown. Mice were sacrificed after 4 days of infection. Bacterial counts obtained from livers (A), hearts (B) and kidneys (C). Statistical analysis was performed using the Student's unpaired t-test. P values denote significant differences from wild type (WT). WT n=10, $\Delta brnQ1$ n=10, $\Delta brnQ3$ n=8, $\Delta brnQ123$ n=7.

Chapter 4- Discussion

The bacterial pathogen *S. aureus* has the ability to cause a wide variety of infections ranging from endocarditis to pneumonia and osteomyelitis. While our knowledge of the genetics and physiology of this pathogen is increasing rapidly, we still have a relatively poor understanding of *S. aureus* virulence *in vivo*.

The study of BCAA uptake has been studied extensively in gram-negative bacteria (74). Through kinetic experiments and genetic studies, it has been shown that BCAAs are transported in *E. coli* (45, 145), *S. typhimurium* (63), and *P. aeruginosa* (55) via numerous transport systems, composed of membrane-bound carriers and periplasmic binding proteins. On the other hand, only one transporter is utilized for the uptake of all three BCAAs in the gram-positive bacterium *Streptococcus cremoris* (31).

Various transport systems responsible for uptake of essential nutrients by *S. aureus* have been identified; however, no such study has yet been conducted to look at the uptake of BCAAs in this bacterium. During infection, the ability of *S. aureus* to scavenge amino acids from the host would emerge as an important adaptation to growth *in vivo* (22, 87). USA300 is not auxotrophic for any of the BCAAs (data not shown), yet intracellular biosynthesis must not be enough to fulfill their nutritional needs in an environment such as an abscess, thus creating a need for BCAA uptake during infection.

Screening, via STM, has allowed for the identification of several genes important for *S. aureus* survival *in vivo* (22, 87). Several virulence genes were identified in these screens; however, the largest gene classes represented by these mutants encoded proteins which played roles in pathways for cellular metabolism and small-molecule biosynthesis as well as cell surface transmembrane proteins involved in small-molecule binding and transport. (14, 22). Furthermore, mutants with defects in the amino acid and peptide transport, such as the Opp transporters, formed a bulk of these attenuated STM isolates (14, 87).

In one study, a *braB* mutant in *S. aureus* identified through STM was attenuated in a mouse model of systemic infection. It was proposed that this transporter might allow the cell to scavenge for specific nutrients or cofactors or, potentially function in transport of solutes in response to osmotic or other environmental stresses (14). This gene is allelic to *S. aureus* gene, *brnQ3*, which encodes a 442-amino-acid branched-chain amino acid carrier protein with 74% amino acid identity to BraB (14). Two other paralogues of *brnQ3* also exist: *brnQ1* and *brnQ2*, however, the role of all three putative BCAAs transporters in *S. aureus* has not been characterized until this study.

Briefly, in another study, more than 200 direct CodY targets in *S. aureus* were identified by genome-wide analysis of *in vitro* DNA binding. Both *brnQ1* and *brnQ2* were shown to be direct targets of CodY and were over-expressed in a *codY* mutant (82). Moreover, branched-chain amino acids play a crucial role in mediating CodY activity, since they act as effector molecules to CodY DNA binding. Hence, a lack of intracellular BCAAs will affect CodY binding to its target sites. As a result, one should observe an over-expression of genes involved in virulence and amino acid biosynthesis and transport, thereby enhancing *S. aureus* pathogenesis by allowing it to adapt to nutrient depleted environments.

Thus, due to the lack of prior studies, our lab was interested in studying the role of three putative BCAA transporters in *S. aureus* and their affect on virulence. In the current study, three tightly regulated and conserved genes encoding putative branched-chain amino acid transporters, BrnQ1, BrnQ2, and BrnQ3, were cloned, and their role in BCAA uptake were functionally characterized. One important question that this research attempted to address was the relative contribution of BrnQ1, BrnQ2, and BrnQ3 to BCAA uptake in *S. aureus*. We first generated mutations in each of the *brnQ* genes and radioactive BCAA uptake assays were conducted to determine the specificity of each transporter. When assays

were performed only by the addition of labeled-BCAA, we saw that there was no difference in uptake between the WT strain and the $\Delta brnQ123$ that was devoid of all three BrnQ transporters. This suggested that even in the absence of the three BrnQ transporters, there is an alternate transporter or transporters that are involved in BCAA uptake. However, since the focus of the research at the outset was on the BrnQ transporters, we proceeded to determine an optimal condition where we can compete out the effects of the alternate transporter and determine the function of the BrnQs. As a result, through the addition of a mixture that contains labeled-BCAA competing with a 1000-fold excess of unlabeled-BCAA, we were able to see a difference in uptake between the WT strain and the $\Delta brnQ123$ strains, whereby the triple mutant was deficient in taking up the labeled-BCAAs but the WT was still capable of uptake. This suggested that in both strains, the alternate transporter of BCAA uptake has a preference for high concentrations of the unlabeled BCAA in the mix, potentially implicating it as a low affinity, possibly less selective transporter. Nonetheless, we were able to determine that the BrnQ transporters do play a role in BCAA uptake.

One of the key findings of the study was the identification of the BrnQ1 transporter as selective for all three BCAAs based on the data showing that the *brnQ1* mutant had a significant deficiency in uptake of all three labeled BCAAs, and that *brnQ1* in trans resulted in uptake of all three BCAAs in both the $\Delta brnQ1$ and $\Delta brnQ123$ mutants. *L. lactis* encodes two BCAA transporters, BcaP and BrnQ, where BcaP was shown to be the major BCAA carrier in this organism and BrnQ played a smaller role in a part of BCAA uptake. Deletion of *bcaP* along with *brnQ*, a second low-affinity BCAA permease, reduced the viability of the strain. Interestingly, the BrnQ1 protein in USA300 demonstrates 61% identity and 78% total similarity to the BrnQ protein in *L. lactis*, suggesting that BrnQ1 possibly plays a similar role to that of a major BCAA carrier. Moreover, we also noted that the $\Delta brnQ1$ strain showed

some uptake of isoleucine, while the triple mutant had a complete deficiency in isoleucine uptake. This suggested that another BrnQ transporter played a role in isoleucine transport. This was resolved with the data demonstrating that overexpression of plasmid-encoded BrnQ2 in $\Delta brnQ123$ resulted in significantly increased uptake of isoleucine. From our uptake assay results, the overexpression of BrnQ3 protein did not have a significant impact on the uptake of any BCAA, suggesting that BrnQ3 doesn't have any major role in BCAA uptake. We hypothesize that it can play a potential role in uptake of some non-BCAA, although this remains to be tested. As a result, our current understanding on the role of BrnQ3 in *S. aureus* is unclear.

Another key finding of this study was that the presence of *brnQs* has a beneficial effect on *S. aureus* growth in CDM containing free amino acids as the sole amino acid source (Fig. 15) and that this ability is lost when the cells are exposed to a complex mixture of peptides (i.e. TSB; see Fig. 14) present in the medium. When peptides are available, these BrnQ transporters are probably unnecessary, since uptake and intracellular cleavage of peptides into free amino acids is possibly enough to maintain intracellular BCAA pools. These peptides can be substrates for highly efficient transport systems encoded by Opp transporters (54).

Our data suggest that the presence of more than one carrier system exists and enables BCAA transport in *S. aureus*. To identify candidates that could play a potential role in the remaining influx of BCAAs, we searched the staphylococcal genome for other putative BCAA uptake system genes. The product of gene SAUSA300_2538, a general amino acid permease, shares homology with BcaP in *L. lactis*, illustrating 65% identity and 80% similarity (25). Moreover, transcription of this gene, like that of the *brnQ* genes, is dependent on CodY, since its identical homolog, SA2619 also encoding an amino acid permease was

shown to be highly overexpressed in a *codY* mutant in *S. aureus* UAMS-1 (82). This gene may therefore be another important BCAA carrier. Future work will aim to identify alternate genes encoding functional proteins and determine whether they are involved in BCAA transport.

In our study, the addition of CCCP inhibited active transport of all three BCAAs by the WT strain, suggesting that the BrnQ transporters function using proton motive force (PMF). This finding is supported by previous research on the effect of proton motive force on function of transporters in several *Streptococcus* species (68, 69). PMF functions as the driving force for the translocation of several solutes. Conclusions about the mode of energy coupling to solute transport are usually drawn from the effects of uncouplers, ionophores, and inhibitors of the membrane-bound H⁺-translocating ATPase and glycolytic enzymes. These compounds usually have an effect on numerous metabolic processes within cells. For example, uncouplers, which increase membrane permeability to protons, dissipate the pH gradient (ΔpH), causing changes in internal pH and a significant depletion in the ATP pool (111). Furthermore, alterations in the ΔpH can result in complex changes that may be due to a regulatory effect of the internal pH **Error! Bookmark not defined.** The CCCP functions by making the cell membrane more permeable to protons, causing a collapse in proton motive force, thereby hampering BCAA transport. Additional experiments can be conducted with the addition of DCCD, which inhibits membrane-bound ATPase and thereby active transport. In *S. agalactiae* for instance, active transport of BCAAs was inhibited by both the addition of CCCP and DCCD. This suggested that energy in the form of ATP is coupled to transport via the action of ATPase, which couples ATP hydrolysis to the extrusion of protons from the cell (85). The subsequent proton extrusion creates a proton motive force that drives the energy-dependent transport of BCAAs in accordance with the chemiosmotic concept of

Mitchell (7, 90).

After elucidating the BrnQ transporters as BCAA uptake systems, we wanted to address the question of the role of these BrnQ transporters *in vivo*. Our results show that these BrnQ uptake systems play a role in nutrient limited environments when the BCAA concentrations are limited. This suggested to us that these transporters may also play a pivotal role within the nutrient-limited host, allowing the cells to scavenge these essential nutrients. In agreement with this hypothesis, *brnQ1* and *brnQ3* mutants were attenuated in some organs during murine infection. Surprisingly, however, the *brnQ2* mutant was hypervirulent. This finding was interesting in light of a recent study demonstrating that a *codY* mutant in *S. aureus* also displayed a hyper-virulent phenotype (93). It was suggested that this hyper-virulence in the *codY* mutant could be attributed to the de-repression of CodY target genes, such as *agr*, where we would expect to see an increase in toxin production (93). Although the BrnQ1 transporter also transports isoleucine, BrnQ2 may play a more important role in isoleucine transport *in vivo*. As a result, we hypothesize that the $\Delta brnQ2$ mutant may be displaying a “*codY* phenotype”, starving the cells for isoleucine and, thus, inactivating the repressor state of CodY. Of note, CodY has highest affinity for isoleucine (a BrnQ2 substrate), and plays the dominant co-regulator role (110).

The $\Delta brnQ123$ also showed higher bacterial burden in both the liver and kidneys, compared to the wild type. We attribute this to what is likely a complex situation stemming from the lack of *brnQ1* and *brnQ3*, which causes attenuation, contrasted with the lack of *brnQ2* in the mutant, which results in hypervirulence. This may explain why 70% of mice injected with $\Delta brnQ123$ still survived but those that survived were still very sick.

Future Directions

While this research has provided important insight into the process of BCAA-uptake in *S. aureus*, there are several possible avenues of study that would be useful in furthering our knowledge of the BrnQ system along with other putative BCAA transporters. One course of study will be to identify which BCAAs are responsible for the prolonged lag-phase in the CDM_{0.05}. In that experiment, all three branched-chain amino acids, including threonine (since it is a precursor for isoleucine synthesis), were depleted in the media and were present in very limited concentration. Further growth assays will be conducted to determine if this growth phenotype by the *brnQ123* strain is due to a depletion of particular BCAAs or the limitation of all three including threonine. Additionally, our lab will also be looking into characterizing other potential candidates for BCAA transport. As previously mentioned, SAUSA300_2538 encodes a putative amino acid permease and it is highly expressed in a *S.aureus* UAMS-1 *codY* mutant. Our lab will be generating a single mutation as well as a strain that carries mutations in all three *brnQ* genes in the USA300 background and its role in BCAA uptake will be further characterized using radioactively labeled-BCAA uptake assays and growth assays. In a strain that is negative for all three *brnQ* genes along with SAUSA300_2538, we would expect to see a complete decrease in its ability to take up labeled BCAA without a competing unlabeled BCAA.

Moreover, our lab is in the process of generating a *codY* mutation in the USA300 genetic background. We are interested in performing real-time PCR to analyze the expression of each of the *brnQ* genes in order to look at whether the transporters are expressed and up-regulated in the USA300 *codY* mutant, due to de-repression of the *brnQ* target genes. We can also look at the expression of a major negatively regulated CodY target gene and compare the expression of this gene in the *brnQ123* mutant versus the expression in the WT strain, thereby allowing us to determine whether CodY activity is affected (over-expressed/ up-

regulated) in the triple mutant due to a defect in BCAA uptake. This can be assessed when cells are grown in CDM with limited concentrations of BCAAs.

Lastly, our lab is also interested in taking a closer look at the hyper-virulent phenotype that is attributed to knocking out *brnQ2*. This includes looking at the secreted protein profile and focusing on the potential increase of toxin production. Moreover, perhaps the lack of *brnQ2* also has a significant effect on isoleucine uptake *in vivo*. Since *brnQ2* is specific for isoleucine uptake, and isoleucine has been shown to be a major ligand for CodY in *S. aureus*, a significant decrease in isoleucine uptake *in vivo* may also have an effect on CodY activity, thereby causing a de-repression of many of its target genes and enhancing virulence.

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