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Examination of BCAA Transport and Synthesis in Staphylococcus aureus

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Microbiology and Immunology

Abstract

Staphylococcus aureus is a human pathogen capable of causing infections that range from mild skin and soft tissue infections to severe infections of the bone, muscle, heart, and lung. To survive and thrive in such diverse host environments, *S. aureus* must maintain sufficient levels of metabolites and cofactors to support virulence determinant production and replication. The branched-chain amino acids (BCAAs; Ile, Leu, Val) represent an important group of nutrients for *S. aureus* metabolism, as they are required for protein synthesis and synthesis of membrane branched-chain fatty acids (BCFAs), which are important for *S. aureus* environmental adaptation. Moreover, the BCAAs are co-repressors for the global transcriptional regulator CodY, which coordinates expression of nutrient scavenging and synthesis systems, as well as virulence genes, upon depletion of both BCAAs and GTP. The requirement of BCAAs for both *S. aureus* replication and niche adaptation necessitates that it either synthesize these nutrients or acquire them from the environment. Although *S. aureus* possesses the genes required for BCAA biosynthesis, it was previously reported as auxotrophic for Leu and Val. Further, BCAAs are presumed to be limited at host infection sites. In this study, we used a genetic approach to examine the mechanisms of BCAA acquisition in *S. aureus* and determine the conditions where BCAA synthesis is turned on. We found that *S. aureus* necessitates two BCAA transporters, BrnQ1 and BcaP, for growth and BCFA synthesis, with the requirement for BCAA transport *in vivo* depending on the infection site. We further determined that *S. aureus* does indeed engage in BCAA biosynthesis, but it represses synthesis in the presence of an exogenous BCAA source. This source preference is regulated by at least two mechanisms that sense exogenous levels of BCAAs, resolving the conundrum of why *S. aureus* appeared auxotrophic for Leu and Val. Altogether, this research defines the major pathways of BCAA acquisition in *S. aureus* and reveals the niche-specific requirement of nutrient acquisition pathways for *S. aureus* virulence, highlighting the impressive adaptability of *S. aureus* to nutrient deprivation and its sophistication at exploiting host nutrient sources.

Keywords

Branched-chain amino acid metabolism, Branched-chain amino acid transport, Isoleucine, Leucine, Valine, *Staphylococcus aureus*, virulence

Co-Authorship Statement

Some data presented in this thesis have been published as peer-reviewed manuscripts. The contributions of JCK to co-authored manuscripts are indicated below.

Chapter 2

The following manuscript was adapted for use in Chapter 2 with the permission of the copyright holder:

Kaiser JC, Omer S, Sheldon JR, Welch I, Heinrichs DE. Role of BrnQ1 and BrnQ2 in branched-chain amino acid transport and virulence in *Staphylococcus aureus*.

SO performed preliminary experiments and the mouse experiments producing Figure 2.12. JRS generated mutant strains. All other data were generated by JCK with guidance from DEH. JCK and DEH wrote the manuscript.

Chapter 3

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Kaiser JC, Sen S, Sinha A, Wilkinson BJ, Heinrichs DE. The role of two branched-chain amino acid transporters in *Staphylococcus aureus* growth, membrane fatty acid composition and virulence.

SS, with guidance from BJW, performed experiments and analyzed data for Table 3.4. AS performed experiments and analyzed data for Figure 3.8. JCK generated mutant strains and performed all other experiments with guidance from DEH. JCK and DEH wrote the manuscript. SS and BJW reviewed and edited the manuscript.

Chapter 4

Data presented in Chapter 4 were completed in collaboration with Dr. Jason C. Grigg and Dr. Michael E.P. Murphy. JCK, with guidance from DEH, performed all experiments and analyzed whole genome sequencing data. JCG, with guidance from MEPM, conducted software and manual analysis of the 5'UTR sequence and identified putative regulatory features, resulting in Figure 4.9.

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List of Abbreviations

˚C	Degrees celsius
ACME	Arginine catabolic mobile element
ACT	Aspartate kinase chorismate mutase TyrA
Agr	Accessory gene regulator
AHAS	Acetohydroxyacid synthase
Ala	Alanine
Ap	Ampicillin
BCAA	Branched-chain amino acid
BCFA	Branched-chain fatty acid
BKD	Branched-chain α-keto acid dehydrogenase
BLAST	Basic local alignment search tool
Bp	Base pair
CA-MRSA	Community-associated methicillin-resistant *S. aureus*
Cna	Collagen adhesion protein
CcpA	Carbon catabolite protein
CDM	Chemically defined medium
CFU	Colony forming units
Clf	Clumping factor
Cm	Chloramphenicol
CoA	Coenzyme-A
CoNS	Coagulase negative staphylococci
Cre	Catabolite-responsive element
Cys	Cysteine
D	Direct
DAP	Daptomycin
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
Em	Erythromycin
EMSA	Electromobility shift assay
FA	Fatty acid
FAME	Fatty acid methyl ester
FAS	Fatty acid synthase
FnBP	Fibronectin binding protein
GAF	cGMP specific phosphodiesterases, adenylyl cyclases, FhlA
GTP	Guanine triphosphate
HA-MRSA	Hospital-associated methicillin-resistant *S. aureus*
Hr	Hour
HYP	Hypothetical
ID	Indirect
Ile	Isoleucine
K_M	Michaelis constant
Kb	Kilobase
LAC	Los Angeles County
Leu	Leucine

LIV	Leucine, Isoleucine, Valine
LIVCS	Leucine, Isoleucine, Valine:cation symporter
Lrp	Leucine responsive protein
LS-B	Leucine-specific binding
M	Molar
Met	Methionine
Mg	Milligram
MIC	Minimum inhibitory concentration
Min	Minute
mL	Milliliter
MRSA	Methicillin-resistant *S. aureus*
MSF	Major super facilitator
ND	Not determined
OD	Optical density
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PLP	Pyridoxal phosphate
PMB	Polymyxin B
pmol	Picomole
PSM	Phenol soluble modulin
PVL	Panton-Valentine leukocidin
qPCR	Quantitative polymerase chain reaction
RLU	Relative light units
RNA	Ribonucleic acid
Rot	Repressor of toxin
s	Second
Sae	*S. aureus* exoprotein expression
Sar	Staphylococcal accessory regulator
SCC	Staphylococcal chromosomal cassette
SCFA	Short-chain fatty acid
SCUFA	Short-chain unsaturated fatty acid
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sm	Streptomycin
SNP	Single nucleotide polymorphism
SSTI	Skin and soft tissue infection
STM	Signature-tagged mutagenesis
Tc	Tetracycline
TCA	Trichloroacetic acid
TD	Threonine deaminase
Thr	Threonine
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UTR	Untranslated region
v/v	Volume per volume
Val	Valine

V_{max}	Maximal velocity
WT	Wild type
µg	Microgram
µL	Microliter
µM	Micromolar
µm	Micrometer

Chapter 1

1 Introduction

Staphylococcus aureus is a significant human pathogen that imparts tremendous burden on human health. This burden is the result of widespread *S. aureus* colonization on the skin and in the nares of the healthy population, the diversity of its infectious capability, from mild skin infections in healthy individuals to invasive infections of nearly every body site in susceptible populations, and the emergence of antibiotic-resistant strains (1–3). Although decades of research have detailed a profusion of virulence strategies important for *S. aureus* pathogenesis (4, 5), an incomplete picture remains of how *S. aureus* maximizes its virulence potential in various host environments, which each present a unique set of challenges for *S. aureus* to overcome. Continued investigation into the basic biology of these aspects of *S. aureus* pathogenesis are therefore needed to uncover how this pathogen responds to changes in its environment and how it coordinates the employment of virulence strategies to adapt to these changes. Such efforts will serve as the foundation for the rational development of novel therapeutics to treat infections and reduce the burden of *S. aureus* disease.

1.1 The Staphylococci

S. aureus is a facultative anaerobe, Gram-positive bacterium that belongs to the *Staphylococcus* genus, which is composed of 47 species and 23 subspecies and includes opportunistic human and non-human pathogens of medical and veterinary importance (6). Staphylococci are divided into two categories based on their ability to coagulate plasma, a phenotypic trait that has proved advantageous in a clinical setting for differentiating between the most pathogenic human-associated staphylococci, *S. aureus*, which is coagulase-positive, from the less pathogenic coagulase-negative staphylococci (CoNS). The test indicates the presence of the coagulase enzyme, which converts fibrinogen to fibrin. *S. aureus* produces two forms, a secreted coagulase and a cell-wall associated coagulase. The phenotypic trait is shared by some animal-associated staphylococci, which can lead to misclassification of isolates as *S. aureus* in a veterinary setting.

CoNS are part of the normal microbiota of animals and human. In humans, the CoNS colonize the skin, with the spatial habitat occupied (i.e. head, arms, legs, axillae, and groin

area) dependent on the species (6). Some species, including *Staphylococcus caprae*, *Staphylococcus epidermidis*, and *Staphylococcus warneri* also colonize the nasopharynx. In a health-care setting, the CoNS are recognized as significant opportunistic pathogens, representing the third most common cause of bloodstream infections (7). Populations at most risk of infection include the elderly, premature newborns, chronically ill/immunocompromised, and patients that have inserted or implanted medical devices, which provide an avenue for CoNS entry into the bloodstream because of a breach in the epithelial barrier and/or colonization of the device (6). Depending on the location of the device, systemic spread of CoNS can lead to abscesses, endocarditis, meningitis or sepsis (8). *S. epidermidis* is the most frequent isolate from CoNS infections, followed by *Staphylococcus hominis*, *Staphylococcus haemolyticus* and *Staphylococcus capitis* (6, 8). *Staphylococcus lugdunensis* represents a serious causative agent of infective endocarditis, and although isolated less frequently than the leading cause, *S. epidermidis*, it causes particularly aggressive infections (9, 10).

1.1.1 *Staphylococcus aureus*

The ecological niche of *S. aureus* resembles that of CoNS, the skin and nasopharynx (2). The most frequent site of carriage is the anterior nares, with approximately 30% of the population carrying *S. aureus* in the nose (11). The carrier status serves as a reservoir for infection of colonized individuals as well as individuals within direct contact, best exemplified by outbreaks in athletes and the military (12–17). The prevalence of *S. aureus* in the healthy population, coupled with its highly invasive capacity, contribute to its success as the leading cause of bloodstream infections, pneumonia, and skin and soft tissue infections (SSTIs) (18).

SSTIs are on the milder side of the severity spectrum of *S. aureus* infections and include infections of hair follicles (i.e. furuncles and carbuncles) or the subcutaneous layer (i.e. cellulitis), which *S. aureus* can invade upon damage to the epithelial barrier. Severe infections can arise, however, if the bacteria disseminate to the bloodstream, which can lead to sepsis, abscesses, infection of muscle or bone, or pneumonia (3). Severe *S. aureus* infections have historically been associated with the hospital setting, where immunosuppression, antibiotic use, surgery, and insertion of medical devices put patients at risk of invasive disease (3, 19). The global emergence of strains resistant to β-lactam

antibiotics in the 1960s (i.e. methicillin-resistant *S. aureus* (MRSA)) severely limited treatment options and gave rise to numerous hospital outbreaks (20, 21). The epidemiology of *S. aureus* infections shifted again in the 1990s to early 2000s, when the incidence of SSTIs in young, healthy individuals increased at an unprecedented level (22). These infections were traced to new strains of community-associated (CA)-MRSA, referring to the origin of these infections in individuals lacking the typical health-care associated risks. Although originally associated with infections with a community onset, CA-MRSA strains, in particular the USA300 strain, now rival hospital-associated (HA)-MRSA strains in causing nosocomial MRSA outbreaks (21).

One of the genetic features distinguishing CA-MRSA from HA-MRSA is the staphylococcal chromosomal cassette *mec* (SCC*mec*) element, which carries the *mecA* gene that confers resistance to β–lactams. The SCC*mec* element in CA-MRSA is missing non-β–lactam resistance genes and is therefore smaller than that in HA-MRSA (23, 24). CA-MRSA infections are also associated with the presence of the Panton-Valentine leukocidin (PVL) (25–27). The USA300 strain can be further distinguished by the acquisition of the arginine-catabolic mobile element (ACME), a 30.9 kb pathogenicity island (28). Given its renowned reputation in the clinic, molecular pathogenesis studies of *S. aureus* have focused on the USA300 strain (29, 30).

Several unique features of USA300 are thought to contribute to its overwhelming burden as the leading cause of SSTIs and severe infections, such as pneumonia and necrotizing fasciitis (21). With acquisition of the ACME cassette, USA300 gained heightened adaptation to acidic conditions, like those present on the skin surface (31), and resistance to polyamines, which increases the persistence of USA300 in abscesses during the healing process (31–33). Although deletion of the ACME cassette reduces the competitive fitness of USA300 in a model of bacteremia, no virulence defect is observed for pneumonia or skin abscesses (34, 35). Rather, higher production of notable toxins, including α-toxin, the phenol-soluble modulins (PSMs), and PVL, likely related to the increased expression of their regulators *agr* (accessory gene regulatory) and *sae* (*S. aureus* exoprotein expression), is believed to contribute to the hypervirulence of USA300 in comparison to other MRSA strains (29, 30, 36), however, the molecular mechanisms governing these differences remain unknown.

1.2 The intersection of metabolism and virulence in *S. aureus*

In general, *S. aureus* pathogenesis involves three processes; adherence to host tissue, evasion of host defenses, and replication. Several surface proteins aid in adhesion of *S. aureus* to extracellular matrix or host cells, including fibronectin-binding proteins A and B (FnBPA/B), clumping factors A and B (ClfA/B), and collagen adhesion (Cna) (37). If *S. aureus* breaches the surface barriers, it employs its repertoire of virulence factors to circumvent the immune response. It secretes exotoxins that kill or subvert host cells (leukocidins, hemolysins, enterotoxins, superantigens, PSMs), produces exoenzymes that destroy host tissue (proteases, phospholipase C, hyaluronidase), detoxifies antimicrobial peptides and molecules (catalase, superoxide dismutase, staphyloxanthin), and expresses surface proteins that subvert immune responses (protein A, Staphylococcal complement inhibitors) (Figure 1.1) (5). The damage of host tissue also liberates nutrients, such as iron sources, peptides, and lipids that *S. aureus* can acquire to support its metabolism. Also fundamental to its growth during infection is the biosynthesis of nutrients and co-factors (38, 39).

The importance of nutrient scavenging and synthesis for *S. aureus* virulence is exemplified by the finding that the majority of genes necessary for infection identified in large-scale signature-tagged mutagenesis studies fall into the categories of metabolism, transport, and biosynthesis (38, 39). The most detailed characterization of *S. aureus* nutrient acquisition is likely that of the iron acquisitions strategies it employs to overcome the metal withholding strategies of the host (i.e. nutritional immunity). By encoding, and selectively utilizing, multiple metal acquisition systems, *S. aureus* exploits host iron reservoirs to meet the nutritional requirements of growth (40, 41). The mechanisms for obtaining other important nutrients, and the regulation of such systems, however, are not well understood.

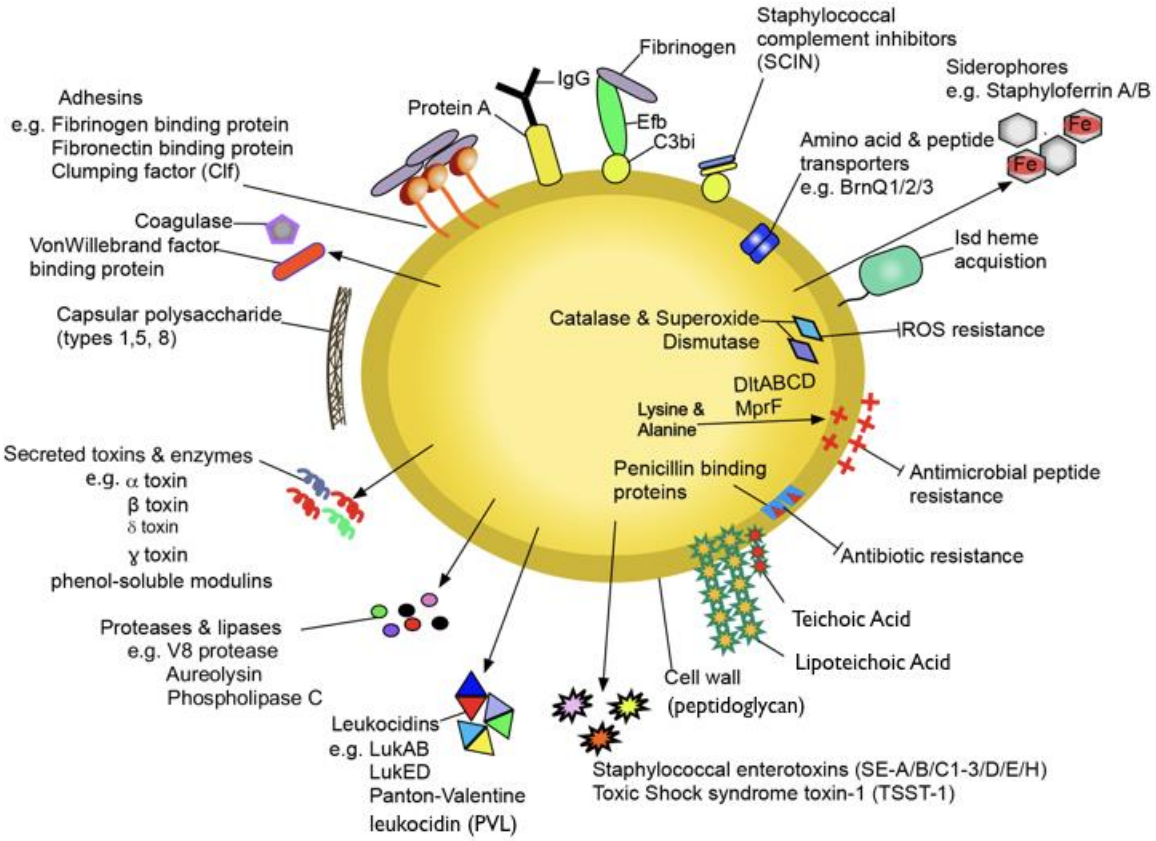

Figure 1.1 *Staphylococcus aureus* virulence factors.

Further linking *S. aureus* metabolism to virulence is the coordination of virulence factor expression in response to metabolic cues. Global metabolic regulators not only directly regulate virulence factor synthesis, but also precede virulence gene regulators in the hierarchy of transcription, indirectly regulating virulence genes in response to changes in the environment (42). The expression of virulence genes is controlled by two central regulators; *agr* and the staphylococcal accessory regulator (*sarA*) (43, 44). The *agr* system is a quorum-sensing system that leads to activation of a two component regulatory system upon accumulation of an autoinducing peptide in the extracellular environment (45). The regulator of the system activates expression of a regulatory RNA, *RNAIII*, which upregulates toxin expression, including hemolysins, PVL, and enterotoxins, and downregulates surface proteins (46). This regulatory system promotes exotoxin expression in response to high population cell density, a characteristic of the growth-phase dependent expression of exoproteins in *S. aureus* (47). SarA regulates expression of exoproteins and cell wall proteins directly and indirectly through *agr*, and although initially considered a DNA-binding

transcription factor, more likely stabilizes target mRNAs by acting as a histone-like protein (48–51). Additional regulators of virulence include the *sae* locus and repressor of toxins (*rot*) (52, 53).

To achieve appropriate virulence factor production, the aforementioned regulatory networks receive input from global transcriptional regulators that respond to environmental cues and induce metabolic reprogramming to support replication and virulence factor synthesis (42). Such regulators include redox-responsive regulators (i.e. Rex) (54), oxygen-dependent regulators (i.e. PerR) (55), metal-dependent regulators (i.e. Fur) (56, 57) and metabolic and nutrient-responsive regulators (i.e. CcpA) (58). Indeed, recent descriptions of novel metabolic regulators or metabolic regulons linking carbon metabolism to virulence in *S. aureus* continue to add to the complexity of virulence gene regulation (Table 1.1). Notably, the branched-chain amino acids (BCAAs) are emerging as nutrients at the intersection of metabolism and virulence in *S. aureus* due to their role as effectors of CodY, a global regulator of metabolism and virulence in *S. aureus* (Table 1.1) (59–61). Obtaining BCAAs is therefore important not only for the growth of *S. aureus*, but also for its adaptation to changes in nutrient availability and the regulation of virulence gene expression. Yet, the strategies *S. aureus* employs to obtain BCAAs and the consequence of BCAA limitation on *S. aureus* environmental adaptation remain unknown.

Table 1.1 Regulators of carbon metabolism and virulence in *S. aureus*

Regulator	Metabolite sensed	Select virulence genes	Reference
CodY	BCAAs, GTP	α-toxin (*hla*), hyaludonidase (*hyl*), coagulase (*coa*), fibronectin-binding protein (*fnBPA*), capsule (*cap*), polysaccharide intracellular adhesion (*ica*), protein A (*spa*)	(59–65)
CcpA	Glucose	Urease, protein A (*spa*), toxic shock toxin (*tst*)	(58, 66)
CcpE	Citrate	Capsule biosynthesis (*capA*), α-toxin (*hla*), phenol-soluble modulins-α (*psmα*)	(67, 68)
RpiRC	Pentose phosphate pathway intermediate	Leukocidins, protein A (*spa*), capsule (*cap*), hemolysins, *RNAIII*	(69, 70)

1.3 Branched-chain amino acids in bacterial physiology

The branched-chain amino acids (BCAAs; isoleucine, leucine, and valine) are small non-polar amino acids that are hydrophobic in nature due to their branched-alkyl side chains (Figure 1.2). Leu is one of the most abundant amino acids in proteins and is typically found in the inner helical core of proteins, as its hydrophobicity is a strong stabilizer of α-helical structures (71). The hydrophobicity of Ile and Val similarly dictates their location in the hydrophobic core of proteins, however, the substitution of the β-carbon with a methyl group on these amino acids creates bulkiness that restricts the conformation of the main chain and destabilizes α-helical structures. Consequently, Ile and Val are preferentially located in β-sheets (72, 73).

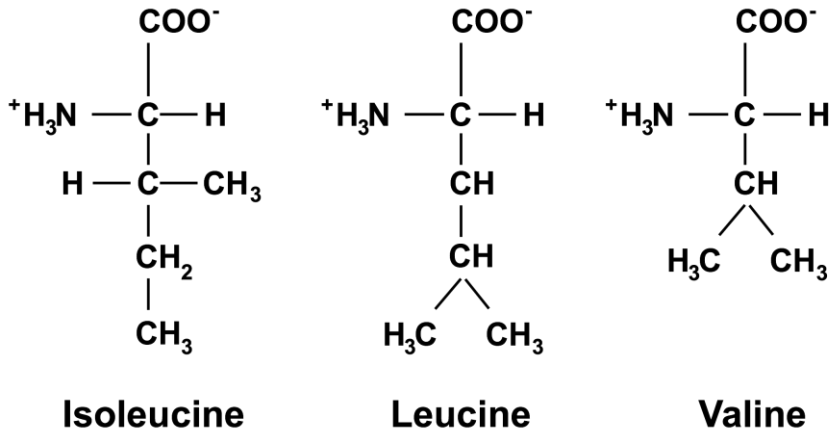

Figure 1.2 Chemical structure of branched-chain amino acids.

Bacteria synthesize BCAAs through a conserved pathway that is present in Fungi and Plants, but absent in Mammals. The level of synthesis is dependent on the availability of metabolites linked to central metabolism, including pyruvate, acetyl-CoA, and α-ketobutyrate, which although not a direct product of central metabolism, is a product of threonine deamination with threonine being synthesized from aspartate, a product of oxaloacetate transamination (Figure 1.3). The biosynthetic pathway also provides intermediates for pantothenate synthesis, which requires α-ketoisovalerate, the α-keto acids of Val (74), and branched-chain fatty acid (BCFA) synthesis, the predominant fatty acids (FAs) in membranes of Gram-positive bacteria (75). Thus, the importance of BCAAs for bacterial physiology stems from

their requirement for maintaining high levels of protein synthesis and their integration with cellular metabolism.

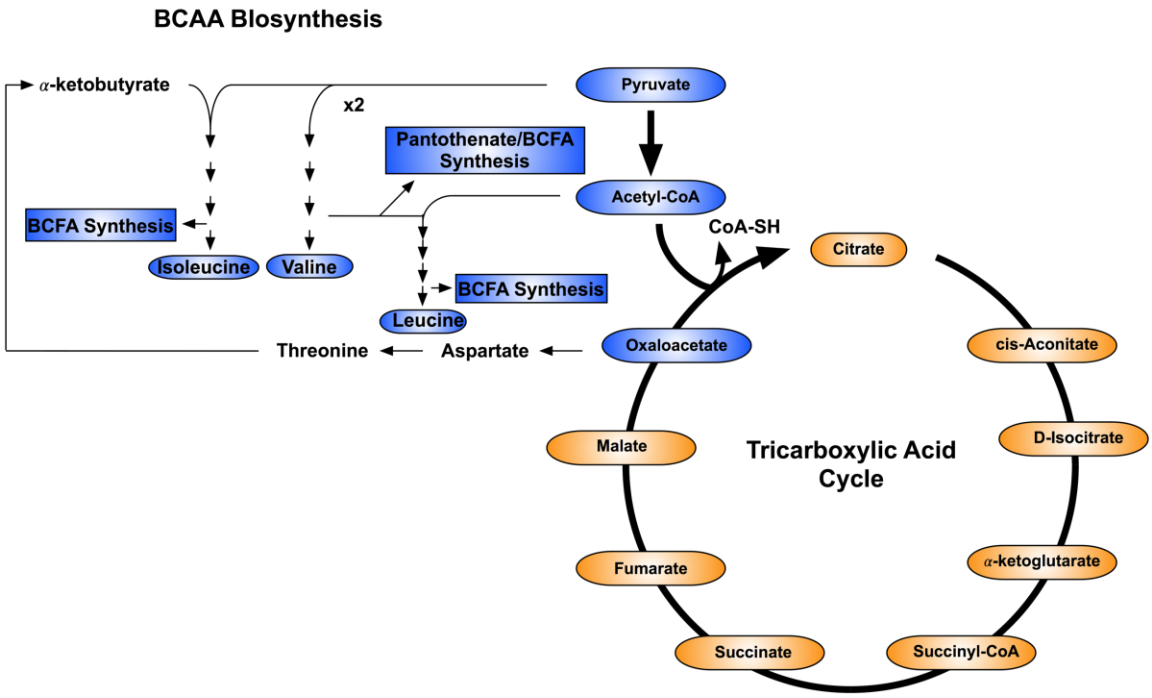

Figure 1.3 Integration of BCAA biosynthesis with cellular metabolism.

Metabolites highlighted in blue are connected to BCAA biosynthesis.

1.3.1 Branched-chain fatty acids

The cytoplasmic membrane of bacteria serves as a barrier to the external environment and is crucial to the adaptation of organisms to physical changes in the environment, including temperature, pH, osmolarity and salinity. The FA composition of the membrane determines its biophysical properties and modification of FAs enable bacteria to adapt to changing conditions. The predominant FAs in Gram-negative bacteria are short-chain fatty acids (SCFAs) and in Gram-positive bacteria are BCFAs (75). The synthesis of SCFAs and BCFAs proceeds through the multi-enzyme fatty acid synthesis (FAS)-II pathway, with acetyl-CoA as the substrate for SCFA synthesis and branched-chain acyl-CoA as the substrate for BCFA synthesis (76). Branched-chain acyl-CoA substrates are produced through the transamination of BCAAs into α-keto-acids, which are then decarboxylated by the branched-chain α-keto acid dehydrogenase (BKD) multi-enzyme complex (77). The substrate an organism uses for

FA synthesis is determined by the substrate specificity of the first enzyme in the FAS-II pathway, FabH, which catalyzes the condensation of either acetyl-CoA or a branched-chain acyl-CoA with malonyl-ACP (78, 79). FabF then elongates the product through the repeated condensation with malonyl-ACP (76). When initiated with branched-chain acyl-CoA substrates, these reactions produce even-chain anteiso-FAs derived from Ile, even-chain iso-FAs derived from Leu and odd-chain iso-FAs derived from Val (Figure 1.4).

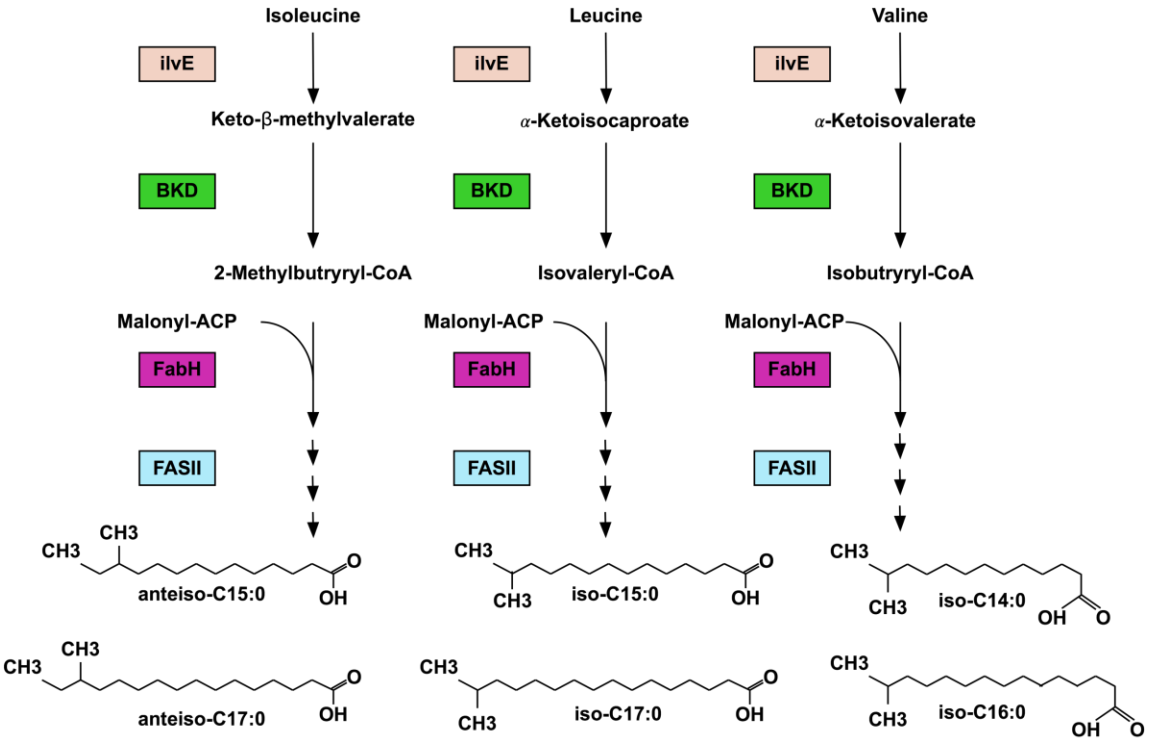

Figure 1.4 Synthesis of branched-chain fatty acids from branched-chain amino acids.

The biophysical properties of membranes composed of SCFAs are determined by the degree of saturation. The FabA and FabB enzymes introduce *cis* bonds in the acyl chains, which disrupt order and increase membrane fluidity (80). Homologs of these enzymes are not found in Gram-positive bacteria, with the exception of *Streptococcus pneumoniae* and *Enterococcus faecalis* (81, 82). Unsaturated FAs are also found in the membrane of *Bacillus subtilis* due to the presence of a cold shock-induced desaturase enzyme, which functions independently of FA synthesis and modifies FAs attached to phospholipids in the membrane (83).

The biophysical properties of membranes composed of BCFAs are determined by FA chain length and the iso:anteiso ratio. These modifications facilitate adaptation to changes in temperature, pH, salinity, and CO_2 (84–86). Of the three BCFAs, anteiso-FAs are the major determinant of membrane fluidity. The positioning of the methyl group on the acyl chain of antieso-FAs disrupts close packing of membrane lipids promoting a more fluid membrane. This property is critical to adaptation to growth at low temperatures, during which the antieso-FA content is increased (84, 87–89). Both the BKD complex and FabH enzyme exhibit substrate preference for anteiso-FA synthesis, with the preference of FabH for anteiso-FA precursors increasing as the temperature decreases (75, 90, 91), emphasizing the fundamental requirement of anteiso-FAs for homeoviscous adaptation in Gram-positive bacteria.

1.4 BCAAs as effectors of global transcriptional regulators

To cope with fluctuations in environmental nutrient availability, bacteria undergo metabolic reprogramming to sustain growth upon nutrient exhaustion. In laboratory growth conditions this follows a pattern of exponential growth under nutrient rich conditions, followed by a period of limited/no growth as a result of nutrient depletion. This transition from feast-to-famine coincides with accumulation of ppGpp (92) and is regulated by global transcriptional regulators; namely the leucine responsive regulatory protein (Lrp) in Gram-negative bacteria and CodY in Gram-positive bacteria (Figure 1.5) (93, 94). BCAAs directly bind and regulate the activity of both regulators.

1.4.1 Lrp

Lrp is a highly conserved transcriptional regulator in enteric bacteria that regulates genes upon transition to stationary phase during bacterial growth (93). Lrp regulates ~10% of the *Escherichia coli* genome and either represses or activates target genes in response to Leu, which can either enhance, antagonize, or have no effect on Lrp activity depending on the target gene (95). Lrp primarily regulates metabolic gene expression, including glutamate, glutamine, BCAA, and serine biosynthesis, glycine degradation, and BCAA and oligopeptide transport, as well as genes involved in pili formation (95). Expression of *lrp* is induced by ppGpp (96), a metabolite synthesized from GTP through a reaction that typifies the stringent response, a response provoked by amino acid starvation (92). The stringent response

coincides with entrance into stationary phase thereby linking Lrp regulatory activity to nutrient limitation.

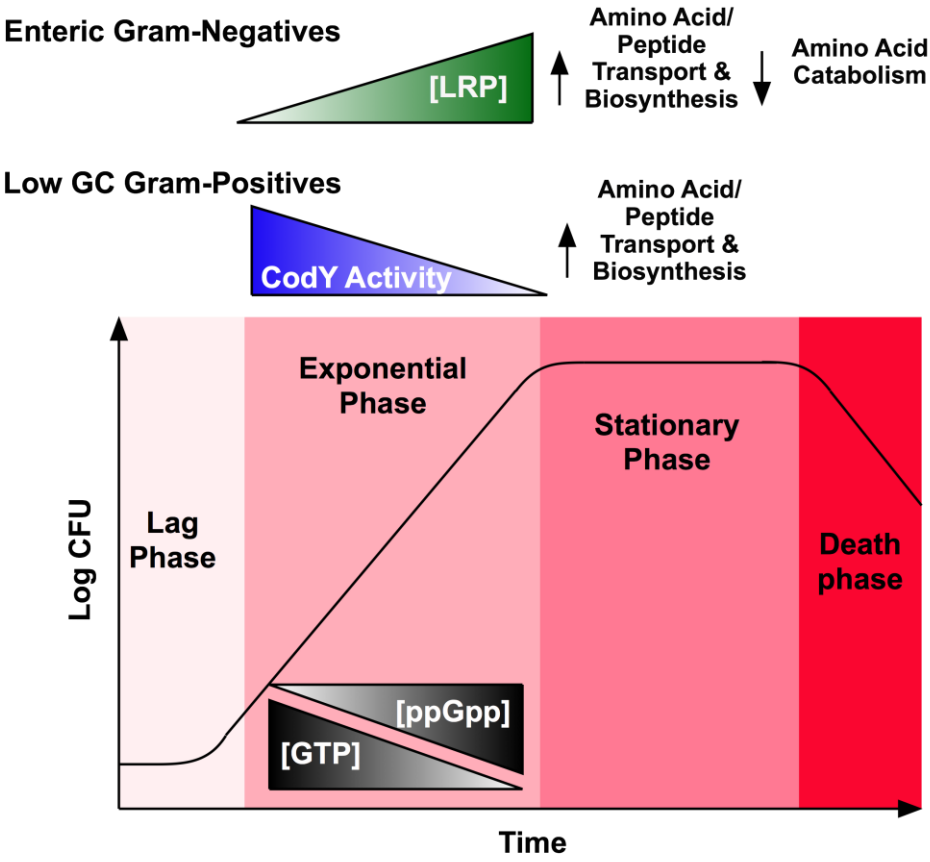

Figure 1.5. Global regulators of stationary phase adaptation in Gram-negative and Gram-positive bacteria.

Exponential growth is associated with nutrient consumption and subsequently, depletion. As amino acids become depleted, ppGpp is synthesized from GTP, correlating with the entrance to stationary phase. As ppGpp accumulates, *lrp* expression in Gram-negatives is induced. Lrp binds to Leu and either activates genes involved in amino acid synthesis and transport or represses genes involved in amino acid catabolism. In Gram-positives, CodY DNA-binding activity decreases as GTP, as well as BCAAs, are depleted. Target genes involved in amino acid biosynthesis and transport are induced.

1.4.2 CodY

CodY is a conserved transcriptional regulator in low GC Gram-positive bacteria (i.e. firmicutes) that senses the metabolic status of the cell and promotes adaptation to nutrient limitation (97, 98). CodY is activated through direct interaction with BCAAs and GTP (97–100), with the exception in *Lactococcus lactis* and *Streptococcus pneumoniae*, where CodY

responds to only BCAAs (101–103). In its active state CodY binds to DNA, typically resulting in transcriptional repression. Approximately half of CodY binding motifs are upstream or overlapping with transcriptional start sites and interfere with transcription initiation by either displacing or occluding binding of RNA polymerase or transcriptional activators (104–107). The remaining CodY binding motifs overlap with the coding region and block transcriptional elongation (104–107). Less frequently, CodY can directly activate transcription through an unknown mechanism (108, 109), with the extent of its role as an activator depending on the species. Thus, in general, CodY target genes are repressed during rapid growth and become expressed upon nutrient deprivation. CodY regulates metabolic genes involved in amino acid synthesis, purine biosynthesis, sugar and amino acid transport, and the Krebs cycle (59, 61, 101, 110, 111), and in some species, it regulates sporulation (97, 112–114) and biofilm formation (115–117).

1.4.2.1 Interaction of CodY with its ligands

The N-terminus of CodY constitutes a GAF domain, a widely distributed domain with roles in intracellular signaling and transcriptional regulation. The C-terminus is composed of a helix-turn-helix motif that confers DNA binding activity and is connected to the GAF domain by a 41 amino acid linker region (118, 119). Ile binds to the GAF domain and triggers a conformational change that is thought to promote DNA binding activity (120–122). The binding of GTP to CodY has proved more difficult to discern. Crystallization of GTP with CodY revealed a GTP binding pocket at the interface of the GAF and the linker region (119), contradicting initial theories that predicted GTP-binding involved three highly conserved GTP-binding motifs in the GAF domain (97, 100). GTP binding triggers a conformation change in the linker region of the protein (123). One group of researchers have proposed a model of CodY activity where in its liganded state, CodY forms a tetramer that conceals the DNA-binding domain, preventing DNA binding, and in the absence of ligand it forms a dimer, exposing the DNA-binding domain (119). Another group has suggested that the tetramer is not a physiologically relevant conformation, and rather ligand binding causes conformational changes in the dimer that promote DNA-binding (122). In addition to conformational changes, autophosphorylation of CodY might contribute to its DNA-binding activity, although this remains controversial. CodY from *Bacillus anthracis* has been observed to autophosphorylate on a serine residue (Ser_{215}) located in the DNA binding

domain (123). Ser_{215} is conserved in CodY across Gram-positives (123), has previously been identified as a site of phosphorylation in *B. subtilis* (124), and is a critical residue for DNA binding activity of *B. subtilis* CodY (118). However, the interaction between CodY and DNA has previously been shown to be independent of GTP hydrolysis (100), thus further studies are required to elucidate whether phosphorylation is relevant to CodY DNA-binding activity.

1.4.2.2 CodY binding strength dictates the hierarchy of target gene expression

CodY binds to a 15-nt binding motif AATTTTCWGAAAATT (105, 125), with a model emerging where a second binding motif that overlaps by 6 nt confers CodY-dependent regulation (107, 126). Motifs with nucleotide substitutions that deviate from the consensus sequence decrease the binding affinity of CodY (105, 107) and in fact, mutating the CodY motif to a perfect match, which is nonexistent in the *B. subtilis* genome (107), results in higher DNA binding activity in the absence of ligands than the binding activity of CodY to an imperfect sequence in the presence of its ligands (127). The binding strength of the motif correlates with the extent of repression, such that upon modest decreases in CodY activity, genes with lower affinity binding motifs are de-repressed and genes with higher affinity binding motifs remain repressed (127, 128). The strength of the binding motif therefore dictates when the gene is expressed in the graded de-repression of CodY target genes (128).

1.5 Mechanisms of maintaining intracellular BCAAs

1.5.1 BCAA synthesis

Bacteria maintain intracellular BCAAs through endogenous synthesis or acquisition of exogenous sources, in the form of free amino acids or peptides. Most bacteria are capable of *de novo* BCAA biosynthesis, with the exception of *Erysipelothrix rhusiopathiae*, *Mycoplasma* spp., *Ureaplasma* spp., *Peptostreptococcus anaerobius*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* (129). BCAAs are synthesized through a conserved pathway in Gram-negative and Gram-positive bacteria (Figure 1.6). Ile and Val synthesis proceed in parallel reactions, apart from the first step. Ile synthesis begins with the deamination of threonine to form α-ketobutyrate, catalyzed by threonine deaminase (IlvA). Next, acetohydroxy acid synthetase (AHAS) (IlvBN) transfers an active acetyaldehyde from pyruvate to either α-ketobutyrate in the case of Ile or a second pyruvate in the case of Val.

The resulting acetohydroxy acids are then converted by aceothydroxy acid isomeroreductase (IlvC) into dihydroxy acids, and then to branched-chain α-keto acids by dihydroxy acid dehydratase (IlvD). Branched-chain amino acid aminotransferase (IlvE) then catalyzes the transamination of the branched-chain α-keto acids to Ile or Val. The pathway for Leu synthesis proceeds from the branched-chain α-keto acid intermediate in Val synthesis. α-isopropylmalate synthase (LeuA) catalyzes the condensation of α-ketoisovalerate with acetyl coenzyme A to form α-isopropylmalate. An isopropylmalate isomerase (LeuBD) then converts this intermediate into β-isopropylamalate, and followed by conversion into α-ketoisocaproate by β-isopropylmalate dehydrogenase (LeuC). The branched-chain keto acid is then transaminated by IlvE to form Leu. The BCAA biosynthetic genes are typically organized in at least one operon, depending on the species. In *S. aureus* the biosynthetic genes are, with the exception of the *ilvE* gene, organized in a nine gene operon (*ilvDBNCleuABCDilvA*) (Figure 1.6).

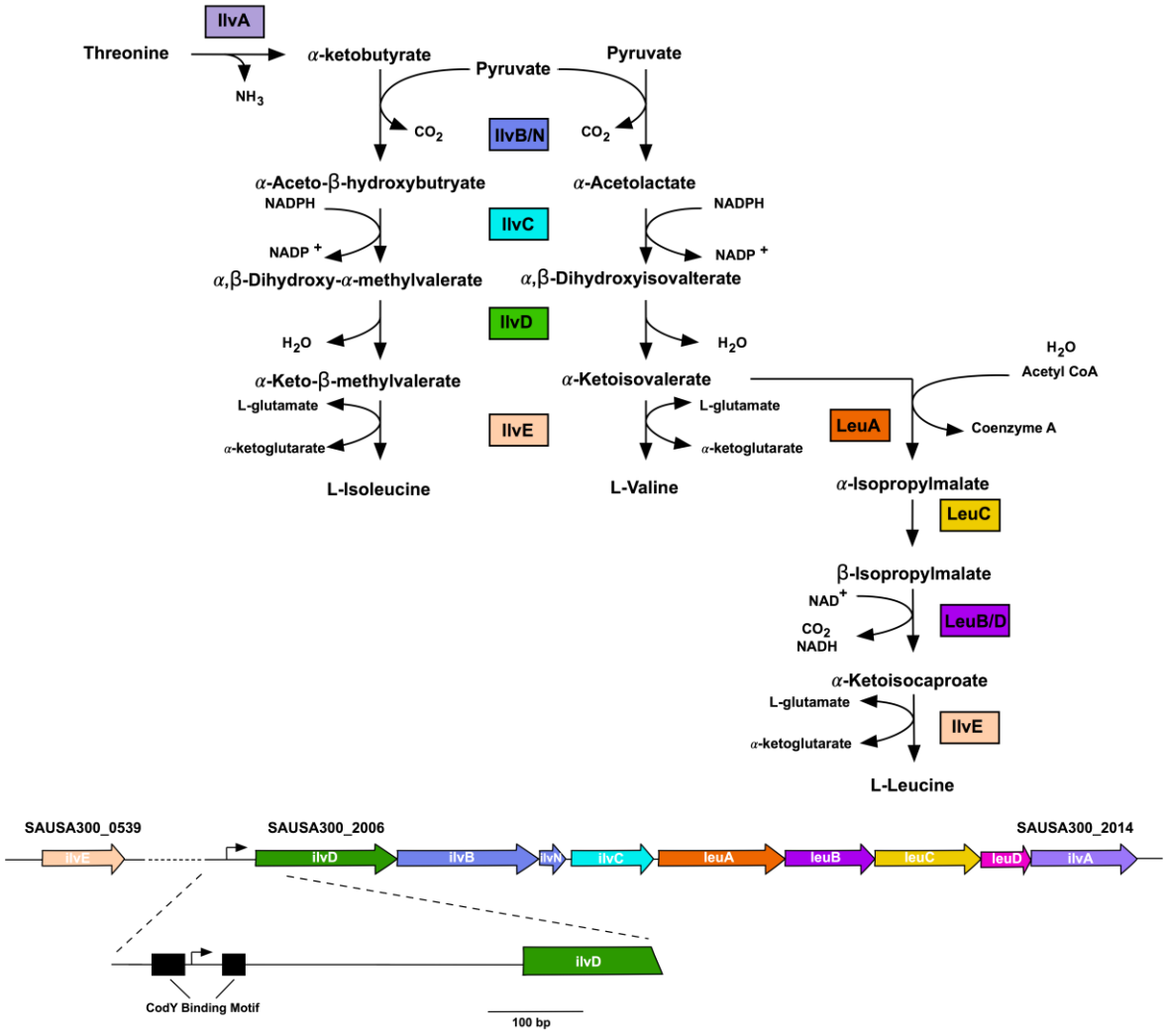

Figure 1.6 Biosynthetic pathway for isoleucine, leucine, and valine and organization of the biosynthetic operon in *S. aureus*.

1.5.1.1 Transcriptional regulation of BCAA biosynthetic operons

The most detailed descriptions of the regulation of BCAA biosynthesis come from studies in *E. coli* and *B. subtilis*. The genes for BCAA biosynthesis in *E. coli* are organized into two operons; *ilvGMEDA* and *leuABCD*. In addition to the *ilvGM* AHAS enzyme encoded by the *ilv* operon, *E. coli* encodes two additional AHAS isoenzymes, encoded by *ilvIH*, and *ilvBN*. Regulation of BCAA biosynthesis in *E. coli* is primarily mediated by transcriptional attenuation. Transcriptional attenuation involves an open reading frame upstream of the gene that is rich in regulatory codons. Depletion of tRNA corresponding to the regulatory codons causes the ribosome to stall. The stalling of the ribosome allows base-pairing of the leader

mRNA to form an anti-terminator stem loop. This base-pairing prevents formation of the terminator hairpin and results in transcriptional read-through (Figure 1.7). High levels of the appropriate tRNA allow the ribosome to continue translation, blocking formation of the antiterminator structure and promoting formation of the terminator hairpin (Figure 1.7). In this way, attenuation couples transcription of the biosynthetic operon with the availability of amino acids. Indeed, the *ilvGMEDA* operon is preceded by a 32 aa peptide that contains 15 BCAAs (130, 131), *ilvBN* is preceded by a 32 aa peptide with 11 BCAAs (132) and the *leuABCD* operon is preceded by a 28 aa peptide containing 4 Leu (133). Attenuation is also the primary mechanism of regulating the *leu* operon in *Salmonella typhimurium* (134, 135) and putative leader peptides and terminator hairpins are found upstream of BCAA biosynthesis genes across various Gram-negative species (136), suggesting that attenuation is a conserved mechanism of regulating BCAA biosynthesis.

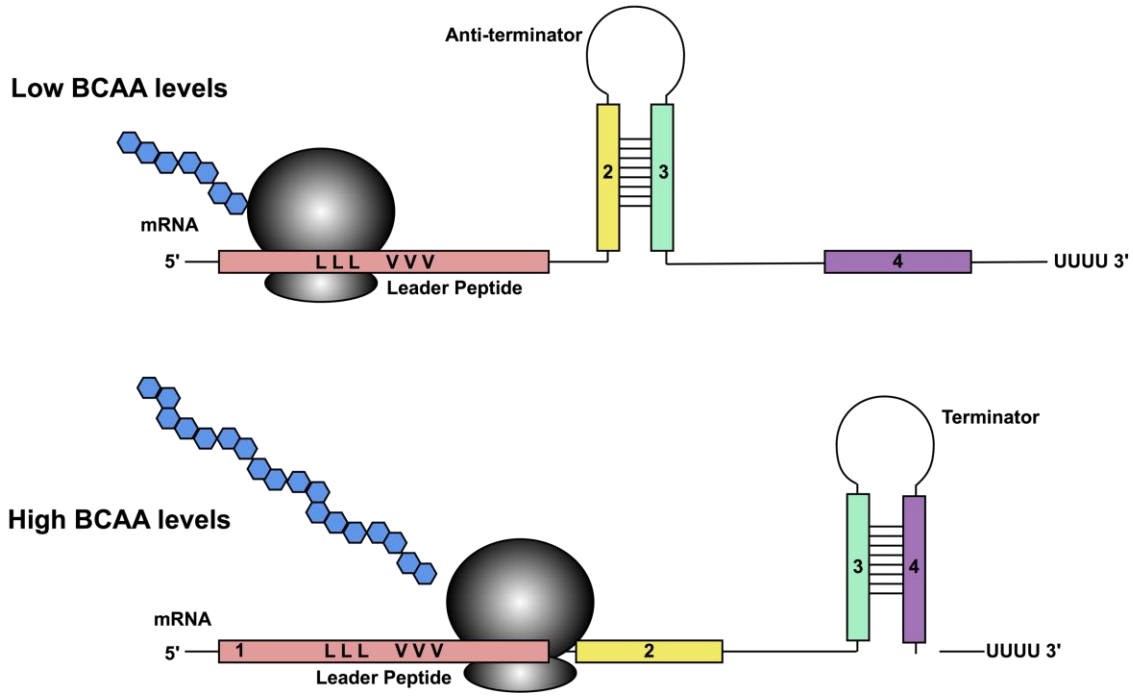

Figure 1.7 Transcriptional attenuation of BCAA biosynthesis.

In conditions where levels of Leu and Val are limited, the corresponding tRNA is not available for translation of the leader peptide that is rich in Leu (L) and Val (V). The ribosome stalls on the mRNA, promoting formation of the anti-terminator stem loop forms. Transcription proceeds. In conditions where Leu and Val are in excess, the corresponding tRNA is available for translation of the leader peptide. The ribosome proceeds along the mRNA, promoting formation of the terminator stem loop. Transcription does not proceed.

The biosynthetic genes are also regulated by Lrp. In the absence of Leu, binding of Lrp to the *ilvIH* promoter activates transcription and binding to the *ilvG* promoter represses transcription (137–139).

The genes for BCAA biosynthesis in *B. subtilis*, are encoded by the *ilvBHCleuABCD* operon and *ilvA*, *ilvD*, and *ybgE*. Regulation of the *ilv-leu* operon in *B. subtilis* involves multiple global regulators that coordinate expression of the operon in response to carbon, nitrogen, and amino acid availability (Figure 1.8) (140). The operon is positively regulated by the carbon catabolite protein A (CcpA) (140, 141), a global transcriptional regulator that regulates carbon utilization in response to a preferred carbon source (142). CcpA activates or represses its target genes by binding to the catabolite-responsive element (*cre*), a *cis*-regulatory element in the promoter region of the target gene, when in complex with the phosphorylated form of the Hpr protein (143). Hpr is a component of the phosphoenol-pyruvate-dependent phosphotransferase transporter system that is phosphorylated by HprK, a kinase that is activated by fructose 1,6-bisphosphate (144). Thus, CcpA activates transcription when there is sufficient carbon for BCAA biosynthesis. When *ccpA* is mutated, the BCAAs are not synthesized (145).

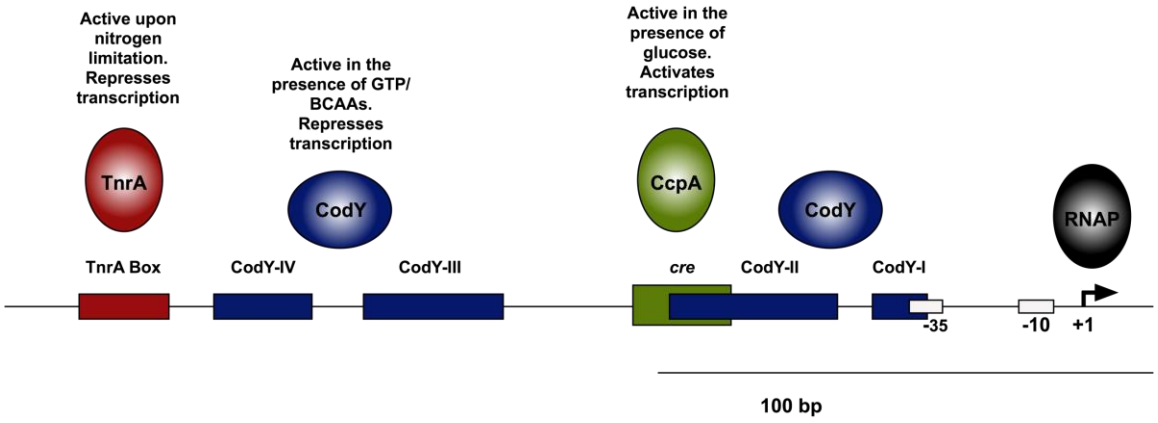

Figure 1.8 Regulation of the *ilvB* promoter in *Bacillus subtilis*. Binding sites for transcriptional regulators of the *ilv-leu* operon in *B. subtilis* are indicated relative to the promoter and transcriptional start site of *ilvB*. TnrA binds to the TnrA Box and represses transcription in the presence of nitrogen. CodY binds to four binding sites and represses transcription in the presence of BCAAs and GTP. CcpA binds to the catabolite response element (*cre*) and activates transcription in the presence of a preferred carbon source (i.e. glucose).

The positive regulation of *ilvB* by CcpA is antagonized by either TnrA or CodY (146). TnrA binds to its target sequence in response to nitrogen limitation and either represses or activates gene involved in nitrogen metabolism (147). Binding of TnrA to the *ilvB* promoter represses transcription, limiting BCAA biosynthesis when nitrogen is scarce (148). CodY represses *ilvB* transcription when cells are replete of GTP and BCAAs (99, 110), allowing for conservation of carbon and nitrogen when exogenous BCAA sources are present. A high-affinity CodY binding site proximal to the *cre* allows CodY to directly compete with CcpA binding to the *ilvB* promoter (140, 141, 146). The mechanism by which TnrA prevents CcpA activation is less clear, as the TnrA box is upstream of the *cre* and therefore does not interfere with CcpA binding. One model proposes that when TnrA binds to DNA it forms a complex with CcpA/P-Hpk and induces DNA bending that prevents RNA polymerase from initiating transcription through steric hindrance (146). Additional fine-turning of *ilv-leu* expression is mediated by a Leu-responsive T-box riboswitch (140, 149–151), as well as mRNA processing (152).

CodY-dependent repression of BCAA biosynthesis is common across Gram-positives, including *S. aureus* (59, 61, 102, 153–155), whereas TnrA homologs are less conserved (156), suggesting that nitrogen levels might not influence BCAA biosynthesis in other species. Additionally, the T-box mechanism of *ilv-leu* regulation has not been observed in any other Gram-positives, and therefore appears to be unique to *B. subtilis*. Rather, bioinformatics analysis of the leader sequence of the BCAA biosynthetic genes in *L. lactis* (157), *Corenybacterium glutamicum* (158, 159), and *Streptococcus* spp. (160) have revealed BCAA-rich peptides and terminator hairpins consistent with attenuation. In *S. aureus*, an additional regulatory mechanism governing BCAA biosynthesis has been described involving repression by the essential Gcp/YeaZ complex (161, 162). Gcp and YeaZ are homologous to proteins in *E. coli* that function as a complex to synthesize t6A, a modification of tRNA that strengthens the A-U codon:anti-codon interaction on the ribosome (163). Consistent with this, reducing levels of Gcp in *S. aureus* results in a decrease in t6A modification (161). Interestingly, YeaZ binds upstream of the *ilv-leu* operon, suggesting possible direct repression of the operon (162). The conditions that regulate its binding are currently unknown.

1.5.1.2 Allosteric regulation of biosynthetic enzymes

In addition to transcriptional regulation, the biosynthetic enzymes involved in BCAA biosynthesis are subject to feedback inhibition and activation. Firstly, threonine deaminase (TD), which catalyzes the first step in Ile synthesis, is inhibited by Ile and activated by Val. TD is a pyridoxal phosphate-dependent enzyme that contains an N-terminal catalytic domain and a C-terminal regulatory domain. The C-terminal regulatory domain contains one or two ACT-like sub-domains. The *E. coli* TD enzyme, which contains two ACT-like sub-domains, is inhibited by Ile and activated by Val (164–166). The *B. subtilis* TD enzyme, which contains one ACT-like sub-domain, is inhibited by Ile and it is proposed that Val activates TD in the presence of Ile and inhibits TD at high concentrations (167). Secondly, the AHAS enzyme, which catalyzes the condensation of pyruvate with either α-ketobutyrate or pyruvate for Ile and Val synthesis, respectively, is also subject to feedback inhibition by BCAAs. *E. coli* encodes three AHAS isoenzymes; AHAS-I (IlvBN), which is inhibited by Val, AHAS-III (IlvIH) which is inhibited by all three BCAAs, and AHAS-II (IlvGM), which is insensitive to feedback inhibition by BCAAs (168). The redundancy in AHAS isoenzymes presumably allows *E. coli* to synthesize Ile even in the presence of the other BCAAs. Lastly, α-Isopropylmalate synthetase, which catalyzes the first step in Leu synthesis is inhibited by Leu (169).

1.5.2 BCAA transport

Repression of BCAA biosynthesis when intracellular levels are sufficient to support growth allows bacteria to conserve carbon and nitrogen, and instead rely on acquisition of exogenous sources of BCAAs to support metabolism. Active transporters specific to BCAAs are common across bacteria and require either ATP or the proton motive force to import BCAAs. BCAA transporters in Gram-negatives (Table 1.2), first described in *E. coli*, include the high-affinity LIV-I system and the low-affinity LIV-II and LIV-III systems (Figure 1.9).

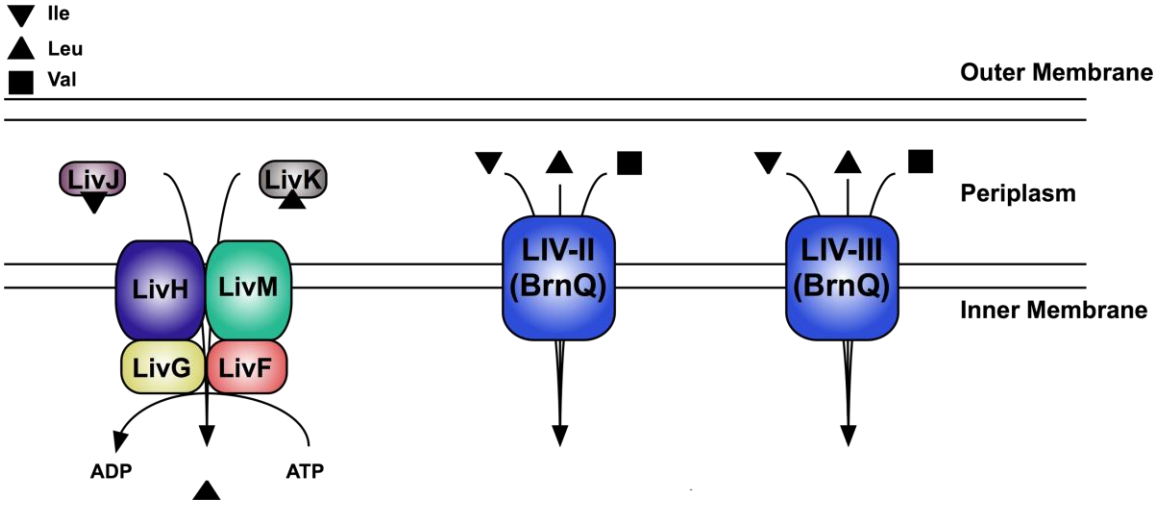

Figure 1.9 Mechanisms of BCAA transport in Gram-negative bacteria.

LIV-I is an ATP-Binding Cassette (ABC) transporter encoded by *livJKHMGF* (170). Two substrate binding proteins mediate BCAA transport through this system; LIV-B (*livJ*), which can bind all three BCAAs, and LS-B (*livK*), which is Leu specific (171, 172). LIV-II, also known as BrnQ, is a permease with 12 transmembrane helices and belongs to the Major Facilitator Superfamily (MFS). LIV-III is a permease homologous to LIV-II, with transport activity that is obviated only in a LIV-II deficient background in *Salmonella typhimurium* and *Pseudomonas aeruginosa* (173, 174). LIV-II and LIV-III use energy from the proton motive force to couple BCAA transport with Na+ across an energy gradient (175). An additional MSF transporter specific to Ile transport and unique to *Francisella tulaneris* has 12 transmembrane helices and belongs to the phagosomal nutrient transport family of MSF transporters identified in *Legionella pneumophilia* (176, 177).

Table 1.2 BCAA transporters in Gram-negative bacteria.

Organism	Transporter	Energy Source	Specificity	Reference
Chlamydia trachomatis	LIV-II (*brnQ*)	PMF	ILV	(176)
Escherichia coli	LIV-I (*livKHMGF*)	ATP	Leu	(170–172, 178)
	LIV-I (*livJHMGF*)	ATP	ILV	
	LIV-II (*brnQ*)	PMF	ILV	
Francisella tularensis	*ileP*	PMF	I	(179)
Pseudomonas aeruginosa	LIV-I	ATP	ILV	(180–184)
	LIV-II (*braB*)	PMF	ILV	
	LIV-III (*braZ*)	PMF	ILV	
Salmonella typhimurium	LIV-I	ATP	ILV	(173, 185–188)
	LIV-II (*brnQ*)	PMF	ILV	
	LIV-II	PMF	ILV	

BrnQ also functions as a BCAA transporter in Gram-positive bacteria, along with a second nonhomologous permease, BcaP (Table 1.3). *L. lactis* acquires BCAAs via both BcaP and BrnQ, with BcaP playing a more predominant role (189, 190). Similarly, BcaP is the predominant transporter in *B. subtilis*, with two additional transporters, BrnQ and BraB (a BrnQ homolog) contributing to Ile and Val uptake, and an unidentified transporter contributing to Leu uptake (191). A LIV-I system with a substrate binding protein able to bind BCAAs has been described in *S. pneumoniae*, although no transport function has yet been ascribed to this system (192). BrnQ also directs BCAA transport in *Lactobacillus delbrücki* and *C. glutamicum* (193, 194). Although *brnQ* homologs exist in *S. aureus*, the transport functions of these gene products have not been formally tested.

Table 1.3 BCAA transporters in Gram-positive bacteria.

Organism	Transporter	Energy Source	Specificity	Reference
Bacillus subtilis	*bcaP*	PMF	ILV	(191)
	braB	PMF	ILV	
	brnQ	PMF	ILV	
Corynebacterium glutamicum	*brnQ*	PMF	ILV	(194)
Lactobacillus delbrückii	*brnQ*	PMF	ILV	(193)
Lactococcus lactis	*brnQ*	PMF	ILV	(189, 190)
	bcaP	PMF	ILV	
Streptococcus pneumoniae	*livAJHMGF*	ATP	ILV	(192)

1.6 Nutritional importance of BCAAs for pathogen growth *in vivo*

Both BCAA biosynthesis and transport have been linked to promoting the virulence of pathogens in host environments, most of which are likely limited for BCAAs. Metabolomics studies indicate that human nasal secretions contain Leu concentrations in the range of 130-287 μM, Val in the range of 13-156 μM, and undetected levels of Ile (195). Human serum contains Leu, Val and Ile at approximate concentrations of 98 μM, 212 μM, and 60 μM, respectively (196). Indeed, pathogens exploit these sources, as large-scale mutant screens have identified BCAA transporters as important genes for the virulence of *Yersinia pestis*, *S. aureus*, and *S. pneumoniae* (192, 197–199). Given the redundancy in BCAA transporters in any given organism (Table 1.2, Table 1.3), single gene mutant screens are likely to underestimate the requirement of BCAA transport for virulence.

The intracellular environment is also presumably limited for BCAAs, as intracellular pathogens up-regulate BCAA biosynthetic genes within host cells (200). To sustain replication in this host niche, intracellular pathogens, including *Burkholderia pseudomallei*, *Mycobacterium bovis*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes* must synthesize BCAAs (201–205). Despite the limiting concentrations of BCAAs in the host cytosol, *F. tularensis* is able to exploit this source and requires the Ile transporter IleP for optimal intracellular replication and infection *in vivo* (176).

If deprived for BCAAs, pathogens not only face challenges in supporting protein synthesis, but also in maintaining the appropriate BCFA content to protect against host defenses that target the bacterial membrane. A role for BCFA synthesis in promoting resistance to host defenses is best highlighted in *L. monocytogenes*, where BCFAs comprise ~90% of the membrane (84, 87). BCFA deficient strains have increased susceptibility to antimicrobial peptide killing, lysozyme digestion, and decreased production of the virulence factor listeriolysin O (206), all of which likely contribute to the decreased intracellular growth and virulence of a strain deficient of BCFAs (206, 207). In *S. aureus*, BCFAs comprise ~65% of

the membrane and a BCFA deficient strain exhibits reduced adherence to host cells and is attenuated *in vivo* (208).

One challenge to understanding the importance of BCFA content to growth *in vivo* is the effect that the growth environment has on fatty acid content (209). For example, some pathogens, such as *S. aureus*, are able to incorporate exogenous host-derived short-chain unsaturated fatty acids (SCUFAs) into the membrane (210). When provided a source of host FAs (i.e. human serum), SCUFAs comprise 25-50% of the *S. aureus* membrane (209, 211), reducing the requirement for endogenously synthesized BCFAs (211). The extent to which exogenous host-derived FAs can replace endogenously synthesized FAs in other pathogens requires further investigation.

1.7 Regulatory importance of BCAAs for virulence gene expression *in vivo*

CodY is recognized as an important regulator of the intersection between metabolism and virulence in a number of Gram-positive pathogens, including *Bacillus anthracis* (212, 213), *Bacillus cereus* (115, 155, 214, 215), *Clostridium difficile* (112, 154, 216), *Clostridium perfringens* (113, 217), *L. monocytogenes* (109, 111, 153, 200), *S. aureus* (59–62, 64, 65), *S. pneumoniae* (102), and *Streptococcus pyogenes* (117, 218–220). CodY regulates between 50-300 genes in these pathogens, and although traditionally considered a transcriptional repressor, an unexpected role for CodY in virulence gene activation has emerged (Table 1.4).

Since GTP and BCAA availability determines the extent of CodY activity, their intracellular and extracellular levels represent an important signal for the adaptation of pathogens to host niches. These levels presumably fluctuate considerably as a consequence of nutritional demand of the pathogen and environmental availability, both of which are difficult to discern in the various host niches that pathogens infect. One approach to resolve this is to mutate the mechanisms for acquiring or synthesizing GTP and BCAAs. This approach has proved insightful to understanding the role of GTP concentrations in regulating virulence gene expression (63, 153, 221). Modulation of intracellular BCAA concentrations, however, is challenging given the redundancy in acquisition pathways. Rather, studies using CodY protein variants that mimic graded BCAA nutrient deprivation or involving limitation of

exogenous sources of BCAAs have generated important insight into the coordination of target gene regulation in response to decreasing BCAA concentrations (60, 111, 200).

Table 1.4 CodY regulation of virulence in Gram-positive pathogens

Organism	Phenotype of *codY* mutant *in vivo*	Notable virulence gene regulation	Reference
Staphylococcus aureus	Hypervirulent in skin abscess and pneumonia. No effect on systemic infection.	ID repression of delta-toxin/RNAIII via repression of *agr* activator, D repression of biofilm synthesis (*icaADBC*), α-toxin (*hla*), hyaluronidase (*hysA*), Panton-Valentine leukocidin (*lukSF-PV*)	(59, 62, 221, 222)
Streptococcus pneumoniae	Reduced colonization. No effect on systemic infection.	D activation of adhesion protein choline-binding protein (*pcpA*)	(102)
Bacillus anthracis	Attenuated virulence	ID activation of anthrax toxin components (*cya*, *lef*, *pagA*); ID/D activation of iron scavenging systems	(212, 213)
Clostridium perfringens (Type D) (Type A)	ND	D&ID activation of epsilon toxin (ETX); Repression of sporulation D/ID activation of sporulation and enterotoxin (CPE)	(113, 217) (223)
Bacillus cereus (F4810/72)	Attenuated virulence	ID activation of cytotoxin (*cytK*), enterotoxin (*nhe*), and hemolysin (*hbl*) via D activation of regulator *plcR*; D repression of cereulide (*cesPTABCD*) and inhibitor metalloprotease 1 (*inhA1*)	(215)
Clostridium difficile	ND	ID repression of toxin A (*tcdA*) and B (*tcdB*) via D repression of *tcdR*	(216)
Listeria monocytogenes	Attenuated virulence	ID activation of listeriolysin O (*hyl*) via D activation of regulator *prfA*; D activation of flagellar biosynthesis and ActA	(111, 200)
Streptococcus pyogenes	ND	ID activation of surface proteins via ID/D activation of regulator *mga*; ID/D activation of regulators *fasX* and pel/*sagA*	(218, 219)

ND = Not determined; ID = indirect regulation; D = direct regulation; ID/D = unknown

1.7.1 Implications of GTP depletion on CodY regulation

Bacteria obtain GTP through biosynthesis or transport, and further regulate intracellular pools by conversion of GTP to (p)ppGpp in response to amino acid starvation (i.e. the stringent response) (92). Two classes of enzymes are involved in the stringent response; monofunctional synthases that synthesize (p)ppGpp from GTP, and bifunctional enzymes that can synthesize (p)ppGpp as well as hydrolyze the molecule back to GDP or GTP (224). In addition to consuming GTP for its synthesis, (p)ppGpp depletes GTP levels by inhibiting an enzyme required for GTP synthesis (225). Of these mechanisms, the synthesis of (p)ppGpp from GTP represents an important link between GTP levels and CodY virulence in Gram-positive pathogens (63, 221).

Mutation of the synthase function of the bifunctional enzyme in *S. aureus* (RSH) and *L. monocytogenes* (RelA) has two consequences on the cell; i. the inability to accumulate (p)ppGpp leads to loss of stringent response gene induction, and ii. the high level of GTP leads to continued repression of CodY target genes (63, 153, 221, 226). Without de-repression of CodY target genes, the synthase mutants of both pathogens are attenuated *in vivo*, a phenotype that is reversed upon subsequent mutation of *codY* in this background (153, 221). Although CodY-regulated products in *S. aureus* include virulence factors, such as capsule genes and the *agr* locus, amino acid starvation does not induce these targets (63). These data suggest that the importance of amino acid starvation for promoting virulence is related to CodY-dependent metabolic reprogramming, and not virulence factor production. They further reveal that depletion of just one CodY effector is not sufficient to completely relieve repression of all CodY target genes.

1.7.2 Implications of BCAA depletion on CodY regulation

Emerging evidence indicates that the consequence of BCAA depletion on virulence varies by pathogen. In the more traditional case, CodY acts as a negative regulator of virulence genes in *S. aureus*. CodY regulates approximately 5% of the *S. aureus* genome, with the majority of its targets (85%) subject to repression by CodY (59, 61). These targets include virulence genes, such as the capsule genes, α-hemolysin and adhesion proteins, as well as regulators of virulence gene expression, including the *agr* locus and *saeRS* two-component system (65). Most of the virulence genes are directly repressed by CodY, whereas others, including the

capsule genes and hemolysins, are indirectly activated by *agr* (59, 61). The genes activated by CodY, which fall into the categories of nucleotide transport/metabolism and adhesion proteins, do not have a CodY binding sequence, suggesting an indirect mechanism of regulation (59). The coordination of virulence gene expression with the environment is crucial for *S. aureus* to limit unwanted host-damage as exemplified by the hypervirulence of a *codY* mutant in a skin abscess and pneumonia model of infection (62). To ensure the appropriate expression of virulence genes, the CodY regulon is expressed as a hierarchy that depends on the extent of CodY activation, and therefore nutrient availability (60). This hierarchy was determined by analyzing the transcriptome of *S. aureus* strains expressing mutated CodY proteins that mimic various levels of Ile deprivation (60). This approach revealed that the graded response prioritizes amino acid and peptide transport over synthesis upon modest nutrient limitation, and reserves hydrolytic enzyme and toxins production for more severe nutrient limitation (Figure 1.10) (60). Also within this spectrum are other virulence gene regulators, including the *agr* and *sae* locus, that together form a regulatory cascade that integrates several environmental cues, such as growth phase and host defenses (60, 227). Together, the graded response and regulatory cascade allow *S. aureus* to maximize nutrient acquisition while limiting host toxicity (60).

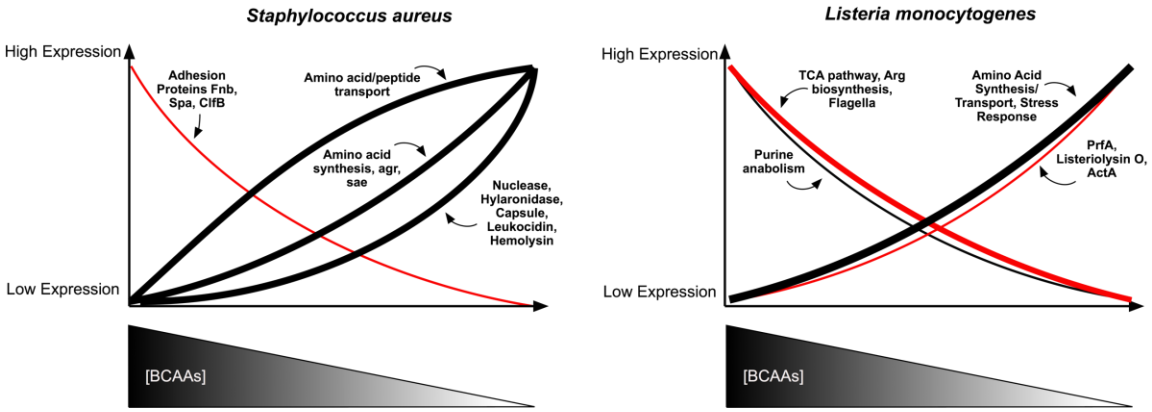

Figure 1.10 CodY regulation of virulence genes in *Staphylococcus aureus* and *Listeria monocytogenes.*

CodY target genes are either activated or repressed by CodY depending on BCAA availability. Black lines indicate genes that are repressed by CodY. Red lines indicate genes that are activated by CodY. The thickness of the line corresponds to the relative proportion of genes in that category.

Limitation of BCAAs in *L. monocytogenes* produces a much more complex response. CodY directly or indirectly regulates approximately 14% of genes in *L. monocytogenes*, with a much greater proportion of genes requiring CodY for activation (111). In nutrient-rich growth medium, approximately 66% of target genes are up-regulated in a *codY* mutant, and in BCAA-limited growth medium approximately 70% of target genes are up-regulated (111). When BCAAs are present in excess, genes primarily involved in nutrient metabolism and transport, stress responses, and some virulence factors are repressed. Under these same conditions, other metabolic genes, including arginine biosynthesis, are activated by CodY. When BCAAs are limited, amino acid and iron transport genes are repressed, whereas *prfA*, the global virulence gene regulator in *L. monocytogenes*, as well as its downstream genes, are activated by CodY (Figure 1.10) (109, 111). Notably, CodY is a direct activator of *prfA* and binds within its coding region specifically under BCAA limiting conditions (109). This results in activation of PrfA-regulated virulence factors, including listeriolysin O and the surface protein ActA, which are important for intracellular replication and cell to cell spread, respectively (200, 228, 229). CodY also directly activates flagellar biosynthesis genes. Consequently, a *codY* mutant is impaired in motility, intracellular replication, and survival *in vivo* (109, 111). The mechanism of *prfA* activation remains uncertain, as it does not appear to involve BCAA-binding and it is unclear how binding of CodY within a coding region would activate transcription (109). These data reveal how the CodY regulatory activity in response to BCAA depletion in *L. monocytogenes* contrasts the predominant negative regulatory activity observed in other organisms.

1.8 Rationale and Hypothesis

S. aureus is a significant human pathogen that exhibits extraordinary replicative capacity in diverse host niches by withstanding changes in host defenses, temperature, pH, oxidative stress, osmotic stress and nutrient availability. How it obtains the nutrients it requires for growth in such hostile environments and what environmental cues it responds to, to regulate its adaptation to these environments are not fully understood. The BCAAs are of interest to study, as they provide the opportunity to investigate both of these aspects of *S. aureus* virulence due to their physiological role (i.e. protein synthesis and FA synthesis) and their regulatory role (i.e. via CodY). Yet, there are no detailed descriptions of the mechanisms *S. aureus* uses to obtain BCAAs to support these functions. Therefore, the objective of this thesis was to characterize how *S. aureus* obtains BCAAs and to investigate the consequences of modulating intracellular BCAAs on *S. aureus* physiology and virulence.

S. aureus encodes an intact BCAA biosynthetic operon, however, it has been reported as auxotrophic for Leu and Val (230, 231). Therefore, in Chapters 2 and 3, I, along with my colleagues, investigate the hypothesis that **BCAA acquisition via specialized transporters is required for *S. aureus* physiology and virulence**. In Chapter 4, my colleagues and I sought to resolve the conundrum of why the intact BCAA biosynthetic operon does not support *de novo* BCAA biosynthesis in the absence of an exogenous source. We hypothesized that **selection for *S. aureus* mutants able to grow in the absence of exogenous BCAAs would enable identification of novel regulators of the biosynthetic operon in *S. aureus***.

An improved understanding of the metabolic requirements of *S. aureus* infection and the role that metabolic cues play in regulating virulence could reveal pathways to target with therapeutics to help reduce the infectious burden of this pathogen.

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Chapter 2

2 The role of BrnQ transporters in BCAA acquisition

2.1 Introduction

Staphylococcus aureus is a highly successful human pathogen that succeeds at infecting virtually every body site, causing skin, soft tissue, respiratory, bone, joint and endovascular infections (1). Maintenance of metabolic homeostasis is important for its infection process, as the majority of genes necessary for infection identified in large-scale signature-tagged mutagenesis (STM) screens fall into the categories of metabolism, transport and biosynthesis (2, 3). Acquisition of host-derived nutrients, specifically amino acids, appears to be an important mechanism of meeting nutritional needs, as a large proportion of attenuated STM strains contain mutations in amino acid transporters (3).

Amino acid transporters are ubiquitous in bacteria and are typically selective for transporting either one amino acid or several amino acids with related structures (4). The branched-chain amino acids (BCAAs; Ile, Leu, Val) are hydrophobic amino acids typically found in the core of globular proteins or in the trans-membrane domain of cell surface proteins (5). The transport mechanisms described for acquisition of the BCAAs in bacteria include secondary transporters belonging to the Leu, Ile, Val:cation symporter (LIVCS) family (e.g. BrnQ, BraB, BraZ, BcaP) (4, 6–9) and the LIV-I ABC transporter (10–13). With the exception of an ABC transporter in *Streptococcus pneumoniae* (14), secondary transport, which couples the movement of an ion, usually Na^+ or H^+, down its concentration gradient with the movement of another molecule against its concentration gradient, is the primary means of BCAA acquisition in Gram-positive bacteria.

S. aureus necessitates BCAA transport for growth, as it exhibits an auxotrophic phenotype for Leu and Val, despite possessing the genes necessary for their biosynthesis (15–17). The mechanisms of BCAA acquisition, however, have not been described in this species. Strategies to maintain intracellular levels of BCAAs are of additional interest in *S. aureus* since the BCAAs also act as co-repressors of the global transcriptional regulator CodY (18–20). CodY, a highly-conserved regulatory protein in low G+C Gram-positive bacteria, uses binding of BCAAs and GTP to sense the metabolic status of the cell, the downstream effect

of this being the adaptation of the cell to nutrient limitation (21, 22). CodY is activated through direct interaction with the BCAAs as well as GTP (18–20, 23) and, in its active state, represses transcription of upwards of 100 genes (17, 24, 25). Depletion of BCAAs and GTP results in de-repression of CodY target genes, the products of which are involved in a range of cellular processes depending on the species, including sporulation (23), biofilm formation (26, 27), protein degradation and utilization (22), and amino acid metabolism and transport (17, 24). More recently, CodY has emerged as a regulator of virulence in *S. aureus* (28–30), implicating CodY as an important link between the nutrient status of the cell and virulence.

Recent characterization of the CodY regulon in *S. aureus* revealed amino acid metabolism and transport genes as a major class of CodY targets, including the *brnQ1 and brnQ2* BCAA transporter genes (17, 24). We therefore hypothesized that BCAA transport is involved in adaptation to nutrient limitation, and might also serve as an important mechanism to maintain intracellular pools of BCAAs that affect CodY activity and, in turn, regulate virulence of *S. aureus*. Despite the potential importance of BCAA transport in regulating virulence in *S. aureus*, no BCAA transporter has, as yet, been functionally characterized for a role in BCAA uptake. In this study we sought to characterize the transport function of the *brnQ* genes in *S. aureus* and investigate their role in nutrient acquisition and virulence in a murine model of *S. aureus* infection.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

Bacterial strains used in this study are described in Table 2.1. MRSA isolate pulsed-field gel electrophoresis type USA300 LAC that has been cured of the erythromycin resistance plasmid was used in all experiments as the wild type (WT) strain. *S. aureus* strains were grown either in tryptic soy broth (TSB) or in a chemically-defined medium (CDM), described previously (31). Complete CDM was composed of the following (final concentrations in μM): 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), thiamine (56), nicotinic acid (10), biotin (0.04), pantothenic acid (2.3), $MgCl_2$ (1000), $CaCl_2$ (100), monopotassium phosphate (40000), dipotassium phosphate (14700), sodium citrate dehydrate (1400), magnesium sulfate (400), ammonium sulfate (7600), glucose (27753). The concentrations of individual amino acids were modified in some experiments as indicated. Where required, chloramphenicol (10 μg mL^{-1}), erythromycin (3 μg mL^{-1}) and tetracycline (4 μg mL^{-1}) were added to growth medium. All growth curves were performed at 37˚C with shaking using a flask:volume ratio of at least 7:1. For growth curves performed in TSB medium, *S. aureus* strains were pre-grown to mid-exponential phase in TSB and sub-cultured into fresh TSB to a starting OD_{600} equivalent of 0.0025. For growth curves performed in CDM, *S. aureus* strains were pre-grown to mid-exponential phase in complete CDM and sub-cultured into fresh CDM to a starting OD_{600} equivalent of 0.0025; as indicated in the Results section, either complete CDM or CDM with altered concentrations of amino acids was used. The optical densities of the cultures were measured until stationary phase was reached, or until desired time points were reached.

Table 2.1 Bacterial strains and plasmids used in this study

Strain/Plasmid	Description [a]	Source or reference
S. aureus		
USA300	USA300 LAC cured of antibiotic resistance plasmid	This study
RN4220	$r_K^- m_K^+$; capable of accepting foreign DNA	(32)
RN6390	Prophage-cured laboratory strain	(33)
Newman	WT clinical isolate	(34)
H2324	Newman *brnQ3*::Tet, Tet^R	This study
H2568	USA300 Δ*brnQ1* (SAUSA300_0188)	This study
H2563	USA300 Δ*brnQ2* (SAUSA300_0306)	This study
H2578	USA300 *brnQ3*::Tet; Tet^R (SAUSA300_1300)	This study
H2589	USA300 Δ*brnQ1* Δ*brnQ2 brnQ3*::Tet; Tet^R	This study
H2994	USA300 *brnQ2*::ΦNΣ	This study
H3001	USA300 *codY*::ΦNΣ	This study
E.coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^-$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169phoA*	Promega
Plasmids		
pKOR1	*E. coli/Staphylococcus* shuttle vector allowing allelic replacement in *S. aureus*	(35, 36)
pDG1513	Antibiotic resistant cassette	(37)
pMAD	Temperature-sensitive *E. coli/S. aureus* shuttle vector	(38)
pRMC2	Anhydrotetracycline-inducible expression vector; Ap^r in *E. coli*; Cm^r in *S. aureus*	(36)
pSO1	pRMC2 containing *brnQ1*; Cm^r	This study
pSO2	pRMC2 containing *brnQ2*; Cm^r	This study
pSO3	pRMC2 containing *brnQ3*; Cm^r	This study

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

2.2.2 Mutagenesis of *brnQ* genes and complementation of mutants.

Clean deletions of *brnQ1* and *brnQ2* genes were constructed using the pKOR-1 plasmid as described previously (35). Primer sequences were based on the published USA300 FPR3757 genome and are displayed in Table 2.2 (39). Briefly, sequences flanking the *brnQ* locus were PCR-amplified and 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. To overcome the restriction barrier of *S. aureus*, these plasmids were first passed through the restriction-defective *S. aureus* strain 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 and confirmed by PCR and DNA sequencing. A schematic representation of the pKOR deletion strategy is depicted in Figure 2.1. 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 to the temperature-sensitive *E. coli/S. aureus* shuttle vector pMAD, and then passaged through *S. aureus* RN4220 at permissive temperatures prior to being transduced using phage 80α into *S. aureus* RN6390. Recombinant RN6390 was cultured at 30˚C to mid-log phase before incubation temperature was shifted to 42˚C. The bacteria were further incubated for 16 hr 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 *brnQ3*::Tc mutation was confirmed by PCR and mobilized to *S. aureus* USA300 by transduction using phage 80α. Transposon insertions in the *brnQ2* and *codY* genes were identified in the Nebraska Transposon Mutant Library with strain IDs of NE605 and NE1555, respectively. The transposons were transduced into our laboratory strain of USA300 using phage 80α and insertion was confirmed by PCR. For complementation vectors, the *brnQ* genes, including their native promoters, were cloned into the shuttle vector pRMC2 Table 1.2 (36).

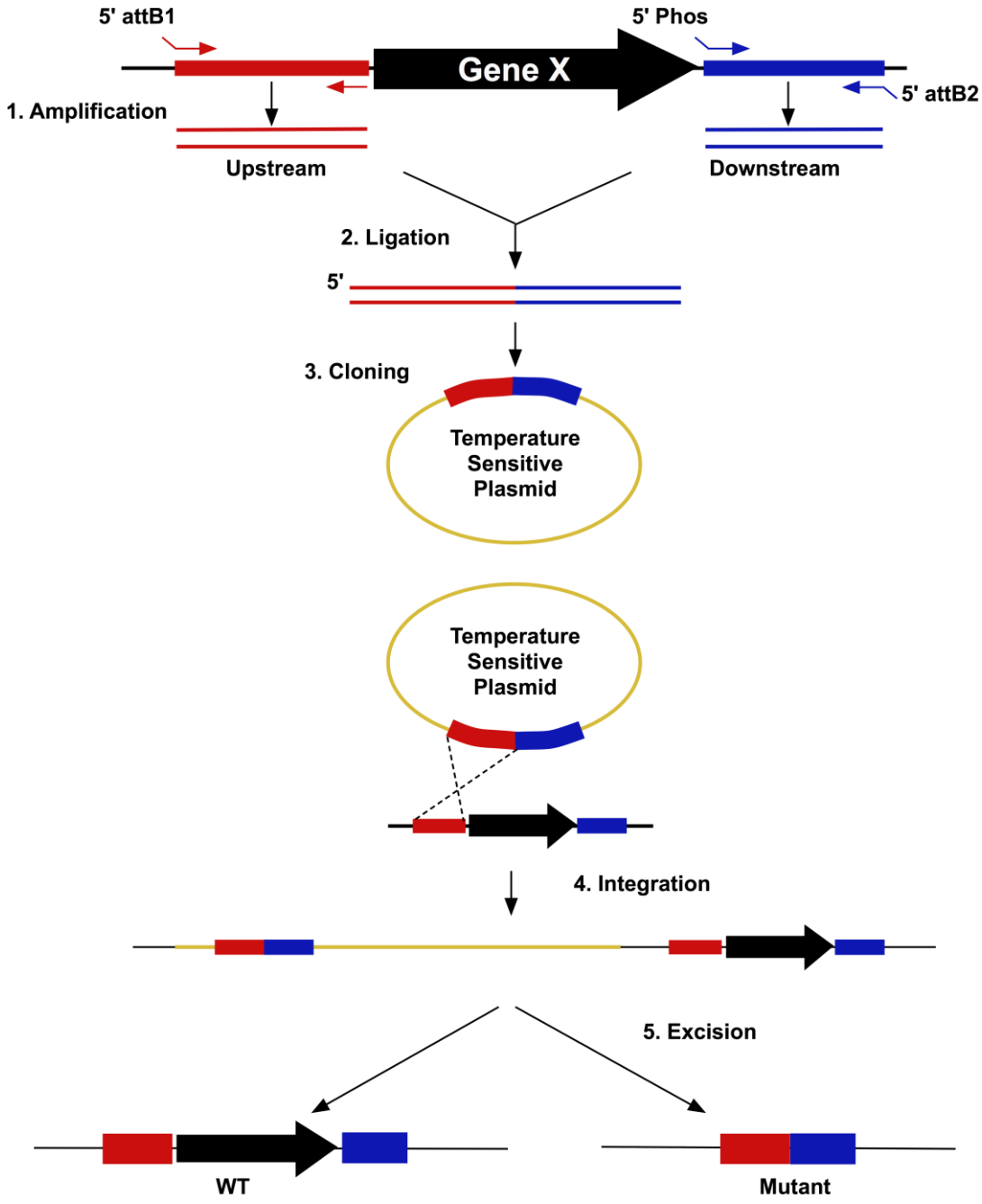

Figure 2.1 Strategy for markerless gene deletion in *S. aureus*.

The pKOR vector replicates extra-chromosomally at 30˚C and integrates at 42˚C. Regions upstream and downstream of the gene of interest, approximately 800-1000 bp in size, are amplified and cloned into pKOR. Cells are grown at 42˚C to promote chromosomal integration via homologous recombination, and then subsequently grown at 30˚C to promote excision. The plasmid is lost via negative selection upon induction of anti-sense RNA, which silences an essential gene in *S. aureus*.

Table 2.2 Oligonucleotide primers used for mutagenesis, cloning, and RT-PCR

Oligonucleotides [a]	
brnQ1 5'F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACCTGCAAGA AGGTATTC
brnQ1 5'R	GGGCGTAAGTTAAGAACGA Generation of 5' arm for deletion of *S. aureus brnQ*1
brnQ1 3'F	/5Phos/AAGGAGGGAGACCGATGAAT
brnQ1 3'R	GGGGACCACTTTGTACAAGAAAGCTGGGTTGGCTAAAGGGT TACCACCA Generation of 3' arm for deletion of *S. aureus brnQ1*
brnQ2 5'F	GGGGACAAGTTTGTACAAAAAAGCAGGCTGCGCCATAGACA GGTTTAGC
brnQ2 5'R	TGGAAATACGTTGGAGCGTTG Generation of 5' arm for deletion of *S. aureus brnQ2*
brnQ2 3'F	/5Phos/ACCCATCGTTACTAGGCTTT
brnQ2 3'R	GGGGACCACTTTGTACAAGAAAGCTGGGTTAAAAGCCGCAG CGATATTC Generation of 3' arm for deletion of *S. aureus brnQ2*
brnQ3 5'F- SalI	TTGTCGACGATTGAACGAGAACAACGAGATGC
brnQ3 5'R- PstI	TTCTGCAGGCGTAAACCCAATGACCCATG Generation of 5' arm for generation of *S. aureus brnQ3*::Tet allele
brnQ3 3'F- PstI	TTCTGCAGCAACGATATTAGGCTATCTAGTCGGC
brnQ3 3'R- SmaI	TTCCCGGGAGCTCGTTGAGCTGCTATTTTCC Generation of 5' arm for generation of *S. aureus brnQ3*::Tet allele
$brnQ1_{SA}$5'F-KpnI	GATCGGTACCGTTCTTAACTTACGCCC
$brnQ1_{SA}$3'R-SacI	GATCGAGCTCCAGTTGTTCAATATAAG Cloning of *S. aureus brnQ1*
$brnQ2_{SA}$5'F-KpnI	GATCGGTACCTTTCCAACTTACTTTCA
$brnQ2_{SA}$3'F-SacI	GATCGAGCTCTCAATAGACAGACGGT Cloning of *S. aureus brnQ2*
$brnQ3_{SA}$5'F-KpnI	GATCGGTACCCATGTGTTTTGTTTCTG
$brnQ3_{SA}$3'F-SacI	GATCGAGCTCTAATGGCGAGACTCCT Cloning of *S. aureus brnQ3*
rpoB-76-F	AGAGAAAGACGGCACTGAAAACAC
rpoB-232-R	ATAACGACCCACGCTTGCTAAG RT-PCR for *rpoB* expression in *S. aureus*

brnQ1-416-Fw	TGCGAGAAAGCCATCAAG
brnQ1-602-Rv	GTATTGTATCCATCTAAAAAGCCG
	RT-PCR for *brnQ1* expression in *S. aureus*
brnQ3-qPCR-F	TTGGGTTTACGCTCTTCG
brnQ3-qPCR-R	CACCTACAATCACACCTAATAATG
	RT-PCR for *brnQ3* expression in *S. aureus*

[a] Restriction sites are underlined.

2.2.3 Radioactive transport assays

The protocol for transport was adapted from a previously-described protocol (40). Cultures were grown overnight at 37°C in complete CDM and sub-cultured into complete CDM at a starting OD_{600} of 0.1. Bacteria were grown to mid-exponential phase (OD_{600} of 1.0), harvested by filtration on 0.45-μm pore-size membrane filters, and washed with PBS before being resuspended in CDM lacking amino acids. Cells were heated at 37°C for 10 minutes prior to the assay. The ^{14}C-labeled amino acid of interest (Perkin Elmer, MA) was added to cells at a final concentration of 1 μM. An aliquot of cells was removed at 20, 40, and 60 s and rapidly filtered through 0.45-μm membrane filters. The filters were immediately washed with 10 mL of 0.1 M $LiCl_2$ at room temperature. Filters were dried and placed in scintillation vials containing 4 mL of Cytoscint™ scintillation cocktail (Fisher Scientific). ^{14}C radioactivity was measured using the LS 6500 scintillation system (Beckman).

To determine the Michaelis-Menten constant, cells, prepared as described above, were incubated with 500 nM, 1 μM, 2 μM, or 4 μM of a ^{14}C-labeled amino acid. An aliquot of cells was filtered as described above after 20 s. The initial velocity of uptake for each substrate concentration was plotted to determine transport kinetics, and the K_m and V_{max} values were extrapolated.

2.2.4 qPCR

qPCR was performed as described previously (31). In brief, RNA was prepared from 5-mL cultures of 3 biological replicates of *S. aureus* strains grown in TSB to an OD_{600} of 0.6, or in CDM to an OD_{600} of 1.0. Cells were collected and RNA was extracted using an Aurum Total RNA Mini Kit as described by the manufacturer (Bio-Rad; Hercules, CA). RNA (500 ng) was reverse transcribed using SuperScript II (Invitrogen; Carlsbad, CA) and PCR-amplified

using SensiFast SYBR (Bioline; Taunton, MA) and the primers described in Table 2.2. Data were normalized relative to expression of the reference gene *rpoB.*

2.2.5 Murine model of systemic *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 BALB/c mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in microisolator cages. Bacteria were grown to exponential phase (OD_{600} of approximately 3.0) in TSB, pelleted by centrifugation, washed twice in PBS, and resuspended in PBS. Bacterial suspensions in 100-µL volumes were administered via tail vein injection (6-7 × 10^6 CFU). Four days post-challenge kidneys and livers were aseptically excised and placed in 4-mL volumes of PBS containing 0.1% (v/v) Triton X-100 in a sterile 15-mL conical tube. Organs were homogenized for 10 s, and bacterial loads were calculated following serial dilution 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.

2.3 Results

2.3.1 Identification of *brnQ* genes in USA300 and generation of mutant strains

The *brnQ* genes identified in the Majerczyk *et al*. and Pohl *et al*. studies (17, 28) are allelic with *brnQ1* (SAUSA300_188) and *brnQ2* (SAUSA300_306) in USA300 FPR375. Bioinformatics analyses revealed a third potential *brnQ* paralog encoded by SAUSA300_1300 with 35% identity to BrnQ1 and 38% identity to BrnQ2; we designated this gene *brnQ3*. BrnQ3 has 100% amino acid sequence identity to the encoded product of a *brnQ* gene previously cloned from *S. aureus* RN450, however its function as a BCAA transporter was never formally tested (41). The three paralogs share significant sequence similarity with the BrnQ1 protein from *Lactobacillus delbüeckii* and one another (Figure 2.2A); BrnQ1 vs. BrnQ2 54%, BrnQ1 vs BrnQ3 54%, BrnQ2 vs BrnQ3 57%. The proteins are similar in length; BrnQ1 (451 aa), BrnQ2 (435 aa), BrnQ3 (447 aa) and TMPred predicts they all have 12 transmembrane domains. Searches of the databases using HHPred revealed the top hits to be transmembrane transporters for a variety of substrates. The genes are located at independent loci in the USA300 genome and a CodY box (i.e. CodY-binding motif) is positioned 117 bp upstream of the *brnQ1* start codon and 85 bp upstream of the *brnQ2* start codon (Figure 2.2B). To ascertain a function for each of the *brnQ* paralogs, in-frame, markerless deletions of the *brnQ1* and *brnQ2* coding regions were constructed and a tetracycline resistance cassette was used to replace the majority of the *brnQ3* coding region. A triple mutant, herein referred to as *brnQ1-2-3*, was also constructed.

2.3.2 BrnQ1 transports Ile, Leu, and Val

To assess the specific role of each of the BrnQ transporters, we performed uptake assays with radiolabeled BCAAs. Cells were grown in a chemically defined media (CDM) to late-exponential phase (OD = 1), a growth phase that we ensured, using qPCR, did not

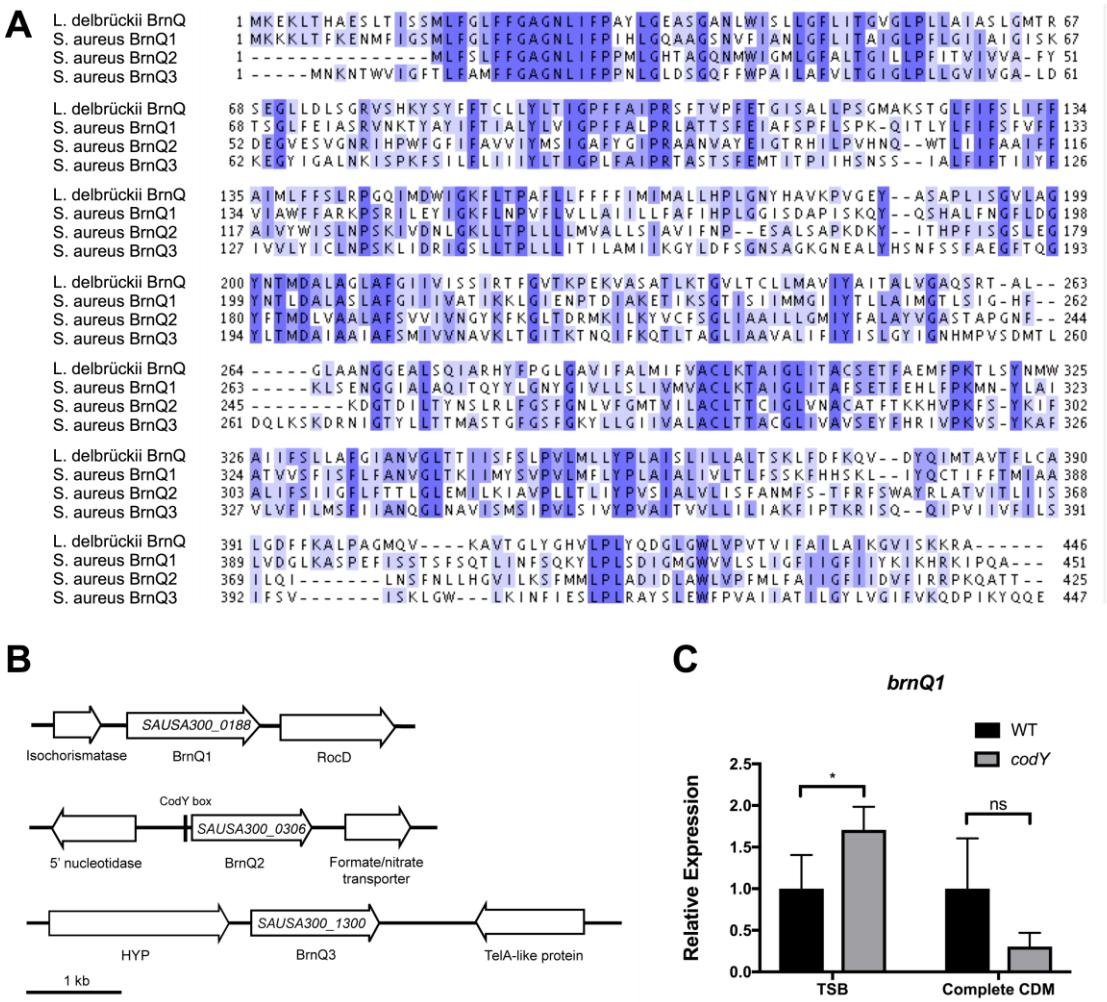

Figure 2.2 Protein sequence alignment and genomic context for the three *brnQ* paralogs in USA300.

A) Amino acid sequence comparison of the BrnQ proteins in USA300 FPR3757 to the BrnQ protein in *Lactobacillus delbrückii* (Accession: P54104.1). Dark blue = 4/4 sequence conservation; Medium blue = 3/4 sequence conservation; light blue = 2/4 sequence conservation; no highlight = no sequence conservation. B) Genomic context of *brnQ* genes in USA300 FPR3757. Abbreviations: HYP = Hypothetical protein. RocD = Ornithine-oxo-acid aminotransferase. C) Expression of *brnQ1* was measured in the WT strain and *codY* mutant grown to mid-exponential phase in either TSB or Complete CDM and normalized to expression of the reference gene *rpoB*. Data are the mean of 3 biological replicates +/- SD. Statistical significant was determined by a Student's *t*-test. * $P < 0.05$; ns = not significant.

demonstrate CodY-dependent repression of the *brnQ* genes (Figure 2.2C). We measured uptake of BCAAs in the single mutants and also the *brnQ1-2-3* mutant complemented with each gene provided *in trans*. We observed that, compared to WT, uptake of each of Leu, Ile and Val was decreased in the *brnQ1* mutant (Figure 2.3). While the *brnQ1* mutant was similar to the *brnQ1-2-3* mutant in terms of Leu and Val transport, *brnQ1-2-3* had decreased Ile uptake compared to the *brnQ1* mutant, suggesting the involvement of one of the other two transporters in Ile uptake. Importantly, expression of *brnQ1* in trans (on pSO1) in the *brnQ1-2-3* mutant resulted in increased uptake of each of Ile, Leu and Val to levels at or above those seen in WT Figure 2.3. Together, these data indicate that BrnQ1 is capable of transporting all three BCAAs.

2.3.3 BrnQ2 is an Ile transporter

Using the approach described in the preceding section, we next determined the substrate specificity of BrnQ2. Relative to WT, transport of Ile, Leu and Val was not decreased in the *brnQ2* mutant but was, in fact, increased (Figure 2.4). Given the just-described data detailing the involvement of BrnQ1 in transport of the three BCAAs, we attribute the transport seen in the *brnQ2* mutant strain to increased expression of *brnQ1* (see below). When we examined uptake in the *brnQ1-2-3* mutant overexpressing *brnQ2 in trans* (on pSO2), we found that Leu and Val transport was not restored, while uptake of Ile was restored (Figure 2.4). These data implicate BrnQ2 as a transporter selective for Ile, with little to no role in transport of Leu and Val, at least under the conditions used in this study.

2.3.4 BrnQ3 is not a functional BCAA transporter

Similar to the assays described in the preceding sections, we assessed BCAA transport in the *brnQ3* mutant to validate its predicted function (41). Our results demonstrate that transport of Ile, Leu and Val were not affected in this strain, relative to WT, and that expression of *brnQ3 in trans* (on pSO3) in the *brnQ1-2-3* mutant background did not appreciably restore transport of any of the three BCAAs (Figure 2.5). Since this was a negative result, we used qPCR to confirm that *brnQ3* was expressed from pSO3 in the

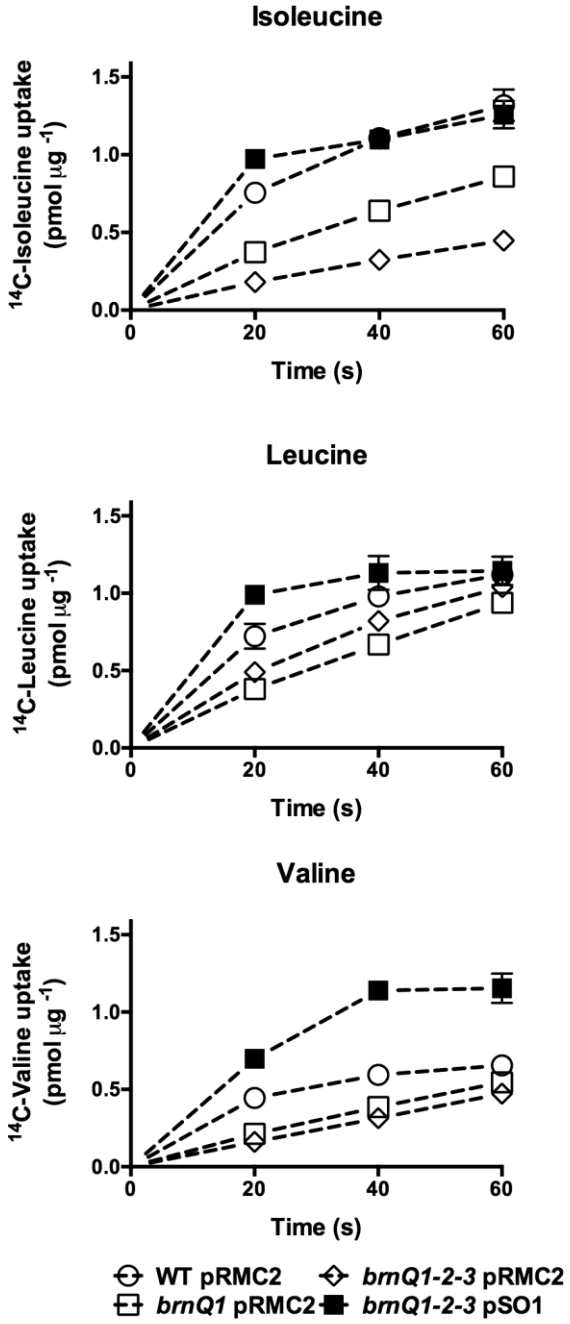

Figure 2.3 BrnQ1 transports isoleucine, leucine and valine.

Cells were harvested from cultures grown to mid-late exponential phase in complete CDM. Transport of ^{14}C-labeled BCAAs, as indicated, was measured at 20, 40 and 60 seconds. pRMC2 = empty vector; pSO1 = pRMC2::*brnQ1*. Data points shown are the mean of three biological replicates +/- SD.

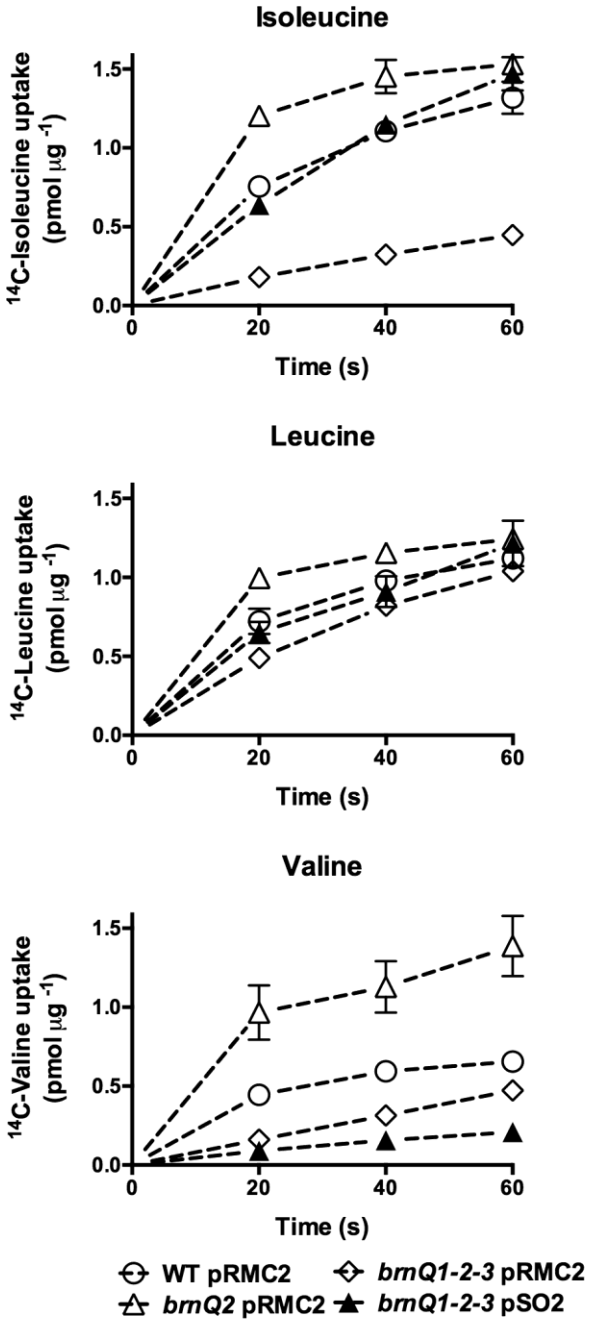

Figure 2.4 BrnQ2 transports isoleucine.

Cells were harvested from cultures grown to mid-late exponential phase in complete CDM. Transport of ^{14}C-labeled BCAAs, as indicated, was measured at 20, 40 and 60 seconds. pRMC2 = empty vector; pSO2 = pRMC2::*brnQ2*. Data points shown are the mean of three biological replicates +/- SD.

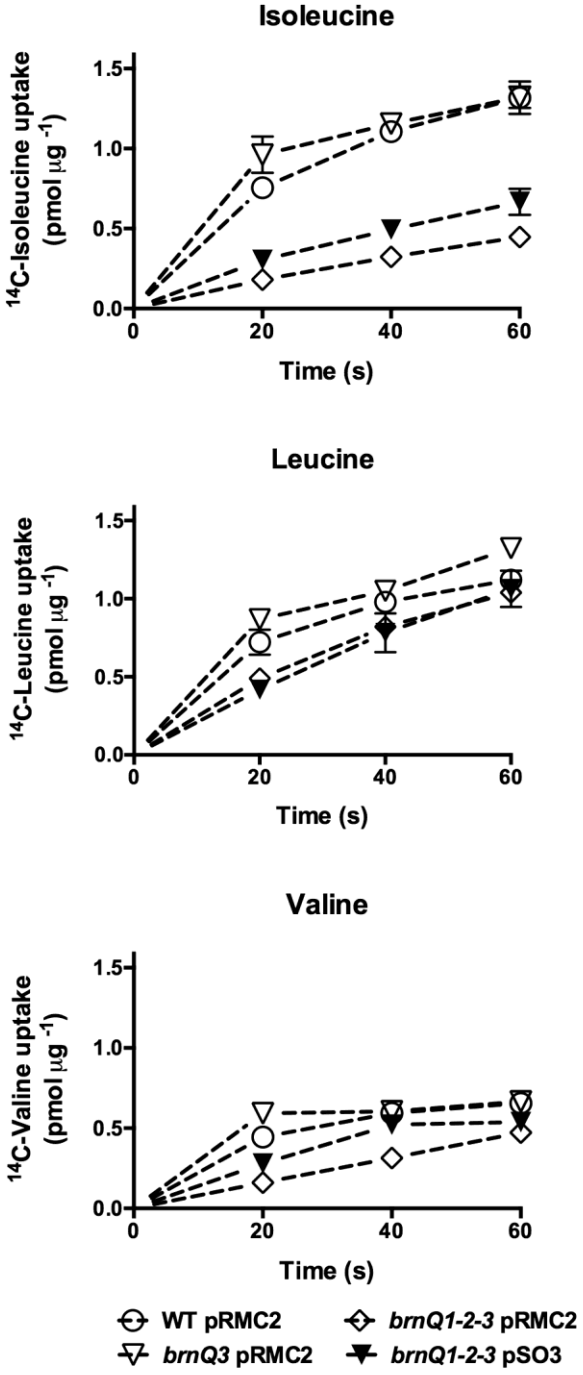

Figure 2.5 BrnQ3 in not a BCAA transporter.

Cells were harvested from cultures grown to mid-late exponential phase in complete CDM. Transport of ^{14}C-labeled BCAAs, as indicated, was measured at 20, 40 and 60 seconds. pRMC2 = empty vector; pSO3 = pRMC2::*brnQ3*. Data points shown are the mean of three biological replicates +/- SD.

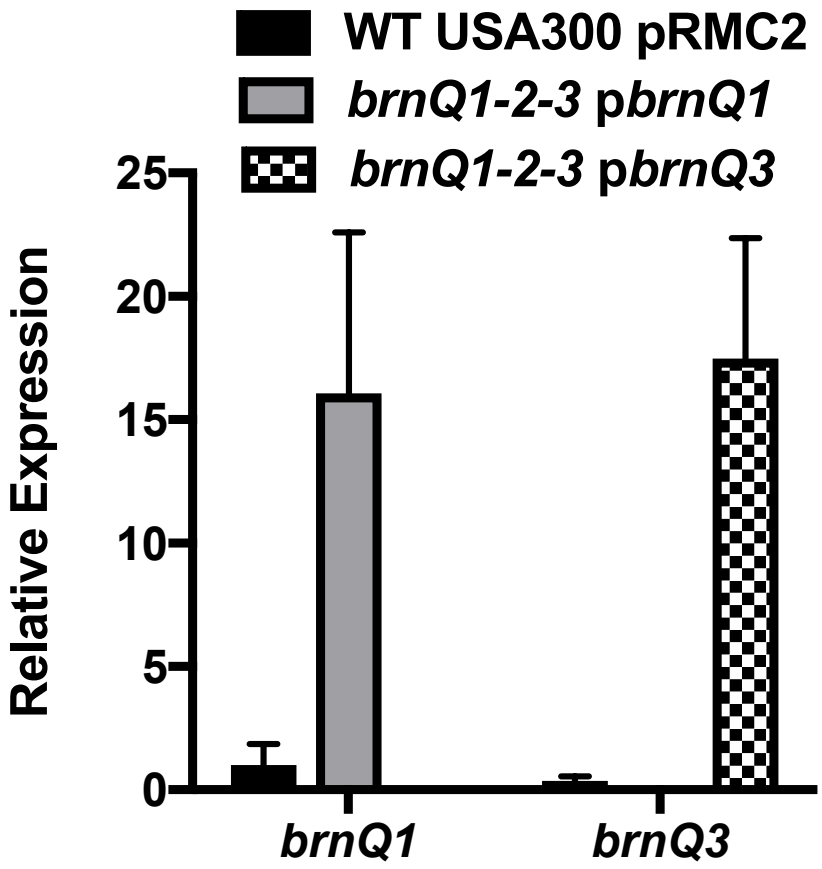

Figure 2.6 Expression of *brnQ1* and *brnQ3* in strains bearing complementation vectors.

Cells were grown to mid-exponential phase in complete CDM and RNA was isolated. Expression of *brnQ1* and *brnQ3* was measured and normalized to expression of the reference gene *rpoB*. Data are the mean of 3 biological replicates +/- SD.

brnQ1-2-3 mutant to levels comparable to those of *brnQ1* expressed from pSO1 (Figure 2.6). Combined, these data led us to conclude that BrnQ3 does not function as a transporter of BCAAs, at least under the conditions used in this study. Mutation of the *brnQ3* gene was previously identified as causing salt-sensitivity in *S. aureus* strain RN450 (42). To examine this phenomenon further, we streaked USA300, along with its isogenic *brnQ1*, *brnQ2* and *brnQ3* mutants on TSB agar and TSB agar containing 2M NaCl. Although all strains grew relatively slower on 2M salt-containing agar plates, mutation of the *brnQ* genes had no effect on the ability of strain USA300 to grow in the presence of high concentrations of NaCl, including the *brnQ1-2-3* mutant (Figure 2.7). It is likely that the absence of the salt-sensitive phenotype in our study is due to strain differences between RN450 and USA300, since the assay was performed using identical media to that previously described (42).

2.3.5 Transporter kinetics

To gain further insight into the role of the three BrnQ paralogs in BCAA transport, we determined the kinetic properties of BCAA transport in strains expressing each of BrnQ1, BrnQ2 and BrnQ3 by measuring the initial velocity of uptake in response to varying substrate concentration. The kinetic plots are shown in Figure 2.8, and apparent K_M and V_{max} data are provided in Table 2.3. The data indicate that BrnQ1 is a major determinant of uptake of all three BCAAs, while BrnQ2 appears specific to Ile, albeit with lower affinity for Ile than BrnQ1.

Table 2.3 K_M and V_{max} values for the BrnQ transporters

Transporter	Leucine		Isoleucine		Valine	
	K_M[a]	V_{max}[a]	K_M	V_{max}	K_M	V_{max}
BrnQ1	1.62	6.85	0.93	10.22	1.79	12.81
BrnQ2			8.94	18.13		

[a] K_M units μM, V_{max} units pmol μg^{-1} min^{-1}; units of activity are expressed relative to total protein

Values are the average of three biological replicates ±SD.

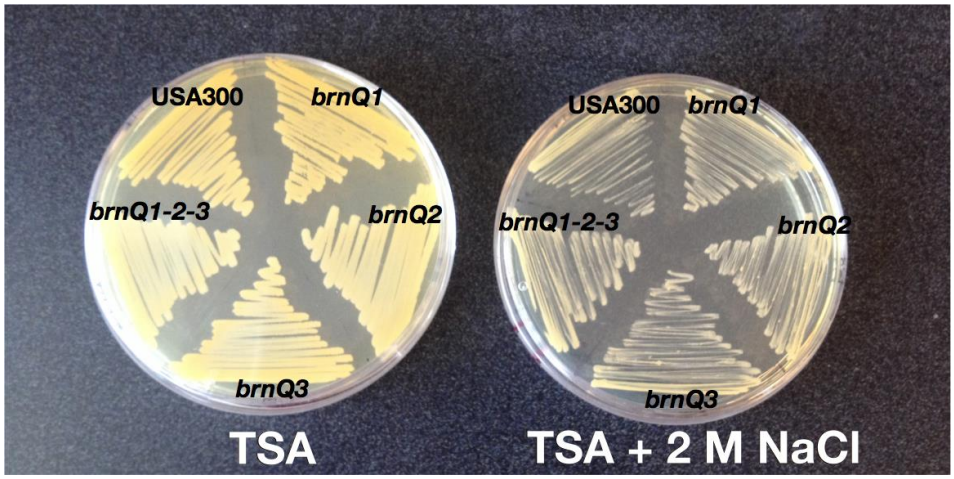

Figure 2.7 Mutation of *brnQ* genes does not render salt sensitivity on USA300.

Single colonies of WT USA300 and the *brnQ* single and triple mutants were streaked from TSA onto either TSA or TSA containing 2 M NaCl. Plates were incubated at 37˚C for 24 hours, at which point growth was observed.

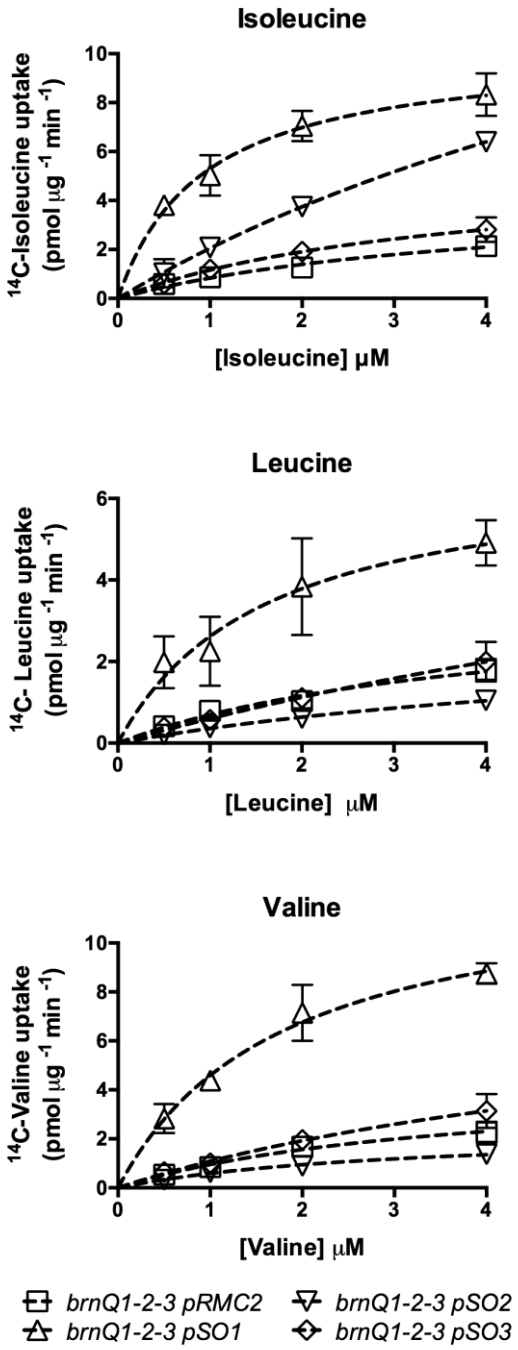

Figure 2.8 Kinetics of BCAA transport in *S. aureus*

Cells were harvested from cultures grown to mid-late exponential phase in complete CDM and incubated with ^{14}C-labeled amino acid at concentrations of a 0.5 µM, 1 µM, 2 µM, or 4 µM for 20 s. Values are the mean of three independent cultures +/- SD.

2.3.6 BrnQ1-mediated Leu and Val acquisition is required for optimal growth of USA300 in CDM

We next sought to investigate the physiological role of the functional BCAA transporters in meeting the nutritional requirements of *S. aureus* growth. To do so, we selected CDM, described previously (31), enabling the limitation or omission of amino acids of interest. In complete CDM, we consistently observed a slight growth delay for the *brnQ1* mutant compared to the WT strain (Figure 2.9A), indicating that BrnQ1 contributes to rapid growth in this medium. In comparison, all strains grew equally well in TSB (Figure 2.10). We next investigated the amino acid specificity of the growth phenotype in CDM. *S. aureus* has been reported to be auxotrophic for Leu and Val despite the presence of BCAA biosynthetic genes (15–17), indicating a critical role for BCAA uptake to support growth. First, to confirm the auxotrophic phenotype of USA300, the WT strain was grown in complete CDM as well as CDM with each BCAA omitted; CDM^{-Ile}, CDM^{-Leu} or CDM^{-Val}. As expected, based on the literature, a growth delay was not observed for WT USA300 grown in CDM^{-Ile} compared to complete CDM, indicating that USA300 is capable of *de novo* synthesis of Ile. In contrast, we observed that growth of WT USA300 in CDM^{-Leu} was significantly delayed for the first 12 hours of incubation, but by 24 hours the cells had attained an equivalent biomass to that obtained in complete CDM (Figure 2.9B). For growth in CDM^{-Val}, we observed an even greater growth defect. A significant growth delay was seen through the initial 24 hours of incubation, followed by growth that resulted in biomass equivalent to WT after 48 hours (Figure 2.9B). This suggests that Leu and Val synthesis is delayed, possibly owing to the repression of the biosynthetic genes by CodY, Gcp and/or unrecognized factors (17, 28, 43). We observed that the growth delay in these media was consistent upon serial sub-culturing, excluding the accumulation of suppressor mutations.

We next assessed the role of BrnQ1 in acquiring Leu and Val to promote early and rapid growth, since the BCAA uptake data implicated BrnQ1 as the dominant Leu and Val transporter. Growth of WT USA300 and *brnQ1* was compared in complete CDM and CDM with varying amounts of Leu and Val limitation. The *brnQ1* mutant was impaired for growth compared to WT USA300 upon limitation of Leu or Val to concentrations

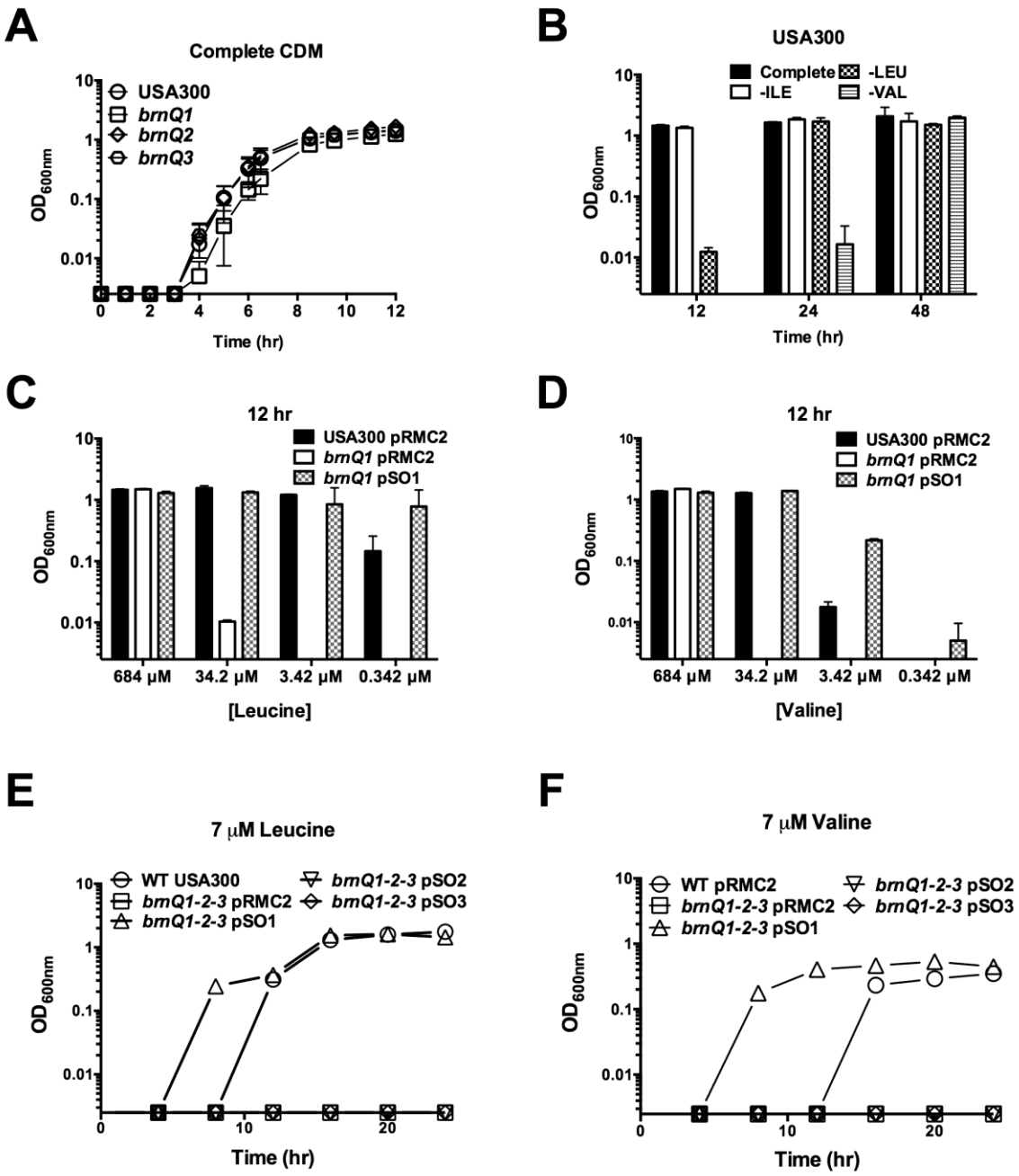

Figure 2.9 BrnQ1 is required for rapid growth in CDM limited for leucine or valine.

A) Strains were pre-grown in complete CDM and sub-cultured into complete CDM. Growth was monitored until stationary phase was reached. B) WT USA300 was grown in complete CDM and sub-cultured into complete CDM, or CDM lacking Ile, Leu, or Val. Growth yield was monitored at 12, 24, and 48 hrs. C-D) Strains were pre-grown in complete CDM and sub-cultured into CDM where all amino acids were present at the same concentration as in CDM, except for alterations to the concentration of Leu (panel C) or Val (panel D). Growth yield was measured at 12 hrs. In panels E and F, growth curves were performed on the strains as indicated. Cultures were pregrown in complete CDM and sub-cultured into CDM where

all amino acids were present at the same concentrations as in CDM, except that Leu was 1% the concentration of that in complete CDM (panel E) and Val was 1% the concentration of that in complete CDM (panel F). pRMC2 = empty vector; pSO1 = p*brnQ1*; pSO2 = p*brnQ2*, pSO3 = p*brnQ3*. Data shown are the mean of three biological replicates +/- SD and were analyzed using the Student's *t*-tests (compared to WT). ** $P<0.01$, *** $P<0.001$.

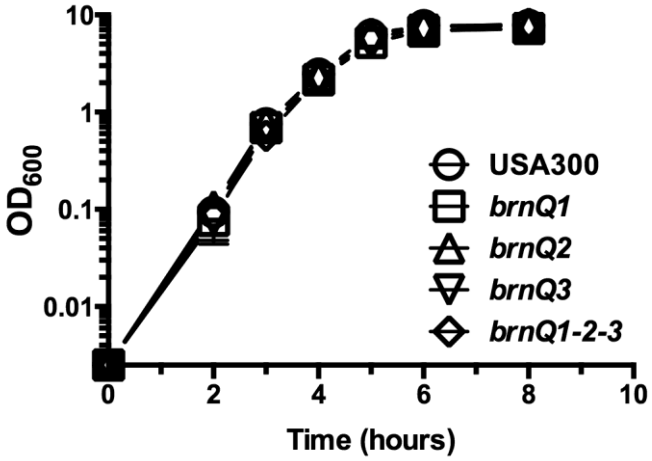

Figure 2.10 The *brnQ* genes are not required for growth of USA300 in TSB.

WT USA300, the isogenic mutants, and the triple mutant were pre-grown in TSB to mid-exponential phase, and then sub-cultured to a starting OD_{600} of 0.0025 nm in TSB. Growth was monitored at the indicated time points until stationary phase was reached. Data are mean +/- SD.

≤5% of that in complete CDM (complete CDM contained 684 μM Leu and 684 μM Val) (Figure 2.9C,D). Growth of the *brnQ1* mutant was fully restored to WT levels upon complementation with *brnQ1 in trans* (Figure 2.9C,D).

To investigate the possible involvement of BrnQ2 and BrnQ3 in the acquisition of Leu or Val for growth, we limited these two amino acids individually to 1% of the concentration in complete CDM, and compared the growth, over 24 hours, of WT to that of the *brnQ1-2-3* mutant carrying empty vector or the *brnQ* genes. We observed that the *brnQ1-2-3* mutant, as expected based on the above-described data, was impaired for growth (Figure 2.9E,F), and neither vector expressing *brnQ2* or *brnQ3* restored this defect. In contrast, expression of *brnQ1* allowed rapid growth such that the strain grew quicker than WT in media limited for Leu (Figure 2.9E). Overexpression of *brnQ1* had an even more pronounced effect on the growth enhancement of *S. aureus* in media limited for Val (Figure 2.9F). Combined, our data indicate that BrnQ1-mediated Leu and Val acquisition is required for rapid growth of *S. aureus* when these amino acids are limited.

2.3.7 Mutation of *brnQ2* results in over-expression of *brnQ1*

The data illustrated in Figure 2.4 demonstrated that a *brnQ2* mutant transported Ile, Leu and Val to levels higher than WT. This was an interesting observation that led us to investigate the possible regulatory effect of a *brnQ2* mutation on *brnQ1* expression, given that our data have shown that BrnQ1 is a major Ile, Leu and Val transporter. As shown in Figure 2.11A, qPCR demonstrated that *brnQ1* expression was over 40-fold higher in the *brnQ2* mutant than in WT. We confirmed that this phenomenon was specifically due to mutation of *brnQ2* and not some secondary mutation in the *brnQ2* mutant since complementation of the *brnQ2* mutant with *brnQ2 in trans* reversed expression of *brnQ1* to levels that were even lower than in WT.

Given that a mutation of *brnQ2* enhances expression of *brnQ1*, we hypothesized that a *brnQ2* mutant would grow quicker than WT in media limited for either Leu or Val. We compared the growth of selected strains in media that contained 1% of the concentration of either Leu or Val found in complete CDM. In support of our hypothesis, we observed that a *brnQ2* mutant demonstrated early and rapid growth, compared to WT, in media limited for

Leu, and more so in media limited for Val. The growth kinetics of this mutant were virtually identical to a *brnQ1* mutant overexpressing *brnQ1* from a plasmid (Figure 2.11B,C).

2.3.8 BrnQ1 is required for USA300 fitness *in vivo*

We next investigated the contribution of BCAA acquisition to the fitness of USA300 *in vivo* using an established bacteremia murine model of infection. Groups of ten female Balb/c mice were infected via tail vein injection with 5-7 x 10^6 CFU of WT USA300 or the isogenic mutant. Mice infected with either WT or the *brnQ1* mutant were sacrificed on day four post-infection and the bacterial burden in livers and kidneys was assessed. Mice infected with the *brnQ1* mutant had significantly lower CFUs in the kidneys and livers compared to WT USA300 (Figure 2.12A,B), suggesting that USA300 encounters BCAA limitation *in vivo* and that BrnQ1 is necessary for their acquisition in order to meet growth requirements.

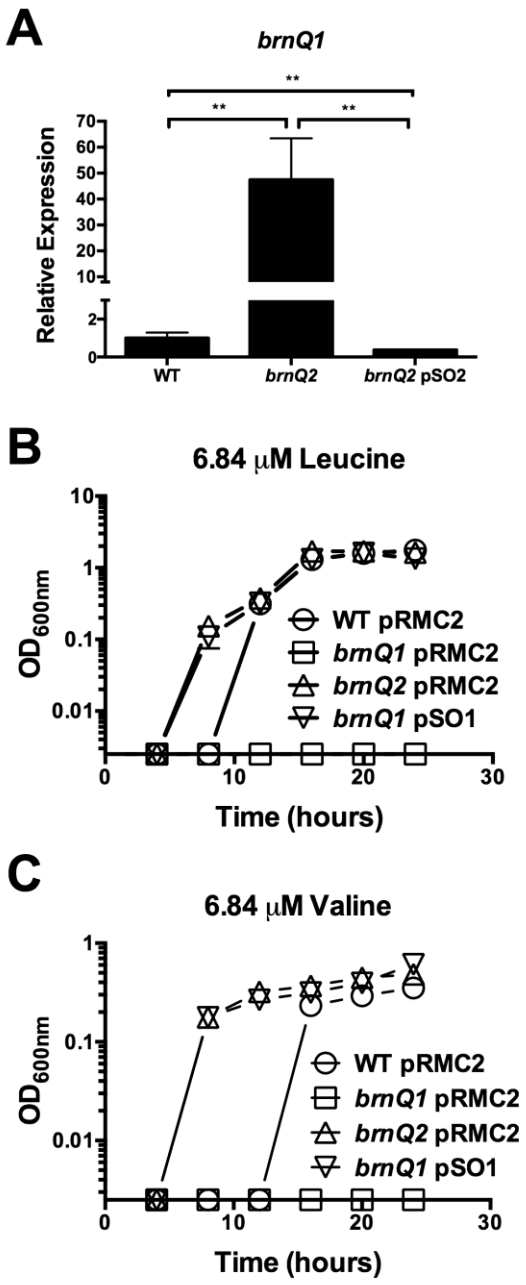

Figure 2.11 Mutation of *brnQ2* results in overexpression of *brnQ1*.

A. The expression level of *brnQ1* was evaluated in *S. aureus* strains, as indicated. Data was normalized relative to the expression of *rpoB*, and expression of *brnQ1* in WT cells was set to 1 as the comparator. B-C. Cultures were pregrown in complete CDM and sub-cultured into CDM where all amino acids were present at the same concentrations as in CDM, except that Leu was 1% the concentration of that in complete CDM (panel B) and Val was 1% the concentration of that in complete CDM (panel C). pRMC2 = empty vector; pSO1 = p*brnQ1*. Data shown are the mean of three biological replicates +/- SD.

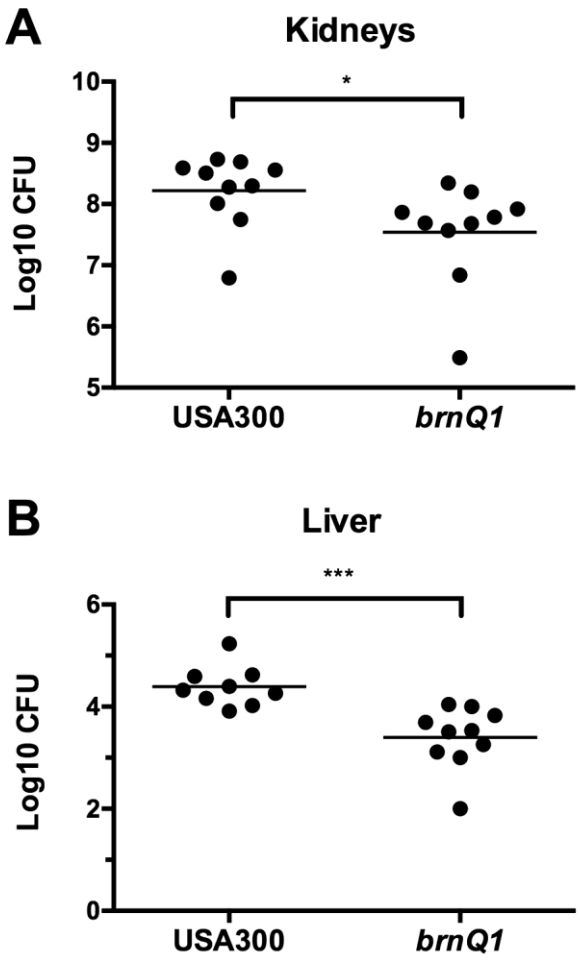

Figure 2.12 BrnQ1 is required for full *S. aureus* virulence in a mouse model of systemic infection.

Balb/c mice were infected with 5-7 x 10^6 CFU of USA300 (n=9) or Δ*brnQ1* (n = 10) via tail vein injection. Mice were sacrificed on day four of infection and A) kidneys and B) livers were harvested, homogenized and dilutions plated for CFU determination. Data were analyzed using the Student's unpaired *t*-test. * $P < 0.05$, *** $P < 0.001$.

2.4 Discussion

S. aureus necessitates expression of transporters for Leu and Val acquisition that would allow for growth, since it has been reported to be auxotrophic for Leu and Val, despite encoding the genes for biosynthesis (15, 16). Here, we demonstrate that BrnQ1 plays a dominant role in meeting Leu and Val nutritional requirements *in vitro*, especially in a medium containing either of these amino acids in the low micromolar range, and postulate also *in vivo* since a *brnQ1* mutant is attenuated *in vivo*. In contrast, BrnQ2 is selective for Ile and BrnQ3 does not function as a BCAA transporter. Although BrnQ3 was previously identified to contribute to the osmotolerance of *S. aureus*, we were unable to replicate this finding. Our ongoing studies will investigate the ligand specificity of BrnQ3, and the importance of this is demonstrated by the finding by *Benton et al.,* that a *brnQ3* mutant is attenuated *in vivo* (44).

Our observation that USA300 is able to grow in the absence of exogenous Leu or Val contrasts previous reports that *S. aureus* is auxotrophic for these amino acids. This is likely explained by the extension of the growth period monitored here, since appreciable growth of USA300 in media lacking Leu or Val is not observed until 24 and 48 hours, respectively (Figure 2.9). We presume the biosynthesis of Leu and Val is de-repressed at the later stages of growth, as we excluded the possibility of development of suppressor mutations. At present, the regulatory mechanisms that govern the regulation of ILV synthesis in these conditions is not known, but plausible mediators of repression include CodY and Gcp, both known repressors of the *ilv-leu* biosynthetic genes in *S. aureus* (17, 28, 43). Although we did not investigate this in further detail, of note, we observed that growth in CDM^{-LEU} and CDM^{-VAL} (i.e. growth dependent on Leu and Val synthesis) is iron-dependent. Indeed, several enzymes involved in BCAA biosynthesis contain Fe-S clusters, including IlvD, LeuC, and LeuD. LeuCD are repressed in *Bacillus subtilis* upon iron starvation (45, 46) and a similar link between iron availability and BCAA biosynthesis might exist in *S. aureus*. This phenotype is relevant when considering the levels of BCAA biosynthesis *in vivo*, as iron is limited during *S. aureus* infection (47) and consequently might impart a greater importance on BCAA acquisition *in vivo*.

BrnQ1 plays a predominant role in BCAA transport, as demonstrated by both radioactive transport assays and the growth assays where Leu and Val are present in low micromolar

concentrations (Figure 2.3, Figure 2.9). However, two lines of evidence suggest the presence of at least one additional BCAA transporter in *S. aureus*. First, a *brnQ1* mutant and a *brnQ1-2-3* mutant are not defective for growth, compared to WT, even at early timepoints, in CDM media containing 100 µM or higher concentrations of Leu or Val, and our transport assays indicate that the *brnQ1-2-3* mutant still transports some Ile, Leu and Val (Figure 2.3, Figure 2.4, Figure 2.5). We are currently investigating additional genes that contribute to BCAA transport, such as a gene homologous to the CodY-regulated transporter BcaP described in *Lactococcus lactis* (9). BcaP functions as the high-affinity BCAA transporter in *L. lactis*, whereas BrnQ is the low affinity transporter (9). Given that BrnQ1 in USA300 is necessary for growth when Leu or Val are in low micromolar concentrations, and not when they are present at higher concentrations in the growth medium, we predict that BrnQ1 is the high-affinity transporter in this species. In line with this, the *brnQ1* mutant is attenuated *in vivo*, suggesting that BrnQ1 functions as a primary means of BCAA transport during infection.

It will be interesting to consider in future studies the effects of mutating *brnQ1* and *brnQ2* on CodY activity. The up-regulation of *brnQ1* in the *brnQ2* mutant, and the subsequent increased ability of *S. aureus* to scavenge Leu and Val, might involve a decrease in CodY activity upon Ile depletion. Ongoing studies in our lab are addressing the effects of manipulating intracellular levels of CodY effector molecules, and their implications on the expression of CodY-regulated genes.

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Chapter 3

3 The role of BcaP in BCAA transport

3.1 Introduction

Staphylococcus aureus, and its methicillin-resistant variant, is associated with significant human infectious morbidity and mortality. It is capable of infecting nearly every body site to cause infections that range from mild skin and soft tissue infections to life-threatening pneumonia, necrotizing fasciitis, and sepsis (1–4). It is also an asymptomatic colonizer of the nares in approximately one third of the population (5). The synthesis and acquisition of nutrients are critical to support *S. aureus* metabolism and virulence factor production and are prerequisites for infection and colonization (6–9). Such nutrients include amino acids, which *S. aureus* is presumably starved for in the host given that genes for amino acid synthesis and transport are up-regulated *in vivo* (6, 10). Yet the specific amino acid requirements of *S. aureus* in diverse host niches and the mechanisms to maintain these nutrients are not fully understood.

The branched-chain amino acids (BCAAs; Ile, Leu, Val) are vital nutrients for bacterial physiology, as their importance extends beyond supporting high levels of protein synthesis. In most Gram-positive bacteria, the BCAAs are precursors for the synthesis of branched-chain fatty acids (BCFAs), which are a major constituent of membrane fatty acids and include Leu/Val-derived iso-fatty acids and Ile-derived anteiso-fatty acids. In *S. aureus*, BCFAs comprise 65% of membrane fatty acids, and straight-chain fatty acids comprise 35% (11). BCFA composition is adjusted to regulate membrane fluidity, an alternative strategy to the incorporation of unsaturated fatty acids, which are toxic to *S. aureus* (12). Of the BCFAs, anteiso-fatty acids are most important for promoting a more fluid membrane and as such, their abundance in the membrane is increased at low temperatures to support growth (11, 13–17). The iso:anteiso ratio is also important for pH stress tolerance, resistance to membrane stressors including antimicrobial peptides, and virulence (11, 14, 18, 19), thus the BCFAs serve an important role in adaptation to various environmental conditions.

Intracellular levels of BCAAs are monitored by the global regulatory protein CodY (20–22). CodY is a highly conserved transcriptional repressor in low %mol G+C Gram-positive

bacteria that regulates >100 genes involved in amino acid and peptide metabolism and transport in response to BCAAs and GTP (23–25). CodY also regulates virulence factors in *S. aureus* (26–29), implicating CodY as an important link between the metabolic status of the cell and virulence. Identifying the mechanisms that maintain intracellular levels of BCAAs is therefore a key step towards understanding how CodY activity is regulated.

S. aureus was previously reported to be auxotrophic for Leu and Val, despite encoding the *ilv-leu* operon required for BCAA synthesis (25, 30). In replicating this, we found that in fact, *S. aureus* is capable of synthesizing Leu and Val, but it represses their synthesis during early stage growth (Figure 2.9). This indicated that *S. aureus* prefers to acquire these nutrients and must therefore be able to scavenge BCAAs from the host. Indeed, free BCAAs are available to *S. aureus* at sites of infection or colonization in the micromolar range (50 – 300 μM except for Ile which is undetectable in nasal secretions) (6, 31). We have therefore focused our studies on investigating mechanisms of BCAA transport. BCAA transporters in other Gram-positive bacteria include the secondary transporters BrnQ, BraB and BcaP (32–34). We previously characterized three BrnQ homologs (BrnQ1, BrnQ2, and BrnQ3) in the community-associated methicillin-resistant *S. aureus* strain USA300. We determined that BrnQ1 transports all three BCAAs, BrnQ2 is a dedicated Ile transporter, and BrnQ3 did not appear to function as a BCAA transporter. Yet a strain lacking all three *brnQ* genes remained capable of BCAA transport (Figure 2.3). BLAST searches revealed that USA300 also possesses a BcaP homolog. In this study, we describe the role of BcaP in BCAA acquisition and demonstrate the importance of BCAA transport to *S. aureus* growth, membrane BCFA content, and fitness *in vivo*.

3.2 Materials and Methods

3.2.1 Strains and growth conditions

Bacterial strains and plasmids used in this study are described in Table 3.1. The wild type (WT) strain in all experiments is the MRSA isolate pulsed-field gel electrophoresis type USA300 LAC that has been cured of the erythromycin resistance plasmid. For experiments involving *S. aureus* grown in tryptic soy broth (TSB) (EMD Millipore, Billerica, MA), two to three colonies were inoculated from TSB agar into TSB and grown to mid-exponential phase at 37˚C, then sub-cultured into fresh TSB to a starting OD_{600} equivalent of 0.0025. For

growth curves performed in chemically defined medium (CDM), described previously (See Section 2.2.1), two to three colonies were inoculated from TSB agar into CDM and were grown to mid-late-exponential phase at 37˚C, then sub-cultured to a starting OD_{600} equivalent of 0.0025 in either complete CDM or CDM with altered concentrations of amino acids. The *brnQ1,brnQ2,brnQ3,bcaP* mutant was treated the same, however media was supplemented with 10 mg mL^{-1} of Bacto™ tryptone (BD Biosciences; Sparks, MD) to support growth. Strains were grown at 37˚C or 25˚C where indicated. Growth curves were performed either in 100-well plates and read using the Bioscreen C visible spectrophotometer (Growth Curves USA; Piscataway, NJ), or in flasks using a flask:volume ratio of at least 5:1. Where required, ampicillin (100 μg mL^{-1}), chloramphenicol (10 μg mL^{-1}), erythromycin (3 μg mL^{-1}), streptomycin (500 μg mL^{-1}) and tetracycline (4 μg mL^{-1}) were added to the growth medium.

3.2.2 Mutagenesis and construction of plasmids

Strains containing a transposon insertion in either the *bcaP* gene (*bcaP*::ΦNΣ) or *crtM* (*crtM*::ΦNΣ) gene were identified in the Nebraska Transposon Mutant Library (35). The *bcaP* transposon was transduced into our laboratory strain of USA300 and into the *brnQ1*, *brnQ1-2-3*, *brnQ1-2* and *brnQ1-3* backgrounds using phage 80α. The same method was used to transduce the *crtM* transposon into WT USA300 and the *brnQ1* mutant. Transposon insertions were confirmed by PCR. All plasmids were first constructed as shuttle vectors in *Escherichia coli* DH5α. Once confirmed, plasmids were subsequently electroporated into *S. aureus* RN4220 as an intermediate host, and then into

Table 3.1 Bacterial strains and plasmids used in this study

Strain/Plasmid	Description [a]	Source or reference
S. aureus		
USA300	USA300 LAC cured of antibiotic resistance plasmid	(36)
RN4220	$r_K^- m_K^+$; capable of accepting foreign DNA	(37)
bcaP	USA300 *bcaP*::ΦNΣ; Em^R (SAUSA300_2538)	(35)
brnQ1	USA300 Δ*brnQ1*	Chapter 2
brnQ1-bcaP	USA300 Δ*brnQ1 bcaP*::ΦNΣ; Em^R	This study
brnQ1-2-3	USA300 Δ*brnQ1* Δ*brnQ2 brnQ3*::Tc; Tc^R	Chapter 2
brnQ1-2-3-bcaP	USA300 Δ*brnQ1* Δ*brnQ2 brnQ3*::Tc *bcaP*::ΦNΣ; Em^R, Tc^R	This study
		This study
brnQ1-2-bcaP	USA300 Δ*brnQ1* Δ*brnQ2 bcaP*::ΦNΣ; Em^R	This study
brnQ1-3-bcaP	USA300 Δ*brnQ1 brnQ3*::Tc *bcaP*::ΦNΣ; Em^R, Tc^R	
codY	USA300 *codY*:: ΦNΣ; Em^R	(35)
crtM	USA300 *crtM*:: ΦNΣ; Em^R	(35)
brnQ1-crtM	USA300 Δ*brnQ1 crtM*:: ΦNΣ; Em^R	This study
E.coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17* ($r_K^- m_K^-$) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169phoA*	Promega
Plasmids		
pRMC2	Anhydrotetracycline-inducible expression vector; Ap^R in *E. coli*; Cm^R in *S. aureus*	(38)
pBrnQ1	pRMC2 containing *brnQ1*; Cm^R	(pSO1 from Chapter 2)
pBcaP	pRMC2 containing *bcaP*; Cm^R	This study
pGY*lux*	Vector harboring promoterless *luxABCDE* operon; Cm^R	(39)
pGY*brnQ1*::*lux*	*lux* reporter vector with *brnQ1* promoter	This study
pGY*bcaP*::*lux*	*lux* reporter vector with *bcaP* promoter	This study

[a] Abbreviations: Tc^R, Cm^R, Em^R and Ap^R designate resistance to tetracycline, chloramphenicol, erythromycin and ampicillin, respectively.

the desired *S. aureus* USA300 strains. Primers used for plasmid construction are listed in Table 3.2.

3.2.3 RT-qPCR

Cultures were grown to mid-exponential phase (OD_{600} of 0.6 in TSB) and RNA was extracted using an Aurum Total RNA Mini Kit as described by the manufacturer (Bio-Rad; Hercules, CA). RNA (500 ng) was reverse transcribed using SuperScript II (Invitrogen; Carlsbad, CA) according to manufacturers instructions and using 500 μg mL^{-1} of random hexamers. cDNA was PCR-amplified using SensiFast SYBR ® (Bioline; Taunton, MA). The primers used and their reaction efficiencies are listed in Table 3.2. Data were normalized relative to expression of the reference gene *rpoB.*

Table 3.2 Oligonucleotide primers used for cloning and qPCR

Oligonucleotides	Sequence[a]
bcaP-KpnI-For	TTTGGTACCGCGTAACGACGCTATTTAAA
bcaP-EcoRI-For	TTTGAATTCTGGATGTTAATCAGATGGGA
	For cloning *bcaP* into pRMC2
rpoB-For	AGAGAAAGACGGCACTGAAAACAC
rpoB-Rev	ATAACGACCCACGCTTGCTAAG
	qPCR for *rpoB* expression in *S. aureus*
brnQ1-For	TGCGAGAAAGCCATCAAG
brnQ1-Rev	GTATTGTATCCATCTAAAAAGCCG
	qPCR for *brnQ1* expression in *S. aureus*
bcaP-For	TTTACGCTACCTGGCATTG
bcaP-Rev	CCCAAGAATAGGCTGAACC
	qPCR for *bcaP* expression in *S. aureus*
brnQ1-lux-For	GATCCCCGGGACATATACTTCCCCCCTTCG
brnQ1-lux-Rev	GATCGTCGACCCGGCACCAAAGAATAAACC
	For cloning *brnQ1* promoter into pGY*lux*
bcaP-lux-For	GATCCCCGGGGCGTAACGACGCTATTTAAAGGC
bcaP-lux-Rev	GATCGTCGACGCCGGATCCTCTTTTCGTGCTA
	For cloning *bcaP* promoter into pGY*lux*

[a] Restriction sites are underlined.

3.2.4 Radioactive transport assays

Cultures were grown overnight at 37°C in complete CDM and sub-cultured into complete CDM at a starting OD_{600} of 0.1. Bacteria were grown to mid/late-exponential phase (OD_{600} of 1.0), harvested by filtration on 0.45-µm pore-size membrane filters, and washed with PBS before being re-suspended in CDM lacking amino acids. Cells were warmed to 37°C for 10 minutes prior to the assay. The ^{14}C-labeled amino acid of interest (Perkin Elmer, MA) was added to cells at a final concentration of 1 µM. An aliquot of cells was removed at 20, 40, and 60 s and rapidly filtered through 0.45-µm membrane filters. The filters were immediately washed with 10 mL of 0.1 M $LiCl_2$ at room temperature. Filters were dried and placed in scintillation vials containing 4 mL of Cytoscint™ scintillation cocktail (Fisher Scientific). ^{14}C radioactivity was measured using the LS 6500 scintillation system (Beckman). Radioactivity was normalized to total protein; 1 mL of culture with an OD of 1.0 corresponded to approximately 115 µg of protein. For competition assays, 1 mM of non-labeled amino acid was added in the presence of 1 µM of either ^{14}C-Leu or ^{14}C-Ile and cells were filtered at 60 s and processed as above.

To determine transport kinetics, cells, prepared as above, were incubated with 500 nM, 1 µM, 2 µM, or 4 µM of a ^{14}C-labeled amino acid. An aliquot of cells was filtered as described above after 20 s. The velocity of uptake for each concentration was normalized to total protein and plotted to determine the Michaelis-Menten equation and the apparent K_M and V_{max}.

3.2.5 Lux reporter gene assays

The WT USA300 strain and *codY* mutant containing either pGY*brnQ1*::*lux* or pGY*bcaP*::*lux* were grown to mid-late exponential phase in complete CDM and sub-cultured to a starting OD_{600} of 0.01 (200 µL/well) of complete CDM in a clear bottom 96-well Microfluor 2 White Plate (Thermo). Plates were incubated at 37˚C with constant shaking. Luminescence and OD_{600} were ready at hourly intervals on the BioTek Synergy H4 Hybrid Reader (BioTek, Winooski, VT) with 1 s of integration and a gain of 200.

3.2.6 Membrane fatty acid analysis and staphyloxanthin quantification

Strains were grown overnight in either TSB or CDM and sub-cultured to an OD_{600} of 0.05 in the same media. Cells were harvested at mid-exponential phase (OD_{600} 0.4-0.6) by centrifugation at 3000 x *g* at 4°C for 15 minutes, and the pellet was washed 3 times with cold sterile distilled water. The fatty acids in the bacterial cells (30 to 40 mg [wet weight]) were saponified, methylated, and extracted for fatty acid methyl ester (FAME) analysis. The resulting methyl ester mixtures were separated using an Agilent 5890 dual-tower gas chromatograph and the fatty acyl groups were identified using the MIDI microbial identification system (Sherlock 4.5 microbial identification system) at Microbial ID, Inc. (Newark, DE) (40, 41). Minor fatty acids (<1% of the total) are not reported in the tables. Quantification of the carotenoid pigment was performed as per the warm methanol extraction protocol described previously (42, 43). Cells were harvested from strains grown overnight in CDM, and carotenoid was extracted with warm (55˚C) methanol for 5 min. The absorbance at 465 nm of the supernatant after centrifugation was measured and expressed relative to the OD_{600} of overnight cultures.

3.2.7 Antibiotic susceptibility assays

The minimum inhibitory concentrations (MIC) for Daptomycin (DAP) and Polymyxin B (PMB) were determined based on growth of *S. aureus* strains in tubes containing 2 mL of complete CDM with a range of 2-fold dilutions of each antibiotic. PMB (Sigma-Aldrich) was resuspended in H_2O to a stock concentration of 50 mg mL^{-1} and DAP (Cayman Chemicals) was resuspended in 100% DMSO to a stock concentration of 5 mg mL^{-1}. The concentration of DMSO did not exceed 5% of the culture volume in any experiment. Cells were grown to mid-late-exponential phase in complete CDM and sub-cultured into CDM containing antibiotics at a starting CFU of approximately 1 x 10^5. Cultures were incubated at 37˚C and were monitored for visible growth after 24 h.

3.2.8 Murine model of systemic infection

All protocols were reviewed and approved by the University of Western's Ontario Animal Use Subcommittee, a subcommittee of the University Council on Animal Care. The systemic infection was performed as previously described (See Section 2.2.5). Briefly, seven-week old BALB/c mice purchased from Charles River Laboratories Canada Inc. were housed in

microisolator cages. Strains were grown in TSB to mid-exponential phase, washed twice in PBS, and resuspended in PBS. Mice were infected with approximately 6 x 10^6 – 7 x 10^6 CFU of *S. aureus* via tail-vein injection. Mice were monitored for activity and weight changes every 24 hr for the duration of the experiment. Mice were sacrificed 96-hours post challenge and kidneys, livers and hearts were removed, homogenized in 0.01% Triton X-100, and plated on TSB agar to determine CFUs.

3.2.9 Murine nasal colonization model

All protocols were reviewed and approved by the University of Western's Ontario Animal Use Subcommittee, a subcommittee of the University Council on Animal Care. To evaluate *S. aureus* nasal colonization, the endogenous murine microbiota, which are non-permissive to *S. aureus* colonization, were depleted with use of streptomycin sulfate, as previously described (44). Since *S. aureus* is not naturally resistant to Sm, Sm resistant colonies were selected for independently in the WT and mutant backgrounds by plating overnight cultures on TSB agar containing 500 μg ml^{-1} of streptomycin sulfate. Sm-resistant clones were confirmed to be free of any obvious growth defects, compared to the Sm-sensitive colonies, during growth in TSB or CDM. Seven-week old C57BL/6 mice purchased from Charles River Laboratories Canada, Inc. were housed in microisolator cages. Mice received 2 mg mL^{-1} streptomycin sulfate in the drinking water 24 hr prior to inoculation with *S. aureus*, which was changed every 3-4 days for the duration of the experiment. Strains were grown in TSB to mid-exponential phase, washed twice in PBS, and resuspended in PBS. Isoflurane-anesthetized mice were inoculated with 5 μL bacterial suspensions into each naris for a total of 10^8 CFU of *S. aureus* and monitored over seven days. On day seven, mice were sacrificed and the snout was excised, homogenized in PBS with 0.01% Triton X-100, and plated on TSB agar for CFUs.

3.2.10 Statistics

All data were analyzed using the statistical tests indicated in figure legends using GraphPad Prism version 6.0.

3.3 Results

3.3.1 Identification of CodY-regulated *bcaP* as a BCAA transporter in *S. aureus*

We hypothesized that one or more BCAA transporter(s) exist in *S. aureus*, in addition to the three *brnQ* genes, since we previously demonstrated that uptake of Ile, Leu and Val is not completely abolished in a *brnQ1-2-3* mutant (Figure 2.3). We prioritized the gene SAUSA300_2538 to investigate since it exhibited 34% and 63% total similarity to BcaP, a BCAA transporter in *Bacillus subtilis* and *Lactococcus lactis*, respectively (32, 34). The SAUSA300_2538 gene, hereafter referred to as *bcaP*, possesses a CodY-binding site (CodY box) 102 base pairs upstream of the start codon (Figure 3.1A). Consistent with this, we observed that expression of *bcaP* in *S. aureus* strain USA300 increased in the absence of CodY activity (Figure 3.1B). This is in agreement with previous findings in *S. aureus* strains Newman and UAMS-1, although the magnitude of *bcaP* up-regulation observed in USA300 is lower than what was previously reported, possibly a consequence of strain variation (25, 45).

To determine whether BcaP functions as a BCAA transporter, we mutated *bcaP* alone and in combination with the *brnQ* genes and measured uptake of ^{14}C-labeled Ile, Leu, or Val in the various mutant strains. The *bcaP* mutation alone had a minor effect on transport of all three BCAAs compared to the WT strain (Figure 3.2). However, when *bcaP* was mutated in the *brnQ1-2-3* mutant background, uptake of all three BCAAs was completely abolished (Figure 3.2A), indicating a clear role for BcaP in BCAA transport. Leu transport was completely abolished in the *brnQ1-bcaP* mutant, suggesting that these are the only genes responsible for Leu transport. Ile transport was reduced in the *brnQ1- bcaP* mutant compared to the *bcaP* mutant alone, and introduction of the *brnQ2* mutation into the *brnQ1-bcaP* mutant resulted no Ile transport, indicating that the *brnQ1*, *brnQ2* and *bcaP* genes all participate in Ile transport.

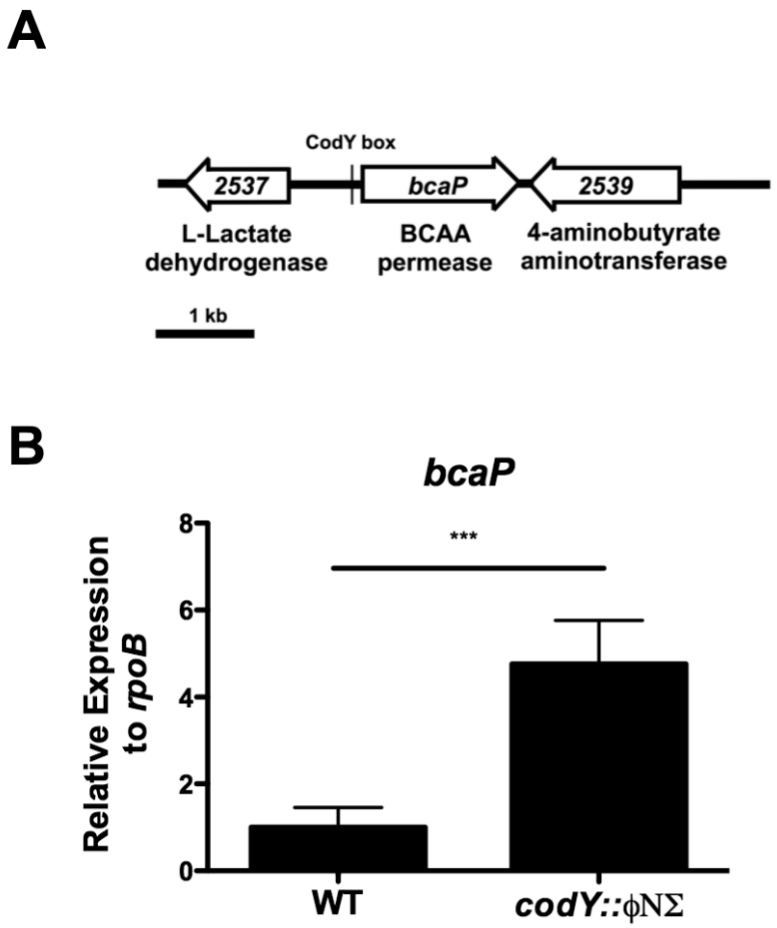

Figure 3.1 BcaP is a CodY-regulated gene in USA300.

A) Genomic context of *bcaP* in the USA300 FPR3757 genome. B) Expression of *bcaP* was evaluated by RT-qPCR in WT USA300 and a *codY* mutant grown to an OD_{600} of 0.6 in TSB. Data were normalized relative to the expression of *rpoB*, and expression of *bcaP* in WT cells was set to 1 as the comparator. Data are the mean of three biological replicates +/- SD. Data were analyzed using a Student's unpaired *t* test. *** $P \leq 0.001$.

Unexpectedly, the *brnQ1-bcaP* mutant retained some Val transport (Figure 3.2A). The addition of the *brnQ3* mutation in the *brnQ1-bcaP* mutant background resulted in no Val transport (Figure 3.2A), uncovering an involvement of BrnQ3 in Val transport that we previously had overlooked, likely due to the transport activity of BcaP.

To confirm a direct role for BcaP in BCAA transport, we tested whether expression of *bcaP in trans* could restore transport in the *brnQ1-2-3-bcaP* mutant. BcaP restored transport of all three BCAAs in the quadruple mutant to levels comparable to WT or the quadruple mutant complemented with BrnQ1 (Figure 3.2B), thus confirming the role of BcaP as a BCAA transporter. Despite our observation that BrnQ3 contributes to Val transport (Figure 3.2B), expression of *brnQ3* from a complementation vector (confirmed using RT-qPCR; see Figure 2.6) was unable to restore Val transport in the quadruple mutant (data not shown). At this point, we have no conclusive evidence that implicates BrnQ3 as a *bona fide* BCAA transporter.

BCAA transporters in *L. lactis* and *B. subtilis* are capable of transporting amino acids structurally similar to BCAAs (32, 34). To test the transport specificity of the BrnQ and BcaP transporters in *S. aureus*, we investigated ^{14}C-Leu uptake with 10-fold, 100-fold, and 1000-fold excess of each unlabeled amino acid. We first determined that 1000-fold excess was required to reduce transport to less than 50%, and selected this as the condition for subsequent experiments. Alanine, cysteine, methionine, and threonine were able to reduce BrnQ1- or BcaP-mediated Leu transport whereas only aspartic acid was able to compete for Ile transport mediated by BrnQ2 (Figure 3.3). The transport kinetics for these amino acids is beyond the scope of this study, however we investigated whether BrnQ1 and BcaP are required for growth in chemically defined media (CDM) with limiting concentrations of Ala, Cys, Thr, or Met. The double mutant did not exhibit a growth delay in these media any more so than in complete CDM (Figure 3.4), suggesting that either i) they bind to, but do not transport these amino acids, ii) there are other transporters for these amino acids, or iii) at least in the case of Ala, Thr, and Met (for which *S. aureus* is not auxotrophic) they are synthesized inside the cell, masking a transport-dependent growth defect.

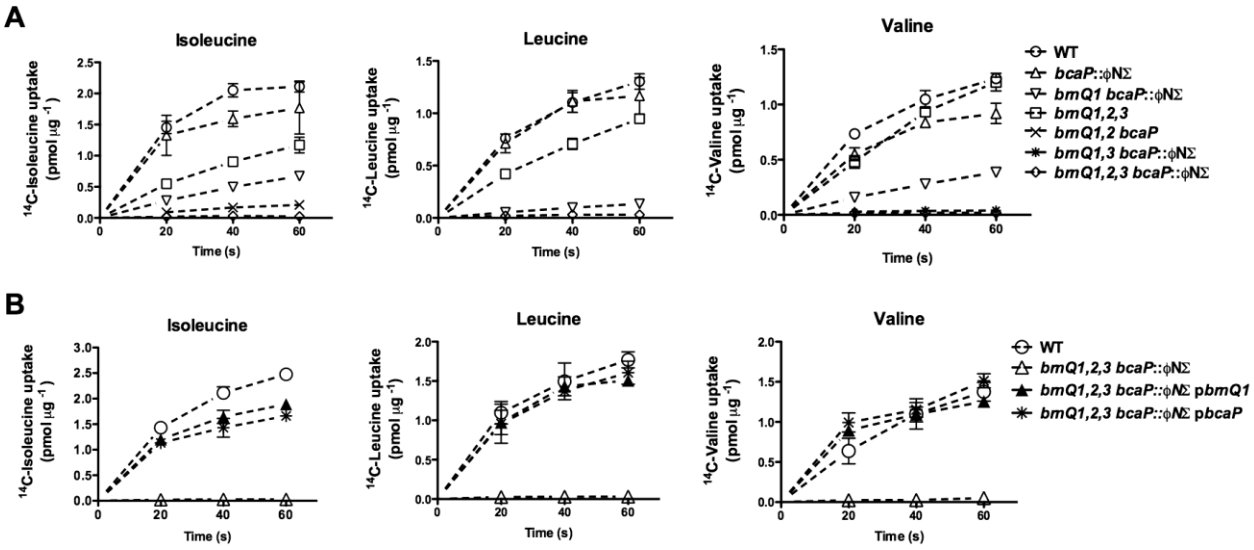

Figure 3.2 BcaP is a BCAA transporter

A,B) Cells were harvested from cultures grown to mid-late exponential phase in complete CDM, or CDM supplemented with 10 mg mL^{-1} tryptone in the case of the *brnQ1,brnQ2,brnQ3,bcaP* mutant. Transport of ^{14}C-labeled BCAA, as indicated, was measured at 20, 40 and 60 seconds and expressed relative to total protein. Data points shown are the mean of three biological replicates +/- SD.

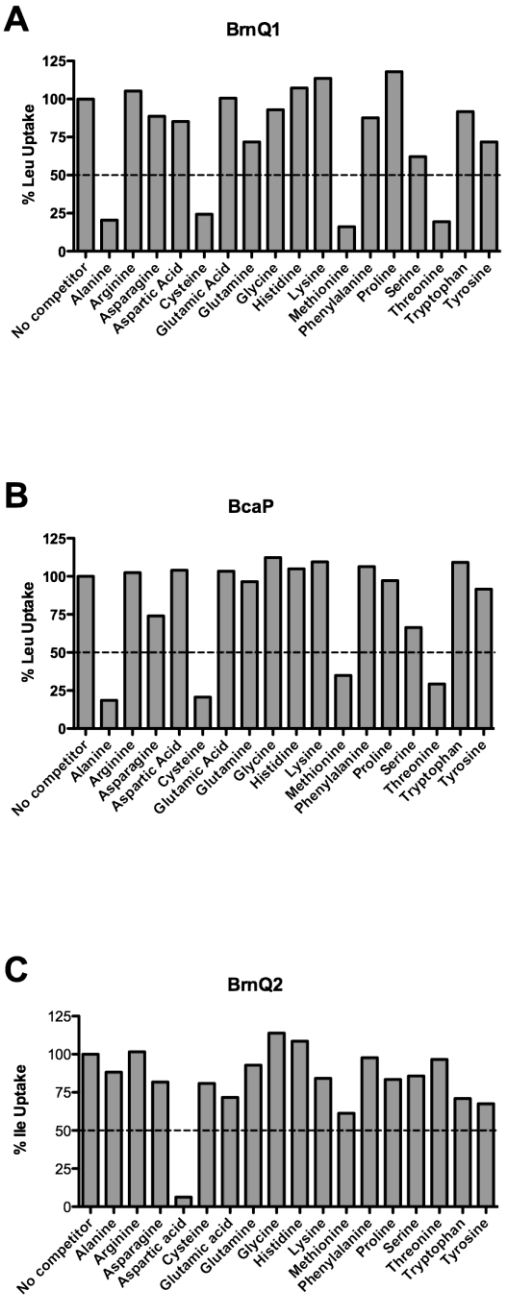

Figure 3.3 Non-BCAAs compete for BCAA transport.

The *brnQ1brnQ2brnQ3bcaP* mutant complemented with a vector expressing either A) *brnQ1* B) *bcaP* or C) *brnQ2* was grown to mid-late exponential phase in complete CDM. Cells were harvested and 1 μM of ^{14}C-labeled A,B) Leu or C) Ile was added in the presence of 1 mM of a non-labeled competing amino acid and uptake was measured at 60 seconds. Data are the percentage of Leu/Ile uptake in the absence of a competitor. Data are the mean of three biological replicates +/- SD.

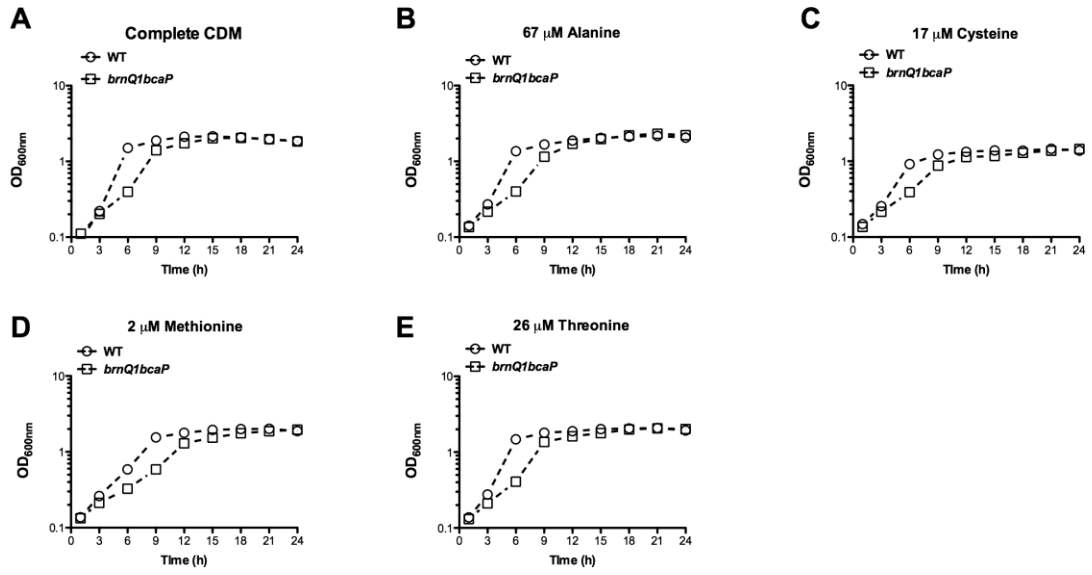

Figure 3.4 BrnQ1 and BcaP do not contribute to growth in media limited for non-BCAAs.

Strains were grown to mid-late exponential phase in complete CDM and sub-cultured to a starting OD_{600} of 0.0025 in either A) complete CDM (670 μM Ala; 170 μM Cys; 20 μM Met; 260 μM Threo) or B-E) CDM with a single amino acid reduced by 10-fold from its concentration in complete CDM, as indicated. Data are the mean of three biological replicates +/- SD.

3.3.2 BrnQ1 is the predominant BCAA transporter required for growth in media containing free amino acids

We previously demonstrated that despite encoding the BCAA biosynthetic operon, USA300 prefers Leu and Val acquisition over their biosynthesis during growth in CDM (Figure 2.9). We therefore determined the biological relevance of each of BcaP and BrnQ1 in *S. aureus* for growth in complete CDM, which contains excess concentrations of Leu and Val (684 μM). A mutation in *bcaP* did not impede growth of USA300 in complete CDM, whereas a *brnQ1* mutation resulted in a reduced growth rate (Figure 3.5A). Combining both mutations further reduced the growth rate (Figure 3.5A). The growth of the *brnQ1-bcaP* mutant did not require a compensatory mutation, as when these cells were plated for isolated colonies, picked and re-inoculated into the same medium, an identical growth phenotype was observed. Thus, either another transporter(s) or induction of BCAA synthesis is able to supply Leu and Val for growth. To minimize the effects of the existence of a potentially low-affinity transporter, we reduced the concentration of Leu or Val in the media by 10-fold. In Leu-limited CDM, the *bcaP* mutant exhibited a growth rate comparable to the WT strain, whereas the *brnQ1* mutant exhibited a growth lag of approximately 12 hr (Figure 3.5B). Addition of the *bcaP* mutation into the *brnQ1* mutant background extended the growth lag to ~14 hr (Figure 3.5B). The *bcaP* mutant also grew comparably to the WT strain in Val-limited CDM, whereas the *brnQ1* and *brnQ1-bcaP* mutants both exhibited a growth lag of ~ 18 hr. Growth of the *brnQ1-bcaP* mutant in all media could be restored to WT levels with the addition of multi-copy complementation vectors expressing either *brnQ1* or *bcaP* (Figure 3.5A-C). Together these data indicate that BrnQ1 is the predominant transporter required for Leu and Val acquisition during growth in media containing free amino acids, and BcaP contributes to Leu acquisition, but only noticeably in the absence of BrnQ1.

To test whether the growth of the mutants in Leu- and Val-limited conditions following an extended growth lag was the result of compensatory mutations, we plated the cultures that eventually grew in these conditions and, using these cells, repeated the growth experiment in the Leu- and Val-limited conditions. The *brnQ1* and *brnQ1-bcaP* mutants

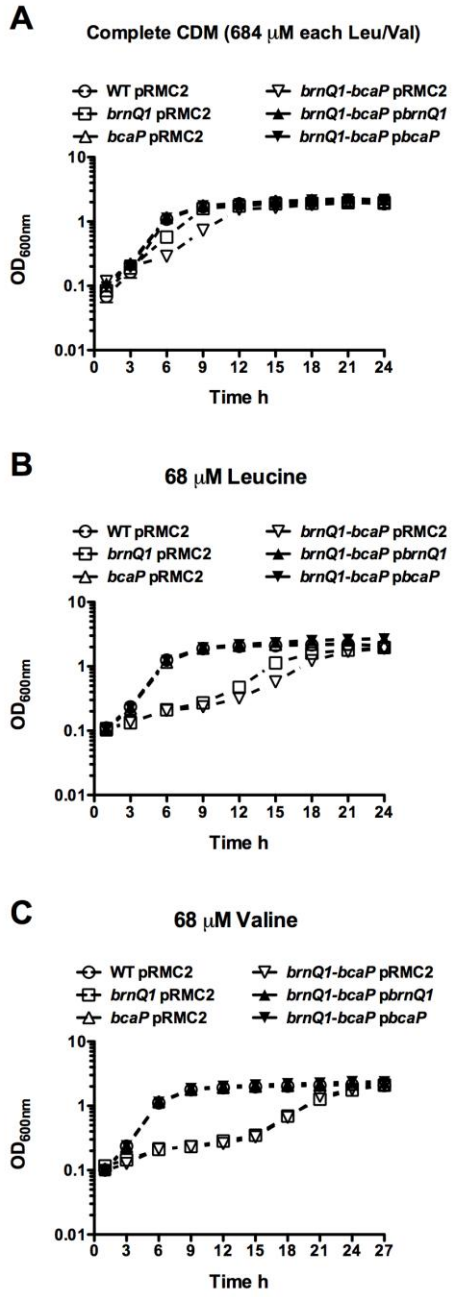

Figure 3.5 BrnQ1 is the predominant transporter required for growth in free amino acid containing medium.

Strains were grown in complete CDM to mid-late exponential phase and sub-cultured to a starting OD_{600} of 0.0025 in either A) complete CDM (684 μM each Leu and Val) or CDM where all amino acids were present at the same concentration as in complete CDM, except for alterations to the concentration of Leu (panel B) or Val (panel C). Data are the mean readable ODs of three biological replicates +/- SD. pRMC2 is the empty vector.

from the Leu-limited CDM exhibited the same growth lag upon sub-culture into Leu-limited CDM indicating that the eventual growth in this condition does not result in a genetically-heritable compensatory mutation. Interestingly, the *brnQ1* and *brnQ1-bcaP* mutant cultures from Val-limited CDM were able to grow without a growth lag upon sub-culture into the same condition and, in fact, both strains exhibited unimpeded growth in CDM with Val omitted (data not shown). These data suggest that the growth of the *brnQ1* and *brnQ1-bcaP* mutants in the Val-limited media resulted in a genetically-heritable compensatory mutation that likely results in Val biosynthesis. The identity of the mutation, or mutations, is currently under investigation.

3.3.3 Predominance of BrnQ1 is dictated by promoter strength

We next investigated the underlying basis for the predominant role of BrnQ1 over BcaP for growth in CDM. We determined the apparent K_M and V_{max} of each transporter using the quadruple mutant complemented with either *brnQ1* or *bcaP*. Both transporters had an apparent K_M for each BCAA in the low micromolar range (1-4 μM) (Table 3.3). V_{max} values further revealed that under the conditions where growth is assessed, both transporters are functioning at maximum velocity, and in fact, the V_{max} of BrnQ1 is lower than the V_{max} of BcaP (Table 3.3). These data indicate that, when over-expressed, both transporters function comparably, and therefore transporter kinetics are not likely sufficient to explain their different roles in growth.

We next turned to a *lux* promoter-reporter assay to determine the promoter activity of each of *brnQ1* and *bcaP* during growth, as we were not able to directly determine protein abundance of each transporter due to lack of antibodies. Promoter activities of both *brnQ1* and *bcaP* were higher in a *codY* mutant compared to the WT strain (Figure 3.6A,B), confirming they are both repressed by CodY. Notably, the promoter of *brnQ1* drove higher expression of the *lux* genes from the reporter plasmid than the *bcaP* promoter, which exhibited little/no *lux* expression (Figure 3.6A,B), suggesting that *brnQ1* expression is higher than *bcaP*. Since CodY promoters do not necessarily respond equally to changes in the level of CodY activity (29, 46), we also assessed promoter activity in the low Leu condition, and observed the same trends in promoter activity as in complete CDM (data not shown). Together, with the observation that over-expression of *bcaP* from a multi-copy plasmid restores growth of the

brnQ1-bcaP mutant, these data suggest that the predominant role of BrnQ1 is due to protein abundance and not differences in transport kinetics between the two transporters.

Table 3.3. Transporter kinetics

Transporter	Isoleucine		Leucine		Valine	
	K_M[a]	V_{max}[a]	K_M	V_{max}	K_M	V_{max}
BcaP	3.4 ±0.8	110 ±12	2.9 ±0.8	28 ±3	3.7 ±0.6	155 ± 12
BrnQ1	1.4 ±0.4	55 ±7	1.1 ±0.7	10 ±2	2.5 ±1.7	92 ± 39

[a]K_M units μM, V_{max} units pmol μg^{-1} min^{-1}; units of activity are expressed relative to total protein
Values are the average of three biological replicates ±SD.

3.3.4 BCFA content in *S. aureus* membranes is dependent on BrnQ1

Branched-chain fatty acids (BCFAs) are synthesized from the BCAAs and comprise 50-65% of total membrane fatty acids of *S. aureus* grown in rich media (11). Ile, Leu, and Val are used for the synthesis of anteiso-fatty acids, odd-chained iso-fatty acids, and even-chained iso-fatty acids respectively, with anteiso-fatty acids being the most abundant (11). Since *S. aureus* relies on transporters to acquire the BCAAs, we hypothesized that transporter mutants would lack the corresponding BCFAs in their membranes. When grown in complex media (TSB), BCFAs made up 43% of total fatty acids in the WT strain and 48% of total fatty acids in a *brnQ1-bcaP* mutant, with no difference in the proportion of anteiso- and iso-fatty acids between strains (Table 3.4). These data suggest that, independent of the free amino acid transporters BrnQ1 and BcaP, *S. aureus* is able to acquire BCAAs from peptide sources present in a rich medium. In contrast, when grown in CDM, the proportion of BCFAs increased to 76% of total fatty acids in the WT strain and 88% in the *brnQ1-bcaP* mutant, with drastic differences in the proportions of anteiso- and iso-fatty acids between the WT and mutant. Whereas BCFAs in the WT strain membrane included both anteiso- and iso-fatty acids, BCFAs in the *brnQ1-bcaP* mutant membrane were derived solely from anteiso-fatty acids, with no detection of Leu-derived odd-chain iso-fatty acids or Val-derived even-chain iso-fatty acids. The absence of Leu- and Val-derived BCFAs in this strain was the consequence of the *brnQ1* mutation since the *brnQ1* mutant alone lacked Leu- and Val-derived BCFAs, whereas the whereas the fatty acid profile of the *bcaP* mutant resembled that

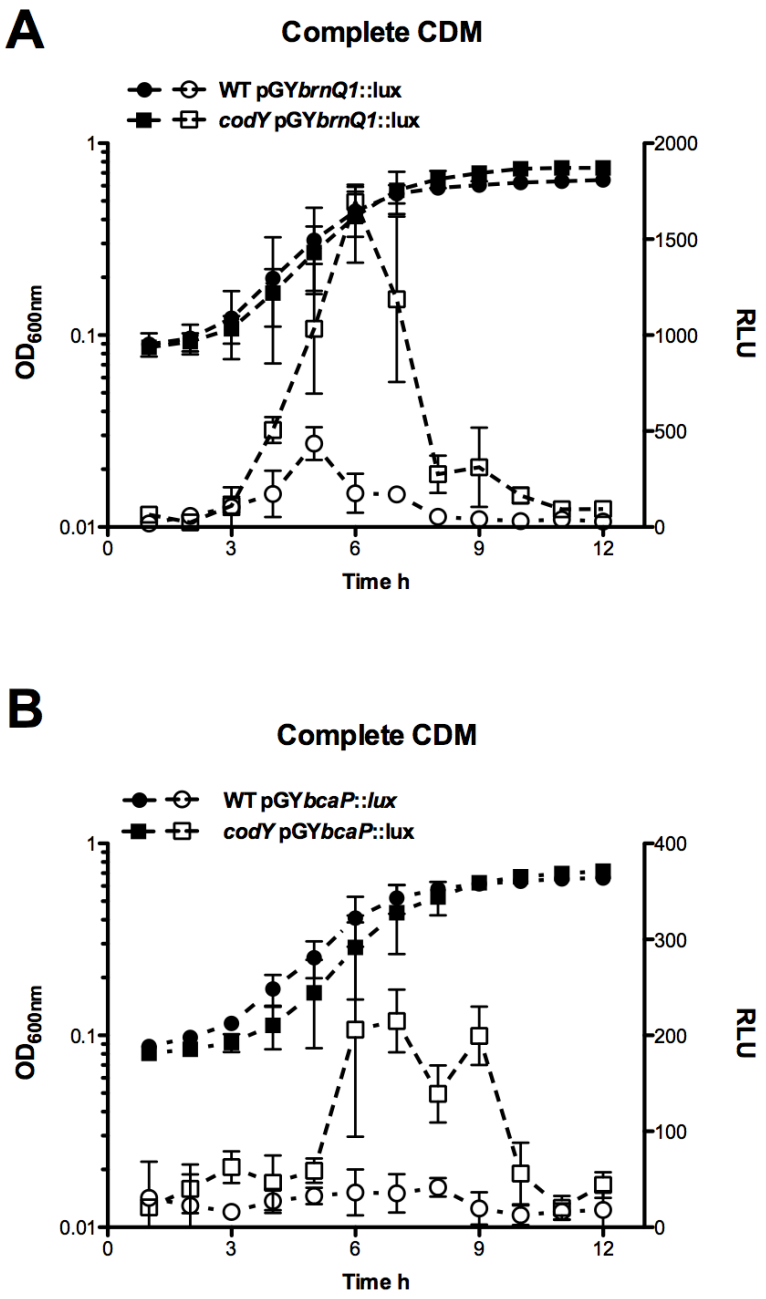

Figure 3.6 Promoter activity of *brnQ1*::*lux* is higher than *bcaP*::*lux*

Growth (OD_{600}; closed symbols) and relative luminescence units (RLU; open symbols) of WT USA300 and the *codY* mutant harboring either A) the pGY*brnQ1*::*lux* reporter vector or B) the pGY*bcaP*::*lux* reporter vector. Strains were grown to mid-late exponential phase in complete CDM and sub-cultured to an OD_{600} of 0.01 in complete CDM. Data are the mean +/- SD of three biological replicates.

of the WT strain (Table 3.4). Notably, the anteiso-fatty acid content was not affected in a strain lacking all Ile transporters (e.g. *brnQ1-brnQ2-bcaP*) in comparison to the WT strain (Table 3.4). Altogether, these data indicate that, in a defined, free-amino acid-containing medium, BrnQ1 is required for iso-fatty acid synthesis, and Ile synthesis is sufficient to maintain anteiso-fatty acid content in the absence of BCAA transport.

Table 3.4 Fatty acid profiles of *S. aureus* BCAA transporter mutants

Fatty Acid	**% (wt/wt) of total fatty acid[a]**						
	WT		*brnQ1brnQ2bcaP*		*brnQ1bcaP*	*brnQ1*	*bcaP*
	TSB	CDM	TSB	CDM	CDM	CDM	CDM
SCFAs	54 ±2	23 ±3	52 ±2	11 ±4	11 ±3	11 ±1	23 ±1
BCFAs	44 ±4	77 ±5	48 ±2	88 ±5	88 ±4	86 ±3	77 ±2
Anteiso	27 ±5	47 ±3	33 ±4	88 ±5	88 ±3	86 ±3	47 ±1
Iso (Odd)	8 ±2	24 ±2	6 ±2	<1.0	<1.0	<1.0	25 ±1
Iso (Even)	9 ±2	5 ±1	10 ±1	<1.0	<1.0	<1.0	5 ±1

[a]Values are the average percentage of fatty acids from three biological replicates ±SD. Fatty acids less than 1% are not reported.

3.3.5 BrnQ1 and BcaP are required for growth in CDM at temperatures below 37˚C

The adaptation of Gram-positive bacteria to growth at low temperature requires an increase in anteiso-fatty acid content to increase membrane fluidity (15, 16, 47). We therefore hypothesized that the *brnQ1* mutant would be better adapted than the WT strain to growth at lower temperatures since anteiso-fatty acids comprise 88% of membrane fatty acids in this strain. In contrast to this hypothesis, the *brnQ1* mutant exhibited growth attenuation relative to the WT strain that was even more evident when grown at 25˚C compared to 37˚C (Figure 3.7A). This temperature-dependent growth phenotype was even more so evident with the *brnQ1-bcaP* mutant (Figure 3.7A). Growth of the *brnQ1-bcaP* mutant could be restored with expression of either *brnQ1* or *bcaP* from a complementation vector (Figure 3.7B), or addition of peptide source to the growth medium (Figure 3.7C). Since BcaP contributes to this growth phenotype, but is not required for BCFA synthesis, we suspect this growth-attenuated phenotype is independent of membrane content, yet nonetheless noteworthy.

3.3.6 Staphyloxanthin abundance is increased in the membrane of the *brnQ1* mutant

In the course of our experiments as described above, we noticed that the cell pellet of the *brnQ1* mutant was brighter yellow than that of the WT strain when grown in CDM (Figure 3.8A). We therefore quantified the abundance of the pigmented carotenoid staphyloxanthin in the membrane of the WT strain and *brnQ1* and *bcaP* mutants. Indeed, the membrane of the *brnQ1* mutant contained more staphyloxanthin than either the WT strain or the *bcaP* mutant, and this increase could be restored to WT levels upon complementation (Figure 3.8B,C). Staphyloxanthin is a polar carotenoid that increases membrane rigidity (48), thus its presence likely helps to counterbalance the high proportion of membrane fluidity-increasing anteiso-fatty acids in the *brnQ1* mutant membrane (16). To determine if the presence of staphyloxanthin masked the growth benefit of the *brnQ1* mutant at low temperatures, we obtained a mutant incapable of producing staphyloxanthin (*crtM*) and mobilized this mutation into the *brnQ1* mutant background to assess the ability of this strain to grow at low temperatures. Staphyloxanthin did not alter growth of the *brnQ1* mutant at 25˚C (Figure 3.8D), supporting our conclusion that this phenotype is not related to membrane BCFAs. Staphyloxanthin is considered a virulence factor in *S. aureus* due to its antioxidant properties which protect cells against oxidative stress, and its proposed role in resisting antimicrobial peptides (42, 49, 50). We therefore investigated whether the *brnQ1* mutant was more resistant to these stressors. We found that the *brnQ1* mutant was equally susceptible, compared to the WT strain, to H_2O_2 (data not shown). The *brnQ1* mutant and WT strain were also equally susceptible to the membrane targeting antibiotics daptomycin and polymyxin B, with MIC values in the range of those typically reported for *S. aureus* (Figure 3.8E,F) (51). Altogether, these data indicate that although staphyloxanthin is more abundant in the *brnQ1* mutant membrane, it is not sufficiently high to impart enhanced resistance to membrane stressors.

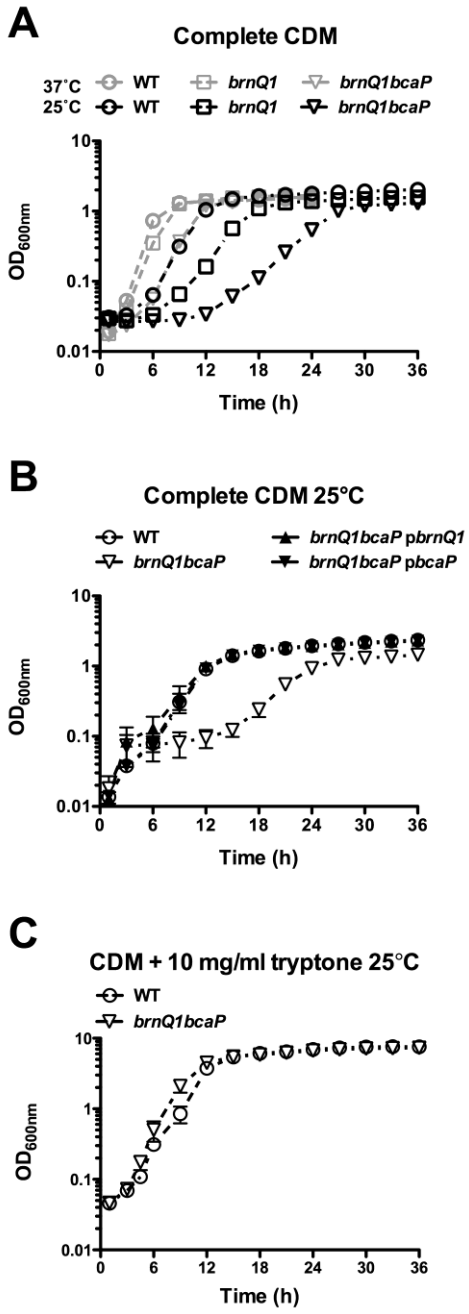

Figure 3.7 BCAA acquisition is required for growth in CDM at 25˚C

A,B) Strains were grown in complete CDM to mid-late exponential phase, sub-cultured to a starting OD_{600} of 0.0025 in complete CDM and incubated at either 25˚C or 37˚C. C) Strains were grown in CDM supplemented with 10 mg mL^{-1} of tryptone to mid-late exponential phase and sub-cultured to a starting OD_{600} of 0.0025 in CDM 10 mg mL^{-1} of tryptone. Data are the mean readable ODs of three biological replicates +/- SD.

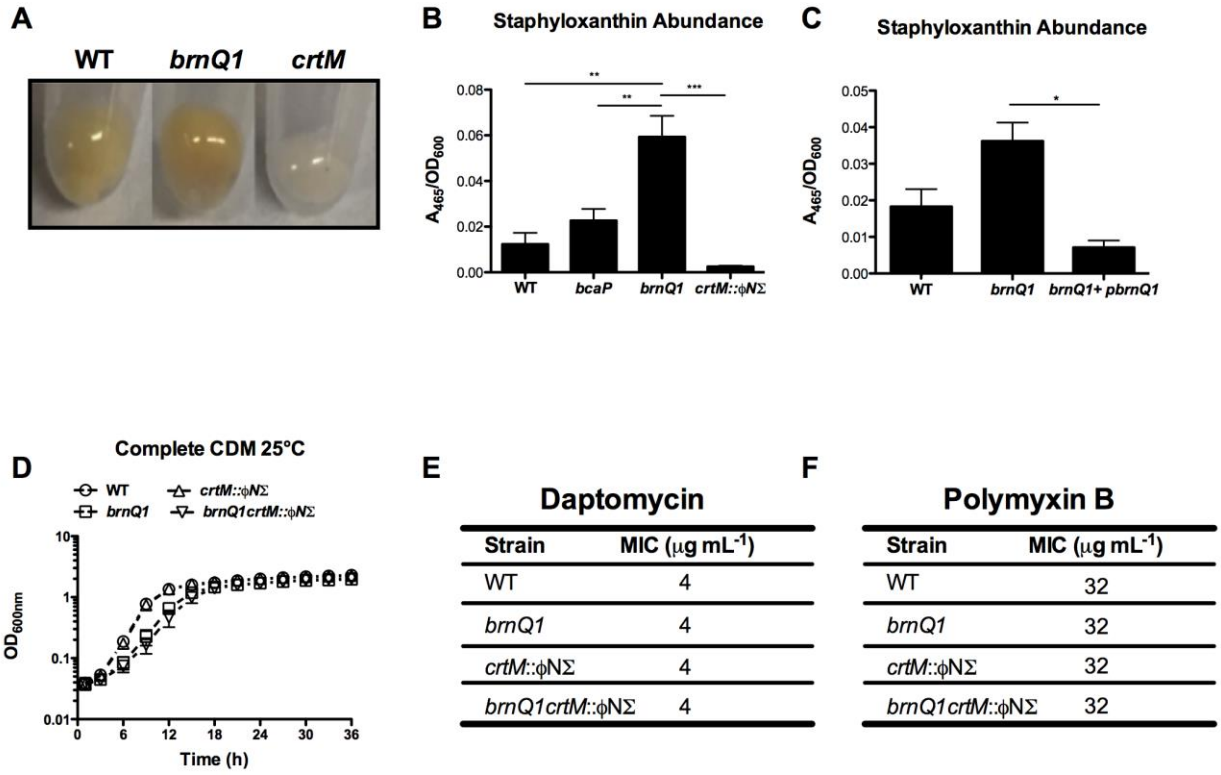

Figure 3.8 Staphyloxanthin abundance is increased in the *brnQ1* mutant membrane

A) Cell pellets of cultures grown overnight in complete CDM. B,C) Staphyloxanthin was extracted using warm methanol from the cell pellets of strains grown to log-phase in complete CDM. Staphyloxanthin was quantified at an absorbance of 465 and expressed relative to the OD_{600} of log-phase cultures. Data are the mean of three biological replicates +/- SD and were analyzed using one-way ANOVA with the Tukey post test. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. D) Cells were grown in complete CDM to mid-late exponential phase, sub-cultured to a starting OD_{600} of 0.0025 in complete CDM and incubated at 25˚C. Data are the mean of readable ODs of three biological replicates +/- SD. E,F) Strains were grown in complete CDM to mid-late exponential phase and sub-cultured to a starting OD_{600} of 0.0025 in complete CDM containing serial dilutions of each antibiotics. The MIC was determined by examining visible growth of two biological replicates after 24 h.

3.3.7 BrnQ1 and BcaP are required for *S. aureus* infection and colonization

High throughput studies have identified amino acid synthesis and transport genes as a major category of genes required for *S. aureus* virulence (8, 9). These genes are also some of the most up-regulated during growth in human nasal secretions (6). We therefore assessed the contribution of Leu/Val transport to USA300 fitness *in vivo* using a murine systemic infection model and nasal colonization model. Mice infected systemically with either the WT strain or the single mutants lost approximately 20% of their body weight by day 4 of infection and exhibited reduced activity and grooming. In contrast, mice infected with the *brnQ1-bcaP* mutant lost on average approximately 5% of their body weight and appeared active and well groomed (Figure 3.9A). Despite these apparent differences in disease severity, we did not observe statistically significant differences in kidney bacterial burden between mice in any of these groups (data not shown). We did, however, observe reduced bacterial burden of the *brnQ1* mutant and the *brnQ1-bcaP* mutant in the hearts (Figure 3.9B) and there appeared to be a trend towards decreased CFUs in the liver for the *brnQ1*, *bcaP*, and *brnQ1-bcaP* mutants (Figure 3.9C).

Using a nasal colonization model, we found that all mice inoculated with either the WT strain or the *brnQ1* or *bcaP* mutants remained colonized on day 7, with comparable CFUs recovered. In contrast, the *brnQ1-bcaP* mutant could not be recovered on day 7 from 27% (8/30) of mice (Figure 3.10). These data suggest that both BrnQ1 and BcaP are conditionally required for fitness of *S. aureus* during nasal colonization.

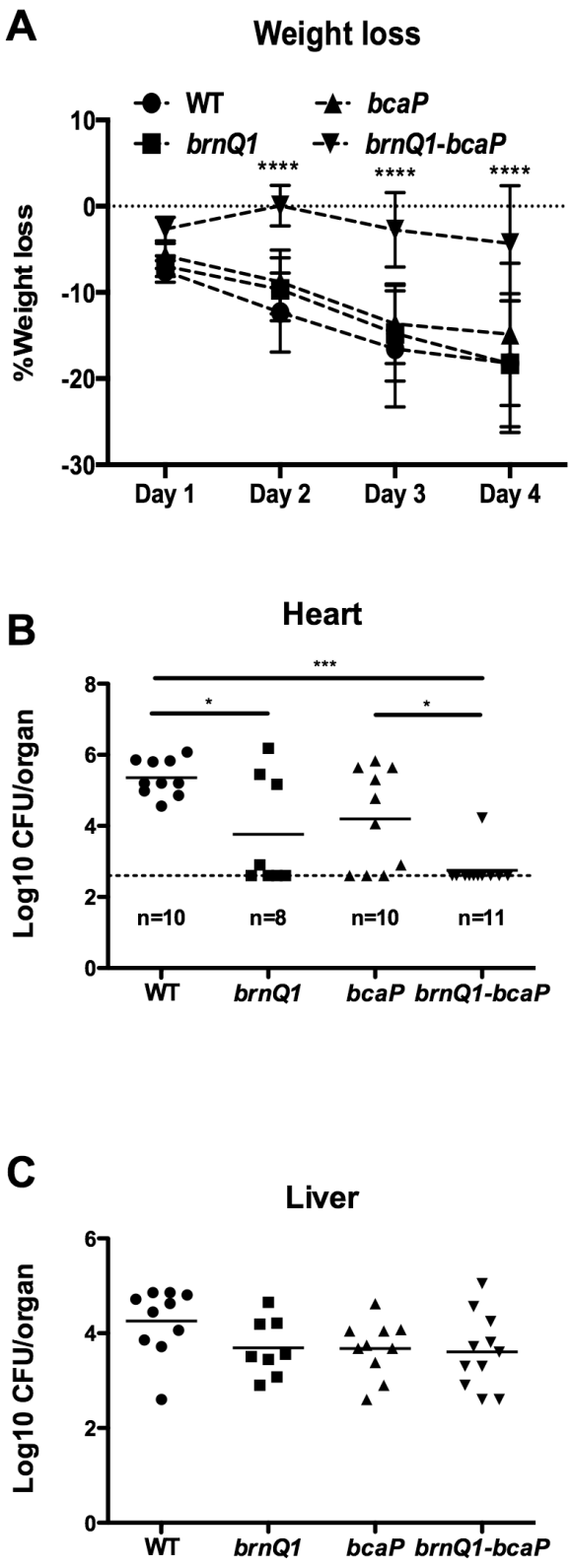

Figure 3.9 BCAA acquisition is required for full virulence of *S. aureus* in a murine systemic infection model

Balb/c mice were infected with 6-7 x 10^6 CFU of the WT strain (n=10), *brnQ1* mutant (n = 8), *bcaP* mutant (n = 10), or *brnQ1-bcaP* mutant (n=11) via tail vein injection. A) Weight loss was monitored every 24 hr post-infection. Data were analyzed for significance relative to the WT strain using two-way ANOVA with the Dunnett's post test. Mice were sacrificed on day four of infection and B) hearts and C) livers were harvested and homogenized, and dilutions were plated for CFU. The dotted line in panel B indicates the limit of detection. Log transformed data were analyzed using one-way ANOVA with the Tukey post test. Data in panel C are not significant. * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

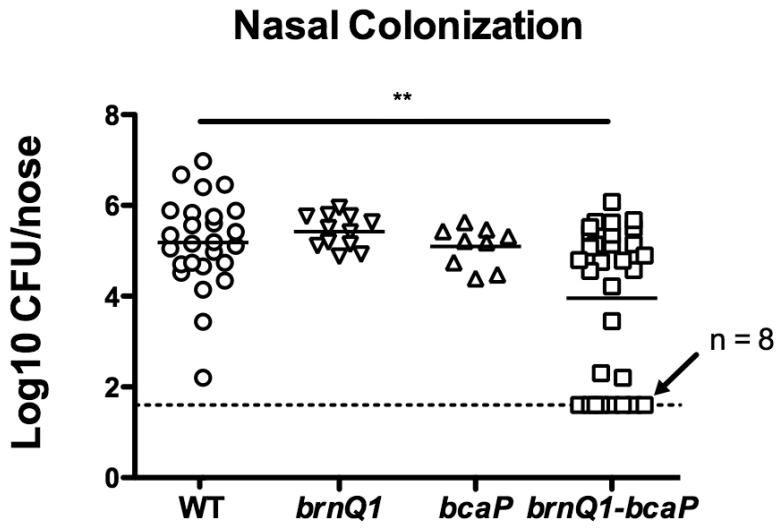

Figure 3.10 BCAA acquisition is required for *S. aureus* murine nasal colonization

C57BL/6 mice were treated with 2 mg mL^{-1} streptomycin sulfate in the drinking water 24 hr prior to inoculation to remove the microbiota. Mice were intranasally inoculated with 1 x 10^8 CFU of the streptomycin resistant WT strain (n=25), *brnQ1* mutant (n=12), *bcaP* mutant (n=9), or *brnQ1-bcaP* mutant (n=30) under anesthesia. On day seven post-inoculation, mice were sacrificed and snouts were excised, homogenized, and plated for CFU. The dotted line indicates the limit of detection. Log transformed data were analyzed using one-way ANOVA with the Tukey post test. ** $P \leq 0.01$.

3.4 Discussion

S. aureus requires BCAAs for several physiological processes, yet it does not readily synthesize Leu or Val and rather relies on the acquisition of these nutrients to support its metabolism (See Chapter 2). We previously identified BrnQ1 as a Leu/Val transporter, however, a *brnQ1* mutation was not sufficient to abolish Leu/Val transport (Figure 2.3). Here, we identify BcaP as an additional Leu/Val transporter in *S. aureus*, which, when mutated in combination with a *brnQ1* mutation, results in abrogation of Leu transport and almost a complete loss of Val transport. Identification of this additional, BcaP-dependent mechanism of Leu/Val transport enabled us to characterize the consequence of Leu/Val limitation on *S. aureus* physiology, and discern which transporters are required for growth, membrane BCFA synthesis, and virulence. Our data indicate that although BrnQ1 is the dominant transporter for supplying Leu and Val for growth and BCFA synthesis *in vitro*, both BrnQ1 and BcaP contribute to *S. aureus* infection and colonization.

We attribute the minimal contribution of BcaP to *S. aureus* growth and BCFA synthesis *in vitro* to its lower expression relative to BrnQ1. Although this was measured indirectly, this is corroborated by the observation that over-expression of *bcaP* in the *brnQ1-bcaP* mutant is sufficient to restore growth to WT levels. This finding contrasts BCAA transport in *L. lactis* and *B. subtilis,* where BcaP serves as the more efficient transporter (32, 34). It should be cautioned that the relative importance of BrnQ1 and BcaP in USA300 should not be generalized across all *S. aureus* strains, since when expressed to similar levels off of a multi-copy vector, BrnQ1 and BcaP in *S. aureus* exhibit comparable K_M and V_{max} values. Thus any differences in transporter gene expression across strains would likely affect the relative importance of BrnQ1 and BcaP.

Despite a contribution of BcaP to growth in CDM in the absence of BrnQ1, BcaP played no role in supplying Leu and Val for BCFA biosynthesis. Instead, the cells replaced iso-fatty acids with anteiso-fatty acids, presumably supported by Ile synthesis. This compensatory mechanism is unique to *S. aureus*, as other Gram-positive bacteria, such as *Bacillus subtilis* and *Listeria monocytogenes*, are able to synthesize all three BCAAs to maintain BCFA content in the absence of exogenous BCAAs (15, 47, 52). Although the mechanism that regulates this is currently unknown, it can be speculated that this ensures Leu and Val are

conserved for protein synthesis, and might be one explanation as to why *S. aureus* has retained Ile synthesis.

This study highlights a paradox in *S. aureus*, in which even upon Leu and Val starvation, endogenous synthesis from the intact BCAA biosynthetic operon does not readily compensate. Our data suggest that BCAA biosynthesis is eventually de-repressed following an extended growth lag of the transporter mutants (14-18 hr) when either Leu or Val are limited, a mechanism that is likely to involve known regulators of BCAA biosynthesis, including CodY, CcpA, or YeaZ (25, 29, 45, 53, 54). Intriguingly, BCAA biosynthesis in the transporter mutants grown in Val-limited conditions requires a compensatory mutation, whereas a Leu-limited growth medium did not result in genetically-heritable suppressor mutations. We had previously made a similar observation with WT USA300 grown in media with either Val or Leu omitted; that is growth in media with Val omitted required a compensatory mutation, whereas growth in media with Leu omitted did not (See Section 2.4). We are currently investigating the regulation of BCAA synthesis and why a compensatory mutation is required for Val but not Leu synthesis.

Although BcaP plays a secondary role in growth *in vitro*, it has an additive function with BrnQ1 *in vivo*. BcaP function might have been underappreciated *in vitro* due to differences in growth conditions and/or gene expression. For example, the activation state of CodY *in vivo* might differ from that *in vitro*, and it is possible that *brnQ1* and *bcaP* expression respond differently to the activation state of CodY (29, 46). Although both are CodY regulated, fine-tuning of expression could occur, and as such the requirement of each transporter would depend on the availability of CodY effectors; BCAAs and GTP. Mechanisms of fine-tuning BCAA transporter expression in *B. subtilis* include multiple upstream CodY binding sites (55) and direct and indirect CodY regulation (56). Additionally, the affinity of CodY for each promoter could determine the timing of expression in relation to nutrient availability (57). A more detailed characterization of the regulation of these genes might provide insight as to how these genes are regulated *in vivo*.

The systemic infection model revealed that infection with a strain with mutations in both *brnQ1* and *bcaP* not only resulted in reduced bacterial burden in the heart, but also in significantly less weight loss and disease severity. The ability of this strain to reach such a

high bacterial burden in the kidneys (e.g. 10^8 CFU, data not shown) but not cause signs of disease could be the result of altered toxin secretion *in vivo*, and will need to be explored in future studies. The ability of the *brnQ1* and *brnQ1-bcaP* mutants to replicate efficiently in some organs (e.g. livers and kidneys) and not others (e.g. heart) could reflect differences in nutrient availability (e.g., peptides sources and/or host-derived fatty acids). Indeed, *S. aureus* encodes a di/tri-peptide permease (*dtpT*) and several oligo-peptide permeases that might supply BCAAs *in vivo* (58), although the requirement for oligopeptide transport for *S. aureus* virulence is not known. Moreover, *S. aureus* is able to incorporate host-derived fatty acids in its membrane (59) and, when grown in serum, BCFAs comprise less than 40% of membrane fatty acids in USA300 (60). Finally, although both *brnQ1* and *bcaP* are up-regulated in human serum and blood, so are the genes for BCAA biosynthesis (10). Thus it is possible that Leu and Val are synthesized *in vivo*.

Our data suggest a dichotomy in the ability of the *brnQ1-bcaP* mutant to colonize murine nares, as the *brnQ1-bcaP* mutant was not recovered from several mice on day 7, yet in some mice was recovered at CFUs comparable to the WT strain. At this point it is unclear whether this is a result of the inability of the *brnQ1-bcaP* mutant to establish colonization in some circumstances, or perhaps it is cleared at a more rapid rate, albeit asynchronously, than the WT strain. We hypothesize the former; cells must maintain a threshold level of BCAAs to support growth in this environment, and BCAA transport is essential to do so. Free amino acid transport might be critical in this environment due to limited availability of peptide sources of BCAAs. The finding by *Krismer et al.,* that an *S. aureus* strain incapable of synthesizing Met has a reduced capacity for nasal colonization suggests that peptides are not sufficient to complement amino acid deficiency in this environment (6). With limited availability of peptides, the transporter mutant must rely on BCAA biosynthesis for growth. Although the BCAA biosynthesis genes are up-regulated when *S. aureus* is grown in media that mimics human nasal secretions (6), the biosynthetic enzymes require iron (61), which is likely limited in this environment (62). Thus, in the absence of transport, cells will only be viable if there is sufficient iron and/or carbon to support BCAA synthesis. The variability in nutrient levels across mice might be sufficient to create this threshold for growth. More studies will be required to test this hypothesis.

Altogether, this study provides a detailed characterization of the role of BCAA transport in *S. aureus* general physiology, and lays a foundation for understanding the link between BCAA metabolism and virulence in *S. aureus*.

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Chapter 4

4 Regulators of BCAA synthesis in *S. aureus*

4.1 Introduction

Staphylococcus aureus is a serious human pathogen capable of causing infections that range from mild skin and soft tissue infections, to severe infections of the bone, muscle, heart and lung (1–4). To survive and thrive in such diverse host environments, *S. aureus* must maintain sufficient levels of metabolites and co-factors to support virulence determinant production and replication (5, 6). The branched-chain amino acids (BCAAs; Ile, Leu, Val) represent an important group of nutrients for *S. aureus* metabolism and virulence, as they are required for protein synthesis and synthesis of membrane branched-chain fatty acids (BCFAs), which are important for *S. aureus* environmental adaptation. Indeed, both BCAA biosynthesis (7–11) and transport (12–15) have been linked to promoting the virulence of pathogens in various host environments. In addition to their nutritional importance, the BCAAs are key regulatory molecules in low GC Gram-positive bacteria, as they are effectors of the global transcriptional regulator CodY. CodY coordinates expression of nutrient scavenging and synthesis systems, as well as virulence genes, upon depletion of both BCAAs and GTP (16–22). The requirement of BCAAs for both *S. aureus* replication and niche adaptation necessitates that it either synthesize these nutrients or acquire them from the environment.

Bacteria acquire BCAAs via dedicated active transporters, including BrnQ (Gram-negative and –positive bacteria), BcaP (Gram-positive bacteria), and the high affinity ATP-Binding Cassette (ABC) transporter LIV-I (Gram-negative bacteria) (23). *S. aureus* encodes three BrnQ homologs (BrnQ1, BrnQ2, BrnQ3), and BcaP. BrnQ1 and BcaP transport all three BCAAs, with BrnQ1 playing a predominant role, and BrnQ2 is an Ile-dedicated transporter (Chapters 2 and 3). Little/no BCAA transport function is associated with BrnQ3 (Chapter 2). Despite encoding the BCAA biosynthetic operon, *S. aureus* relies on the acquisition of BCAAs, most importantly Leu and Val, for rapid growth in media with excess or limiting concentrations of BCAAs, indicating that BCAA biosynthesis is typically repressed (Chapters 2 and 3). Paradoxically, biosynthesis remains repressed even in the absence of an exogenous source of Leu or Val, with growth of *S. aureus* observed only after a prolonged

period of 24 h and 48 h respectively (Figure 4.1), which is why previous studies have been misled to conclude that *S. aureus* is auxotrophic for Leu and Val (24, 25). The molecular explanation for this in *S. aureus* has remained elusive.

Both Gram-positive and Gram-negative bacteria repress BCAA biosynthesis when intracellular levels are sufficient to support growth. In the Gram-negative organisms, *E. coli* and *S. typhimirium*, this is regulated by attenuation, which couples translation of a BCAA-rich peptide upstream of the biosynthetic genes with transcriptional read-through (26–31). In Gram-positives, this is mediated by CodY-dependent repression of the biosynthetic genes (17, 18, 32–35). Additional layers of regulation have also been described in the Gram-positive bacterium *B. subtilis* where, in addition to CodY regulation, the *ilv-leu* operon is regulated by global regulators that coordinate expression of the operon in response to carbon (CcpA) and nitrogen (TnrA) (36). Additional fine-turning of *ilv-leu* expression is mediated by a Leu-responsive T-box riboswitch (36–39), as well as mRNA processing (40).

The BCAA biosynthetic genes in *S. aureus*, encoded by the *ilvDBNleuABCDilvA* operon and *ilvE*, are similarly repressed by CodY, which binds to two regions upstream of *ilvD* proximal to the transcriptional start site and two regions within the operon, preceding *ilvC* and *leuC* (Figure 4.1) (16–18). Repression is also mediated by the essential genes *gcp* and *yeaZ* through an unknown mechanism (41, 42). Yet even with this knowledge, the growth phenotype of *S. aureus* in the absence of Leu or Val remains unaccountable. We therefore investigated the mechanisms governing these phenotypes in *S. aureus* to resolve this and to identify the signals required to induce synthesis. We identify a CodY-dependent and -independent mechanism of repression and identify the corresponding metabolic cues regulating each mechanism, therefore revealing how *S. aureus* regulates its preference for exogenous BCAAs and the environments where endogenous synthesis is induced.

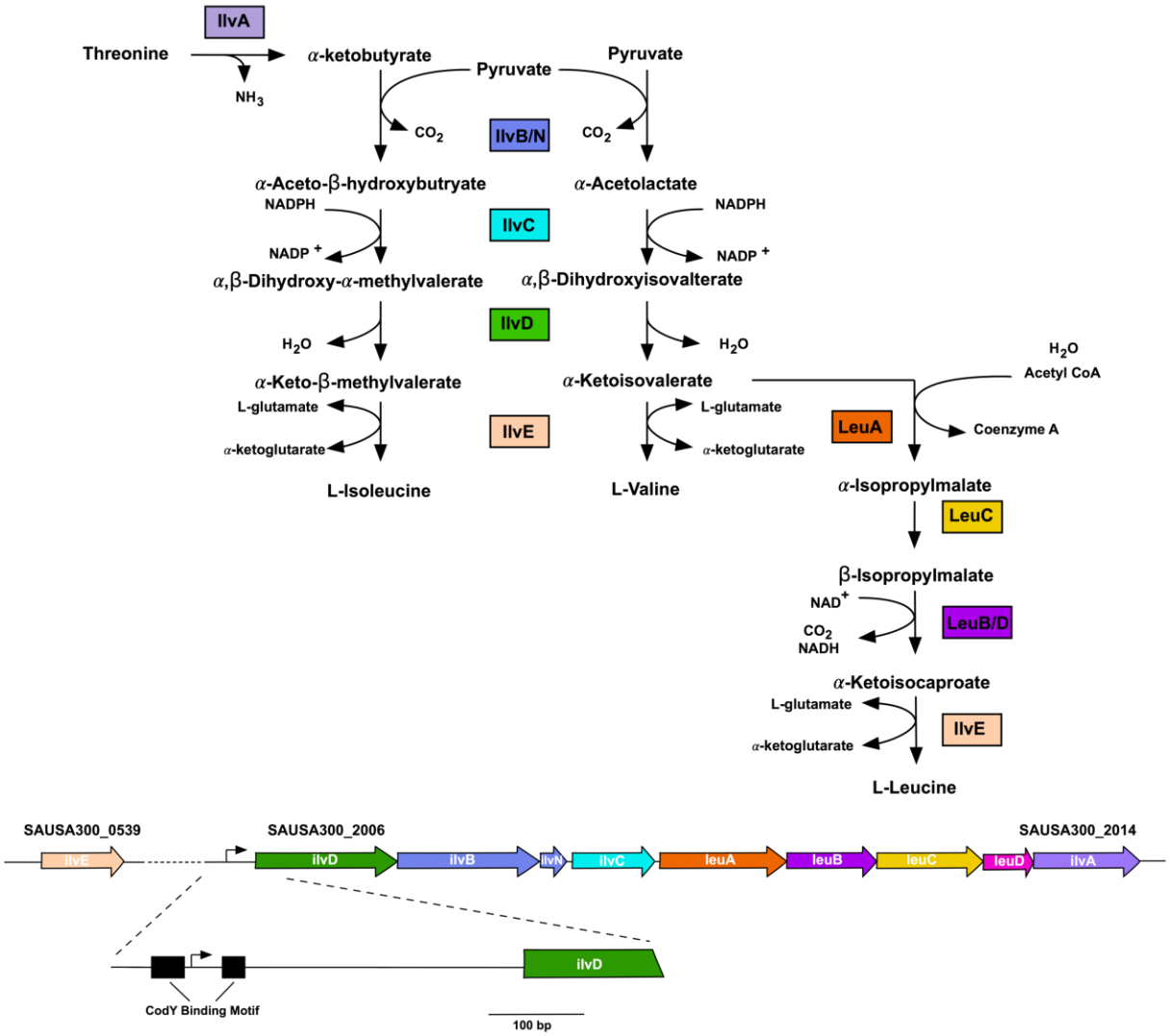


Figure 4.1 Organization of the *ilv-leu* operon in *S. aureus.*

Biosynthetic pathway of BCAA biosynthesis in *S. aureus* and the genomic context of the biosynthetic genes in the USA300 FPR3757 genome.

4.2 Materials and Methods

4.2.1 Growth conditions and plasmid construction

All strains and plasmids used in this study are described in Table 4.1. Methicillin-resistant *S. aureus* (MRSA) isolated pulsed-field gel electrophoresis type USA300 LAC that has been cured of the erythromycin resistance plasmid was used in all experiments as the wild-type (WT) strain. *S. aureus* strains were grown in either tryptic soy broth (TSB) (EMD Millipore, Billerica, MA) or in a chemically defined medium (CDM), described previously (See Section 2.2.1). Final concentrations of Ile, Leu and Val in complete CDM were 228 μM, 684 μM, and 684 μM, respectively. Final concentrations were adjusted to 10% of their concentration in complete CDM in some experiments, as indicated. For growth experiments in TSB, *S. aureus* strains were pre-cultured in TSB until mid-exponential phase was reached, and then sub-cultured into fresh TSB to a starting OD_{600} of 0.01. For growth experiments in CDM, *S. aureus* strains were pre-cultured in CDM until mid-exponential phase was reached, and then sub-cultured into fresh CDM to a starting OD_{600} of 0.05 in either complete CDM or CDM where BCAA concentrations were limited or omitted, as indicated. Growth curves were performed in 100-well plates containing 200 μL/well of media and were read using the Bioscreen C visible spectrophotometer (Growth Curves USA; Piscataway, NJ). End point growth assays were performed in tubes with a 7:1 tube:volume ratio. All growth experiments were performed at 37˚C with shaking. Growth media were supplemented with chloramphenicol (10 μg mL^{-1}), ampicillin (100 μg mL^{-1}), or erythromycin (3 μg mL^{-1}), where required.

4.2.2 Mutagenesis and construction of plasmids

Deletion of *bcaP* was constructed using the pKOR-1 plasmid as described in Chapter 2. Primer sequences were based on the published USA300 FPR3757 genome and are displayed in Table 4.2. The *bcaP* deletion was introduced into the markerless *brnQ1* deletion mutant, described previously (See Section 2.2.2). The pGY*lux* vectors were constructed using primers described in Table 4.2. *lux* plasmids were further used as templates for site-directed mutagenesis, using primers described in Table 4.2. Briefly, PCR reactions containing the Phusion ® High-Fidelity DNA Polymerase (ThermoFisher, Waltham, MA) were set up such that half of the reaction mixture contained the forward primer and the remaining half

contained the reverse primer. These reactions proceeded for 3 cycles of 98˚C for 10 s, 60˚C for 30 s, and 72˚C for 12 min. After 3 cycles, the forward and reverse primer reactions were mixed together and the reactions proceeded for an additional 17 cycles. Plasmids were treated with DpnI (New England Biolabs, Ipswich MA) for 1 hr at 37˚C and were then transformed into *E. coli* DH5α. Mutations were confirmed by PCR. All plasmids were first constructed in *E. coli* DH5α and subsequently electroporated into the restriction-defective *S. aureus* strain, RN4220, prior to electroporation into the desired strain.

4.2.3 Selection for Val^S mutants and whole genome sequencing

To select for genetic mutations that permit adaptation to growth in media lacking Val, twelve independent colonies of WT *S. aureus* were grown in complete CDM to mid-exponential phase and sub-cultured into CDM lacking Val. Cells that grew were harvested and plated onto TSB agar and grown overnight at 37˚C. Isolated colonies were grown in complete CDM to mid-exponential phase and sub-cultured into CDM lacking Val to confirm the occurrence of a heritable mutation. Genomic DNA was isolated from all twelve mutants, referred to as Val^S-mutants, as well as from two biological replicates of our laboratory WT USA300, using the Invitrogen PureLink™ Genomic DNA Preparation Kit (ThermoFisher Scientific, Boston MA) per the manufacturer's instructions. Primers used for the targeting sequencing of the region upstream of *ilvD* and the *codY* gene are listed in Table 4.2. Samples were sent to the London Regional Genomics Center for sequencing on the MiSeq platform. Libraries were prepared using the Nextera™ XT DNA Library Preparation kit (Illumina, San Diego, CA). 150 bp reads were mapped to the USA300 FPR3757 (NC_007793.1) genome using the BWA-MEM aligner (43) and variants were determined using SAMtools (44).

Table 4.1 Strains and plasmids

Strain or Plasmid	Description[a]	Source or reference
Strains		
S. aureus		
USA300	USA300 LAC cured of antibiotic resistance plasmid	(45)
RN4220	r_K^- m_K^+; capable of accepting foreign DNA	(46)
H3001	USA300 *codY*::ϕNΣ; Em^R	(47)
H2568	USA300 Δ*brnQ1*	Chapter 2
H2563	USA300 Δ*brnQ2*	Chapter 2
H3386	USA300 Δ*bcaP*	This study
H3584	USA300 Δ*brnQ1*Δ*bcaP*	This study
E. coli		
DH5α	F^- ϕ80d*lacZ*ΔM15 *recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^-) *supE44 relA1 deoR* Δ(*lacZyA-argF)U169 phoA*	Promega
Plasmids		
pRMC2	Anhydrotetracycline-inducible expression vector; Ap^R in *E. coli*; Cm^r in *S. aureus*	(48)
p*codY*	pRMC2 containing *codY*; Cm^R	This study
pGY*lux*	Vector harboring promoterless *luxABCDE* operon; Cm^R	(49)
pGY*ilvD*^{WT}::*lux*	*Lux* reporter vector with *ilvD* promoter from WT USA300; Cm^R	This study
pGY*ilvD*^{ValS1}::*lux*	*Lux* reporter vector with *ilvD* promoter mutated to contain the Val^S-1 SNP; Cm^R	This study
pGY*ilvD*^{ValS7}::*lux*	*Lux* reporter vector with *ilvD* promoter mutated to contain the Val^S-7 SNP; Cm^R	This study
pGY*ilvD*^{ValS9}::*lux*	*Lux* reporter vector with *ilvD* promoter mutated to contain the Val^S-9 SNP; Cm^R	This study
pGY*ilvD*^{P}::*lux*	*Lux* reporter vector with only the CodY binding motifs in the *ilvD* promoter; Cm^R	This study
pGY*ilvD*^{C}::*lux*	*Lux* reporter vector with the *ilvD* promoter from WT USA300; Cm^R	This study

[a]Abbreviations: Em^R, Ap^R, Cm^R, designate resistance to erythromycin, ampicillin and chloramphenicol respectively.

Table 4.2 Oligonucleotides used in this study.

Oligonucleotides [a]	
bcaP Ups F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAGTCTTCGTATTCACCTGC
bcaP Ups R	CTTCCCATAAACTTTCCTCC
	For generating upstream arm for *bcaP* deletion
bcaP Dwn F	5' /Phos/ ACGTAGCTGAATACCACCC
bcaP Dwn R	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTACCTGCTGACGAAGTAG
	For generating downstream arm for *bcaP* deletion
$ilvD^{C}$ F	GATCCCCGGGACCTGCTCCTAAATCTCCG
$ilvD^{C}$ R	GATCGTCGACACTTCTTGCTGGTGCTTGG
	For cloning *ilvD* 5'UTR into pGY*lux*
$ilvD^{P}$ F	GATCCCCGGGGTACGTCTTACACCAAG
$ilvD^{P}$R	GATCGTCGACAGTTGTCGGTTGATGTTC
	For cloning partial *ilvD* 5'UTR into pGY*lux*
$ilvD^{ValS-1}$ F	CAA ATA TTA TTA TTT TAT aAT ACT CTT TAG GAC TCG
$ilvD^{ValS-1}$ R	CGA GTC CTA AAG AGT ATt ATA AAA TAA TAA TAT TTG
	For site directed mutagenesis of pGY*lux*::*ilvD*
$ilvD^{ValS-7}$ F	CTA AAC GCT TTA AGT CaT ATT TCT GTT TGA ATG
$ilvD^{ValS-7}$ R	CAT TCA AAC AGA AAT AtG ACT TAA AGC GTT TAG
	For site directed mutagenesis of pGY*lux*::*ilvD*
$ilvD^{ValS-9}$ F	CTA AAC GCT TTA AGc CCT ATT TCT GTT TG
$ilvD^{ValS-9}$ R	CAA ACA GAA ATA GGg CTT AAA GCG TTT AG
	For site directed mutagenesis of pGY*lux*::*ilvD*
codY F	GATCGGTACCCCGAATGCAGTTGTAGATATTACC
codY R	GATCGAGGCTCTTATGTCCCAGACTCATCGAC
	For cloning *codY* into pRMC2
codY Seq F	GCAATTACTCGCTTAGCTGAG
codY Seq R	GTGTGTATTGGCTTTATAGCCG
	For target directed sequencing of *codY*
ilvD qPCR F	GCTATCTTTTGCTCTGGTGG
ilvD qPCR R	AGGGCAGGCATTTTGTTCC
	For qPCR of *ilvD*
ilvC qPCR F	CAAGATGTAAAAACGGACGC
ilvC qPCR R	GTCAAAAGAACGACCTGGG
	For qPCR of *ilvC*

[a] All primer sequences are based on the USA300 FPR3757 genome; restriction sites are underlined; nucleotide mutated in site directed mutagenesis is indicate in lower case.

4.2.4 TCA precipitation of proteins and SDS-PAGE

Strains were pre-grown in TSB to mid-exponential phase and then sub-cultured into TSB to a starting OD_{600} of 0.01 and grown overnight. Optical densities (OD_{600}) of stationary phase cultures were determined and a supernatant volume equivalent to 5 OD units was harvested and incubated with tricholoracetic acid (TCA) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 20% overnight at 4˚C. Precipitated protein samples were run on a 12% acrylamide gel and stained with Coomassie-Blue.

4.2.5 *lux* reporter assays

Kinetic *lux* reporter experiments were performed in flat, clear-bottom 96-well white plates (Thermo Fisher Scientific) and read using a BioTek Synergy™ H4 Hybrid Multi-Mode Microplate Reader (BioTek Instruments Inc, Winooski, VT). Pre-cultures were inoculated into either complete or limited CDM to a starting OD_{600} of 0.01 in 200 μL/well. Luminescence and OD_{600} values were read at hourly intervals. For end-point *lux* reporter experiments, pre-cultures were sub-cultured into either complete or limited CDM to a starting OD_{600} of 0.05 in tubes with a 7:1 tube:volume ratio. At hourly intervals, aliquots of 200 μL were transferred to flat, clear-bottom 96-well white plates (Thermo Fisher Scientific) and luminescence and OD_{600} values were read. Samples of strains containing the *lux* construct with the complete *ilvD* promoter region were supplemented with 0.1% (vol/vol) decanal in 40% ethanol and luminescence was measured immediately. Data presented are the relative light unit (RLU) values normalized to the OD_{600} of the sample when the cultures reached mid-exponential phase (OD_{600} 0.6-0.8).

4.2.6 RT-qPCR

RNA was isolated from cells grown to mid-exponential phase (OD_{600} of 0.6-0.8) in complete CDM using the Aurum Total RNA Mini Kit (Bio-Rad; Hercules, CA) per the manufacturer's instructions. RNA (500 ng) was reverse transcribed using SuperScript II (Invitrogen, Carlsbad, CA) per the manufacturer's instructions using 500 μg mL^{-1} of random hexamers. cDNA was PCR-amplified using SensiFAST™ SYBR® No-ROX Kit (Bioline, Taunton, MA). Data were normalized to expression of the *rpoB* reference gene. Primers used are listed in Table 4.2.

4.2.7 Bioinformatics

Putative terminator structures in the *ilvD* 5'UTR were identified using the predictive software RibEx and RNAfold. Mfold was used to identify putative antiterminator sequences. RNAfold and Mfold were used to predict Class 1 and Class 2 T-box Stem 1 features. Multiple sequence alignments of T-box sequences were conducted with all known T-box sequences in *S. aureus* subsp. *aureus* N315 (NC_002745.2) and aligned with the *ilvB* T-box from *B. subtilis* (NC_000964.3) and predicted *Clostridium* spp. *ilvB* T-box (NC_001898.1). The *ilvD* 5'UTR from USA300 FPR3757 was aligned to 168 *S. aureus* complete genomes using BLAST (see Appendix A).

4.3 Results

4.3.1 Growth adaptation of *S. aureus* to BCAA deprivation

S. aureus has previously been reported as auxotrophic for Leu and Val (24, 25), despite possessing an intact BCAA biosynthetic operon. We therefore followed up on these reports in an attempt to understand the molecular basis for this observation. Indeed, we observed no growth of *S. aureus* after 12 hr of growth in media lacking either Leu or Val (Figure 4.2A). However, prolonged incubation allowed us to observe growth in media lacking Leu after 10-16 hr and growth in media lacking Val after 24-36 hr (Figure 4.2A) and (Figure 2.9). The extended growth lag led us to question whether these conditions select for mutations that result in adaptation to the absence of exogenous Leu or Val. To test this, we took cells having grown in media lacking either Leu or Val, and subcultured them back into the same medium from which they were recovered. Cells recovered from CDM^{-Leu} medium exhibited the same growth delay upon sub-culture into the same medium, indicating that these cells had not acquired a heritable mutation. When we recovered cells from CDM^{-Val} medium, plated them, and then repeated the experiment, we found that upon sub-culture, they grew readily, suggesting that they were synthesizing Val (Val^{S}) (Figure 4.2B). These results suggest that growth in the absence of exogenous Leu requires a regulatory adaptation, whereas growth in the absence of Val selects for a heritable mutation. We hypothesized that identification of the genetic mutations permitting growth of *S. aureus* in the absence of exogenous Val would reveal important regulators of the BCAA biosynthetic operon.

CodY is a known repressor of the BCAA biosynthetic operon in *S. aureus* and binds to two regions upstream of *ilvD* proximal to the transcriptional start site and two regions within the operon, preceding *ilvC* and *leuC* (16–18) (Figure 4.1). To confirm its role in regulating BCAA biosynthesis in USA300, we evaluated the growth of a *codY* mutant in CDM lacking each individual BCAA. The *codY* mutant did not have a growth defect in any of the BCAA depleted media, indicating that endogenous synthesis, uncoupled from CodY repression, is sufficient to support growth in the absence of an exogenous source in this strain (Figure 4.2C).

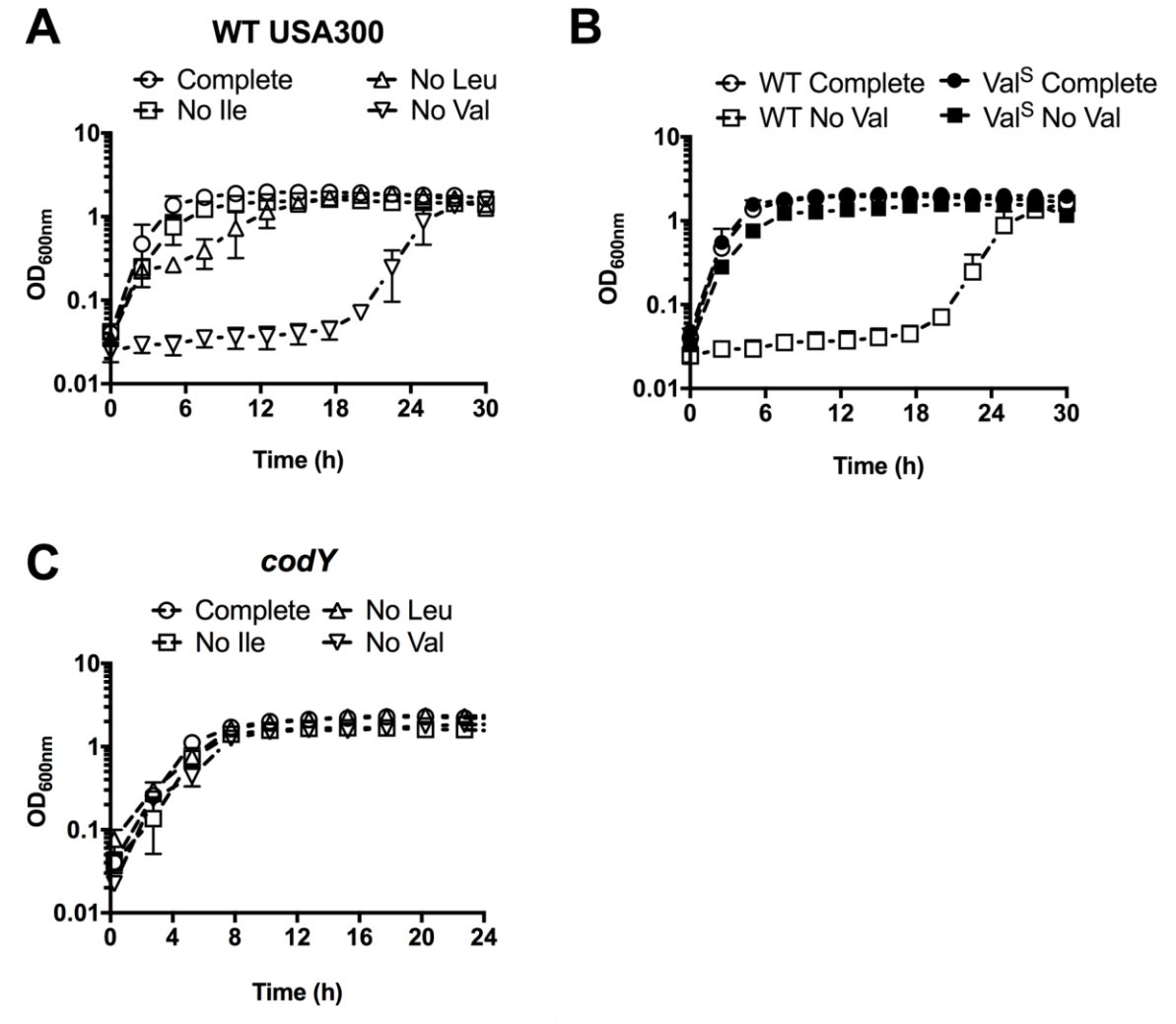


Figure 4.2 Growth adaptation of *S. aureus* to BCAA depletion

A) WT USA300 was pre-grown in complete CDM to mid-exponential phase and then sub-cultured into either complete CDM or CDM with BCAAs omitted, as indicated. B) Cells recovered from the CDM with no Val in panel A were plated. A single colony was selected (Val^S) and subjected to growth in complete CDM and CDM with no Val (filled symbols). Growth was compared to the parental WT strain (open symbols) in the same conditions. C) The *codY* mutant was pre-grown in complete CDM to mid-exponential phase, and then sub-cultured into either complete CDM or CDM with BCAAs omitted, as indicated. Data are the mean +/- SD of three biological replicates.

4.3.2 Growth in media lacking Val selects for mutations in *codY*

Since a *codY* mutant synthesizes BCAAs, we next investigated whether growing WT *S. aureus* in the absence of Val selects for mutations in *codY*. To do this, we isolated mutants from twelve independent cultures of the WT strain in media lacking Val (referred to as Val^S mutants) and amplified the *codY* gene. Five of the twelve mutants contained mutations in *codY*; one had a point mutation resulting in a premature stop codon, two had a 60 bp deletion, and two had independent point mutations resulting in nonsynonymous mutations (Figure 4.3A). We mapped the mutations to the CodY structure to identify their localization in the protein (PDB ID:5EY0) (50). All mutations occur in the linker region between the metabolite sensing domain and the DNA-binding domain Figure 4.1B.

Table 4.3 Mutations identified in CodY

Mutants	Position[a]	Mutation
Val^S-2	1260149-1260208	ΔArg_{167}-Ala_{186}
Val^S-3	1260119	Glu_{157} to Stop
Val^S-4	1260149-1260208	ΔArg_{167}-Ala_{186}
Val^S-8	1260188	Ser_{180} to Pro
Val^S-10	1260230	Leu_{194} to Phe

[a]Position in the USA300 FPR3757 genome (NC_007793.1)

To confirm that these mutations result in an inactive CodY protein, we assessed the secreted protein profile of these mutants, as CodY represses several secreted proteins (51) and therefore the secreted protein profile of a *codY* mutant differs substantially from WT. The secreted protein profiles of the Val^S mutants with confirmed mutations in *codY* resembled the protein profile of the *codY* mutant, except for Val^S-10 mutant (Figure 4.3C), indicating that all but one of our *codY* mutations result in an inactive CodY protein. Complementation of all Val^S mutants with confirmed *codY* mutations with a copy of the *codY* gene *in trans* reverted the growth phenotypes of theses mutants in media lacking Val to WT (Figure 4.3D), confirming that mutations in CodY enable Val synthesis.

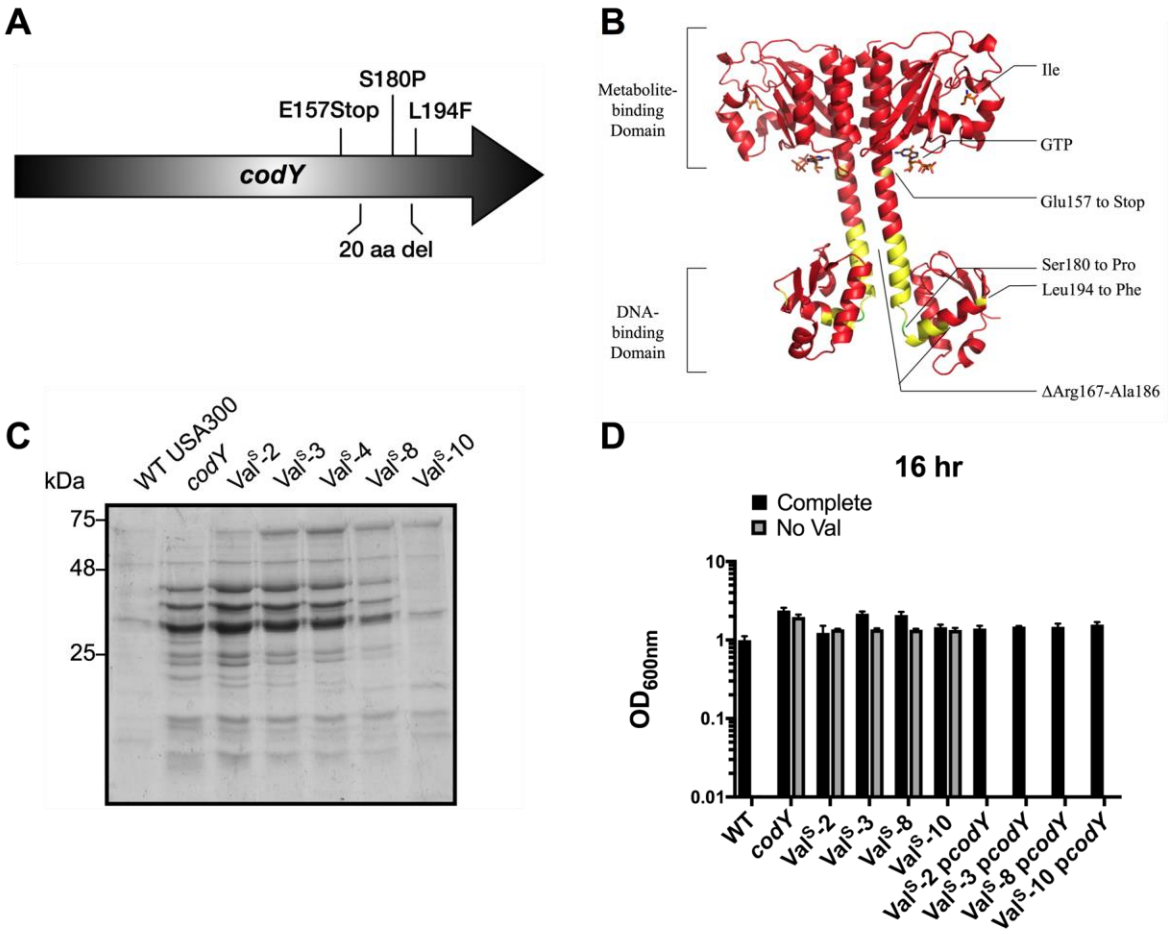


Figure 4.3 The absence of exogenous valine selects for mutations that inactivate CodY.

A) Schematic representation of the mutations identified in CodY. B) Mutations identified are indicated on the CodY structure (PDB ID:5EY0) in yellow, except for the Ser180 to Pro mutation, which is indicated in green. CodY ligands, Ile and GTP, are coloured based on atomic composition. C) Strains were pre-grown in TSB to mid-exponential phase, then sub-cultured into TSB for 16 hr. Supernatants were collected and proteins were precipitated using TCA. Protein samples were normalized to the equivalent of 5 ODs and run on a 12% SDS-PAGE gel. D) Strains were pre-grown in complete CDM to mix-exponential phase, then sub-cultured into either complete CDM or CDM with Val omitted. OD_{600nm} was read after 16 hr of growth. Data are the mean +/- SD of three biological replicates.

4.3.3 Growth in media lacking Val selects for mutations in the 5'UTR of *ilvD*

We next investigated whether the secreted protein profiles of the remaining seven Val^S mutants, which were confirmed to not have mutations in the *codY* gene, were altered to rule out mutations that might indirectly affect the ability of CodY to regulate the *ilv-leu* operon (i.e. mutations in GTP synthesis). The protein profiles of the seven mutants resembled the WT strain (Figure 4.4), suggesting the mutations affect a CodY-independent mechanism of regulation. We sequenced the region upstream of the first gene in the *ilv-leu* operon, *ilvD*, reasoning that mutations might occur in the binding sequence of transcriptional regulators. This approach revealed that all seven of these Val^S mutants had mutations in the 5'UTR of *ilvD*, with a total of three unique point mutations and one 27 bp deletion (Table 4.4) and (Figure 4.5A).

Table 4.4 Mutations identified in the 5' UTR of *ilvD*

Mutants	Position[a]	No. of bp upstream of *ilvD* start codon	Mutation
Val^S-1	2164689	184	T -> A
Val^S-5	2164762-2164788	85	27 bp deletion
Val^S-6	2164762-2164788	85	27 bp deletion
Val^S-7	2164741	132	C -> A
Val^S-9	2164739	134	T -> C
Val^S-11	2164762-2164788	85	27 bp deletion
Val^S-12	2164739	134	T -> C

[a]Position in the USA300 FPR3757 genome (NC_007793.1)

In order to conclude that these mutations were the single basis for the observed ability of the mutants to grow without delay in media lacking Val, we sequenced the genomes of the mutants and the parent USA300 strain. This indeed confirmed these mutations to be the single difference between the mutants and the parental strain. To confirm that mutations in the 5'UTR of *ilvD* result in an increase in expression of the *ilv-leu* operon, we generated a luminescence reporter of the *ilvD* promoter region by cloning the 5'UTR of *ilvD* into the pGY::*lux* vector, and subsequently mutated the 5'UTR to contain each of the point mutations identified. All three mutant sequences resulted in an increase in *ilvD* promoter activity

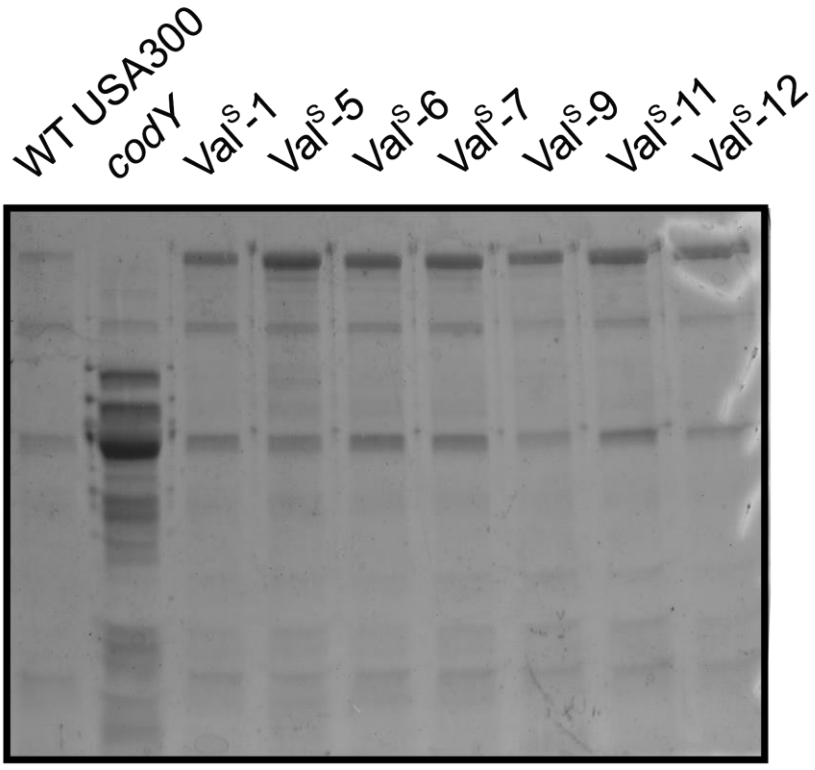


Figure 4.4 Secreted protein profiles of Val^S-mutants with WT *codY* sequence

Strains were pre-grown in TSB to mid-exponential phase, then sub-cultured into TSB for 16 hr. Supernatants were collected and proteins were precipitated using TCA. Protein samples were normalized to the equivalent of 5 ODs and run on a 12% SDS-PAGE gel.

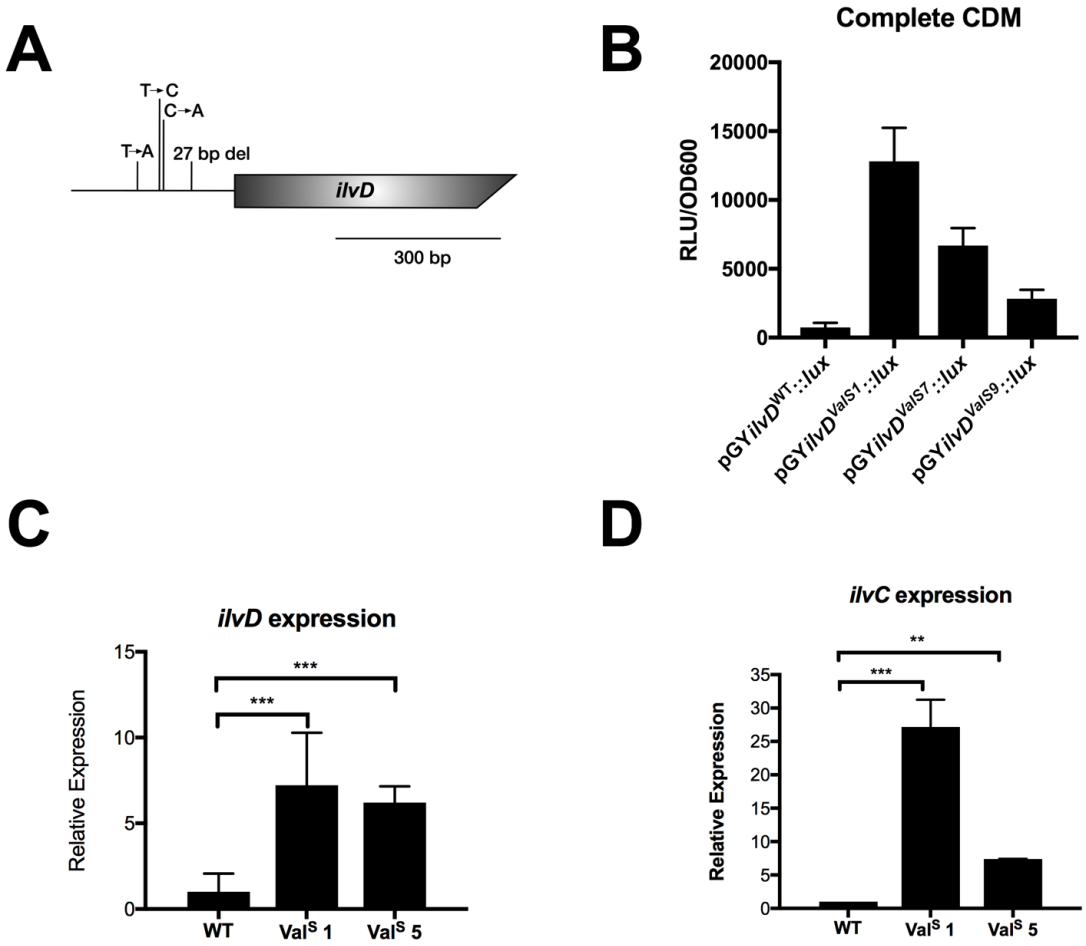


Figure 4.5 Mutations in the *ilvD* 5'UTR result in an increase in promoter activity and *ilv-leu* operon expression

A) Schematic representation of mutations identified in the *ilvD* 5'UTR. B) WT USA300 with the pGY::*lux* plasmid, containing either the WT or mutant *ilvD* promoter region, was grown in complete CDM to mid-exponential phase and then sub-cultured into complete CDM. Luminescence was read at mid-exponential phase and normalized to the OD_{600nm}. Data are the mean +/- SD of three biological replicates. C,D) Strains were grown in complete CDM to mid-exponential phase and then sub-cultured into complete CDM. Cells were harvested at mid-exponential phase and RNA was isolated. Expression of *ilvD* and *ilvC* was normalized to expression of *rpoB*. Data are the mean +/- SD of three biological replicates.

(Figure 4.5B). Furthermore, we selected two of the mutants, Val^S-1 (SNP) and Val^S-5 (deletion) and measured expression of *ilvD* and *ilvC* in the *ilv-leu* operon, and found that both genes were up-regulated in the mutant strains compared to the WT strain (Figure 4.5C,D).

4.3.4 Exogenous BCAAs differentially regulate the *ilv* operon

Although *in vitro* studies (i.e. EMSAs and DNAase footprinting studies) of CodY suggest that all three BCAAs increase its DNA binding activity (22, 52, 53), our results call in to question the relative importance of each BCAA for regulating CodY activity *in vivo* (i.e. during growth). That is to say, if Val was an important effector of CodY, removal of Val from the growth medium would result in a decrease in CodY activity and subsequently de-repression of the ILV operon. Instead, we observe that this condition selects for a mutation producing a null CodY protein. This indicated to us that perhaps each individual BCAA does not affect CodY equally during growth. We therefore used the promoter:reporter system to investigate CodY regulation of the *ilvD* expression. To study CodY-dependent promoter activity in isolation of putative regulatory elements in the *ilvD* 5'UTR, we generated two reporter constructs; one containing the complete *ilvD* leader region, including both the CodY binding sequences and the putative regulatory element (pGY*ilvD*^{C}::*lux*), and one containing a partial *ilvD* leader, including only the CodY binding sequence (pGY*ilvD*^{P}::*lux*) (Figure 4.6A). We first confirmed that both constructs responded to CodY and, indeed observed higher promoter activity in the *codY* mutant compared to the WT strain that peaked during mid-exponential growth (Figure 4.6B,C). All endpoint pGY::*lux* experiments from this point on are therefore the luminescence normalized to the optical density of mid-exponential phase cells (RLU/OD_{600}).

We next assessed promoter activity in response to depletion of each BCAA. Since complete omission of Leu and Val from the growth medium significantly attenuates *S. aureus* growth, we instead limited their concentrations to 10% of that in complete CDM to minimize differences in growth. *ilvD* promoter activity of the pGY*ilvD*^{P}::*lux* construct increased to levels comparable to the *codY* mutant only upon Ile limitation, and limitation of Leu or Val in combination with Ile did not alter *ilvD* promoter activity any further (Figure 4.7A). *ilvD* promoter activity of the pGY*ilvD*^{C}::*lux* construct also increased upon Ile limitation, yet in contrast to the pGY*ilvD*^{P}::*lux* plasmid, we observed a further increase in promoter activity upon limitation of both Ile and Leu (Figure 4.7B). These data suggest that Ile is the

predominant BCAA to affect CodY activity, at least on the *ilvD* promoter region, and Leu contributes to *ilvD* promoter activity via the 5'UTR regulatory element. To test CodY activity under conditions where cells are not nutrient deprived, we also assessed promoter activity of the pGY*ilvD*^{P}::*lux* construct in media containing excess amounts of each BCAA, expecting a repressive effect on CodY activity. Indeed, we found that whereas excess amounts of Ile in the growth medium repressed *ilvD* promoter activity (Figure 4.7C), Leu had no effect (Figure 4.7D), and intriguingly, excess Val had the opposite effect of increasing promoter activity (Figure 4.7E). We further confirmed a predominant role for Ile in regulating CodY activity on the *brnQ1* promoter region as well (Figure 4.7F-H), suggesting that Ile is the predominant BCAA to regulate CodY activity on its other target genes.

We previously identified mechanisms of BCAA transport in *S. aureus*, including BrnQ1 and BcaP, which transport Ile, Leu and Val, and BrnQ2, a dedicated Ile transporter (Chapter 2 and 3). We therefore assessed *ilvD* promoter activity in strains mutated in these transporters to observe the contribution of each transporter to either CodY-dependent or CodY-independent regulation of BCAA biosynthesis. *ilvD* promoter activity of the pGY*ilvD*^{P}::*lux* construct increased only in the *brnQ2* mutant (Figure 4.8A), whereas the pGY*ilvD*^{C}::*lux* increased in the *brnQ1* and *brnQ1bcaP* mutants (Figure 4.8B). These data indicate that BrnQ2-dependent Ile transport is linked to CodY activity and BrnQ1/BcaP-dependent Leu transport is linked to the 5'UTR regulatory element. Notably, using the complete 5'UTR, we did not observe a change in promoter activity in the *brnQ2* mutant. We have previously shown that *brnQ1* is up-regulated in a *brnQ2* mutant and consequently a *brnQ2* mutant takes up more Leu and Val permitting enhanced growth in media limited for these amino acids (Figure 2.11). We thus postulate that in a *brnQ2* mutant, the increased Leu uptake causes repression of *ilvD* promoter activity via the regulatory element and overrides the CodY-dependent Ile response.

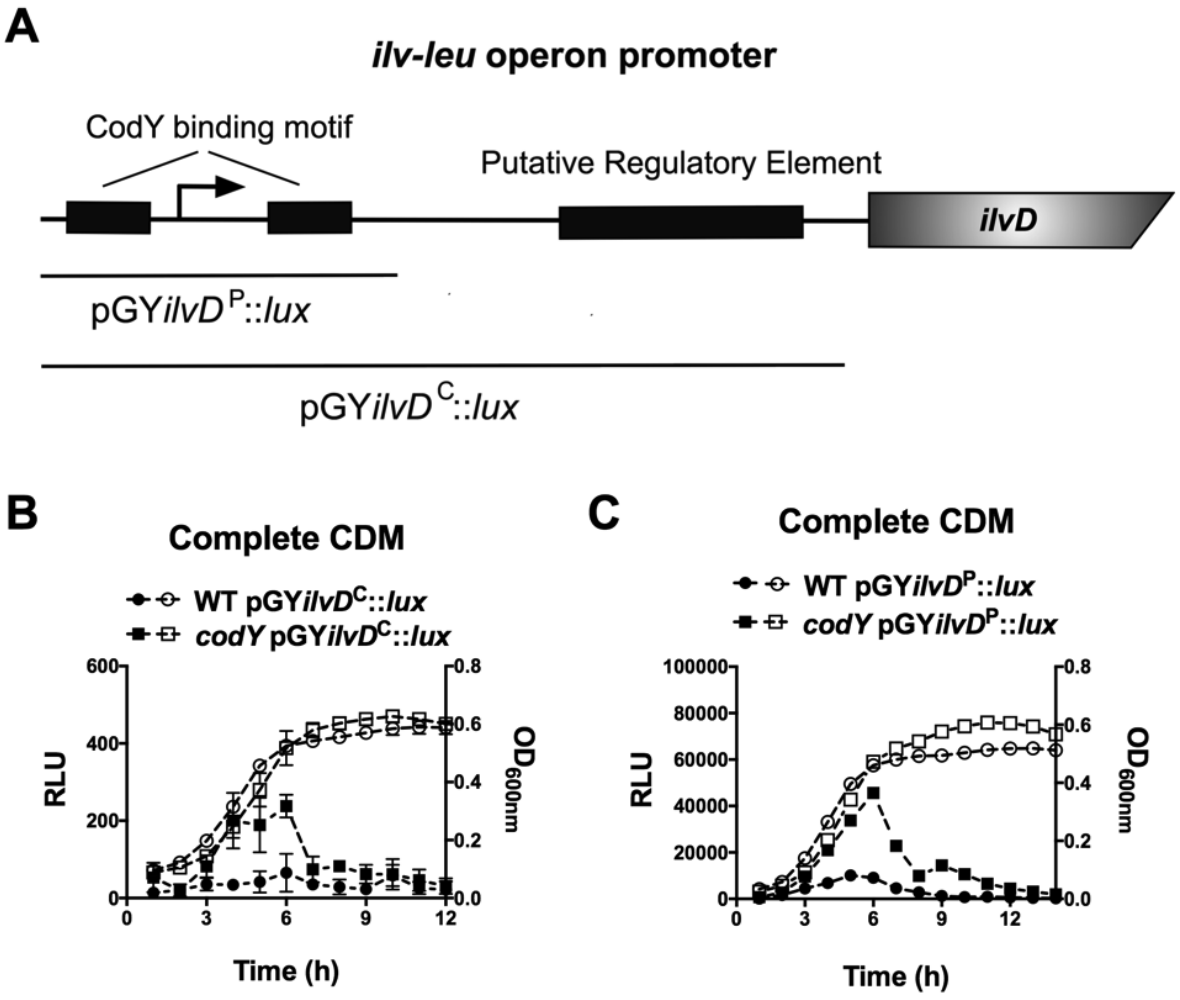


Figure 4.6 Construction of the *ilvD* promoter:reporter vectors.

A) Diagram of the promoter regions cloned into the pGY::*lux* vector to generate the partial promoter construct (pGY*ilvD*^{P}::*lux*) and the complete promoter construct (pGY*ilvD*^{C}::*lux*). B,C) Strains were pre-grown in complete CDM to mid-exponential phase and then sub-cultured into complete CDM in a 96-well plate. Luminescence (left axis, filled shapes) and OD_{600nm} (right axis, open shapes) were read hourly. Data are the mean +/- SD of three biological replicates.

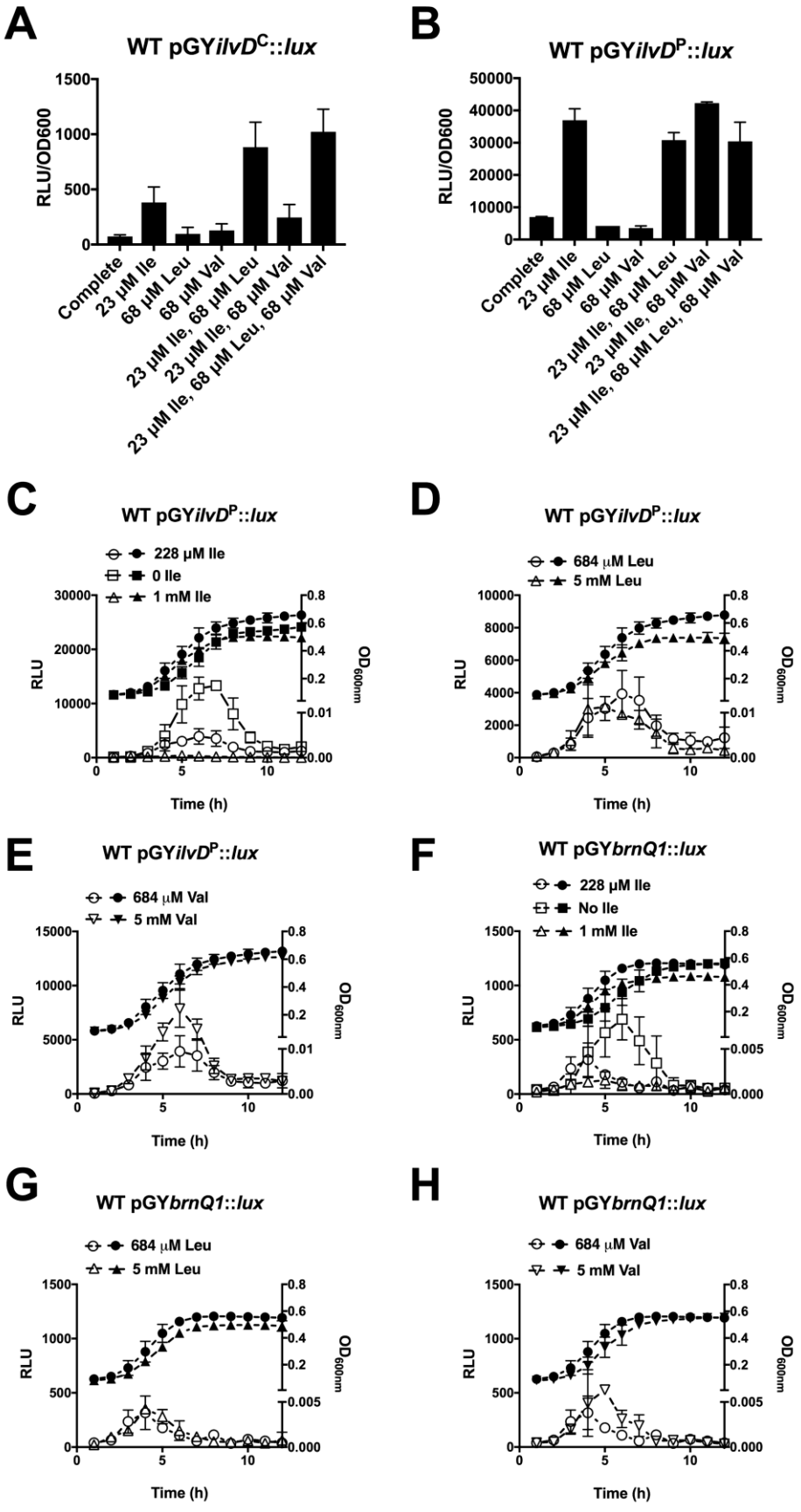

A
WT pGY*ilvD*^{C}::*lux*
RLU/OD600
1500
1000
500
0
Complete
23 µM Ile
68 µM Leu
68 µM Val
23 µM Ile, 68 µM Leu
23 µM Ile, 68 µM Val
23 µM Leu, 68 µM Val
B
WT pGY*ilvD*^{P}::*lux*
RLU/OD600
50000
40000
30000
20000
10000
0
Complete
23 µM Ile
68 µM Leu
68 µM Val
23 µM Ile, 68 µM Leu
23 µM Ile, 68 µM Val
23 µM Leu, 68 µM Val
C
WT pGY*ilvD*^{P}::*lux*
228 µM Ile
0 Ile
1 mM Ile
RLU
OD600nm
Time (h)
D
WT pGY*ilvD*^{P}::*lux*
684 µM Leu
5 mM Leu
RLU
OD600nm
Time (h)
E
WT pGY*ilvD*^{P}::*lux*
684 µM Val
5 mM Val
RLU
OD600nm
Time (h)
F
WT pGY*brnQ1*::*lux*
228 µM Ile
No Ile
1 mM Ile
RLU
OD600nm
Time (h)
G
WT pGY*brnQ1*::*lux*
684 µM Leu
5 mM Leu
RLU
OD600nm
Time (h)
H
WT pGY*brnQ1*::*lux*
684 µM Val
5 mM Val
RLU
OD600nm
Time (h)

Figure 4.7 Ile is the predominant BCAA to regulate CodY activity on select CodY target promoters

A,B) Strains were pre-grown in complete CDM to mid-exponential phase and then sub-cultured into either complete CDM or CDM with limiting concentrations of BCAAs, as indicated. Luminescence values were read when cells reached mid-exponential phase and normalized to the OD_{600nm}. C-E) WT USA300 possessing the lux vector containing only the CodY binding site of the *ilvD* promoter (pGY*ilvD*^{P}::*lux*) or containing the CodY binding site from the *brnQ1* promoter (pGY*brnQ1*::*lux*) was grown in complete CDM to mid-exponential phase and then sub-cultured into complete CDM in a 96-well plate. Luminescence (left axis, open shapes) and OD_{600nm} (right axis, filled shapes) were read hourly. Data are the mean +/- SD of three biological replicates.

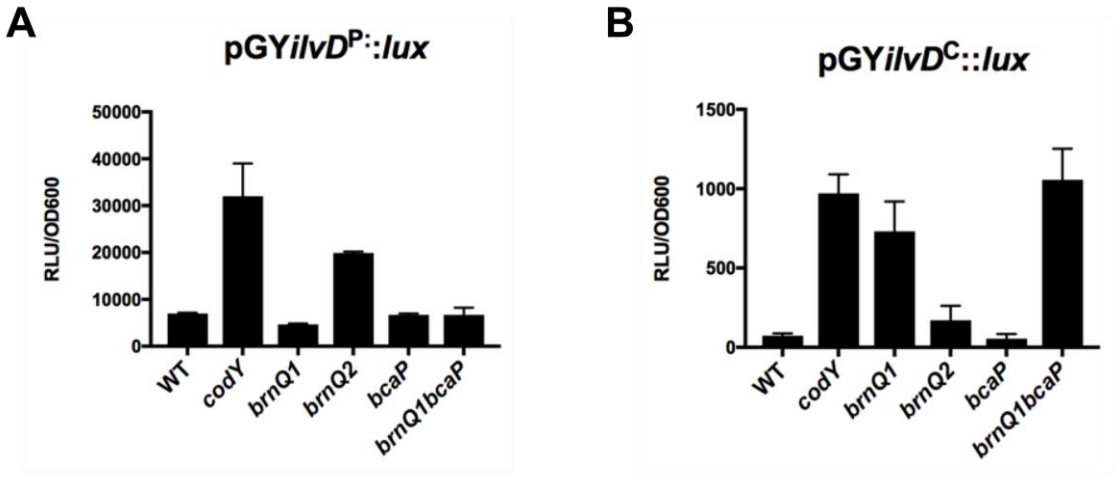


Figure 4.8 BCAA transporters link exogenous BCAAs to regulation of BCAA synthesis

S. aureus strains possessing either the *lux* vector containing the partial *ilvD* promoter (pGY*ilvD*^{P}::lux) or the complete *ilvD* promoter (pGY*ilvD*^{C}::lux) were pre-grown in complete CDM to mid-exponential phase and then sub-cultured into complete CDM. Luminescence values were read when cells reach mid-exponential phase and were normalized to the $OD_{600nm.}$ Data are the mean of three biological replicates +/- SD.

4.3.5 Identification of an attenuator and terminator in the 5'UTR of *ilvD*

We next analyzed the 5'UTR of *ilvD* for putative structural elements that could dictate the regulatory mechanism, which responds to Leu. We initially considered that the 5'UTR might function as a T-box riboswitch, since the *ilv-leu* operon in *B. subtilis* is regulated by a $tRNA^{Leu}$-responsive T-box riboswitch (37, 39). Predictive structure analysis and sequence comparison of the *ilvD* 5'UTR to other known T-box sequences revealed that although the *S. aureus ilvD* T-box contains features that resemble other T-boxes (Figure 4.9), the variability in the distal loop and long insertions between features are not consistent with Class 1 or 2 Stem 1 structures essential to T-box function (54). We next considered translation-dependent transcriptional regulation (i.e. attenuation), since BCAA-rich leader peptides have been found to regulate BCAA biosynthetic genes in *E. coli* (29), *S. typhimurium* (30, 31), and are predicted to regulate BCAA synthesis in *L. lactis* sp. *lactis* (55), *C. glutamicum* (56, 57), and *Streptococcus* spp. (58). A search for ORFs in the *ilvD* leader sequence revealed a 26 amino acid peptide that contains a string of three Ile codons followed by two Leu codons, and an additional three interspersed Leu codons (Figure 4.10). A ribosome binding site was also identified 9 bp upstream of the start codon. We also identified a terminator hairpin structure consistent with transcription termination (Figure 4.10). We therefore predict that, in *S. aureus*, Leu deprivation is sensed via attenuation.

We next considered how the mutations we selected for in media lacking Val might disrupt these features. Intriguingly, the 27 bp deletion in Val^S-5/6/11 deletes the predicted terminator stem-loop (Table 4.5), supporting that this is a biologically relevant structure, and the T153A mutation (Val^S-1) changes a Leu codon in the leader peptide to a stop codon (Table 4.5). The T203C (Val^S-9/12) and C205A (Val^S-7) mutations occur in a region where we would predict the anti-terminator to form. If our model is correct, these mutations likely destabilize the terminator formation, leading to transcriptional read-through.

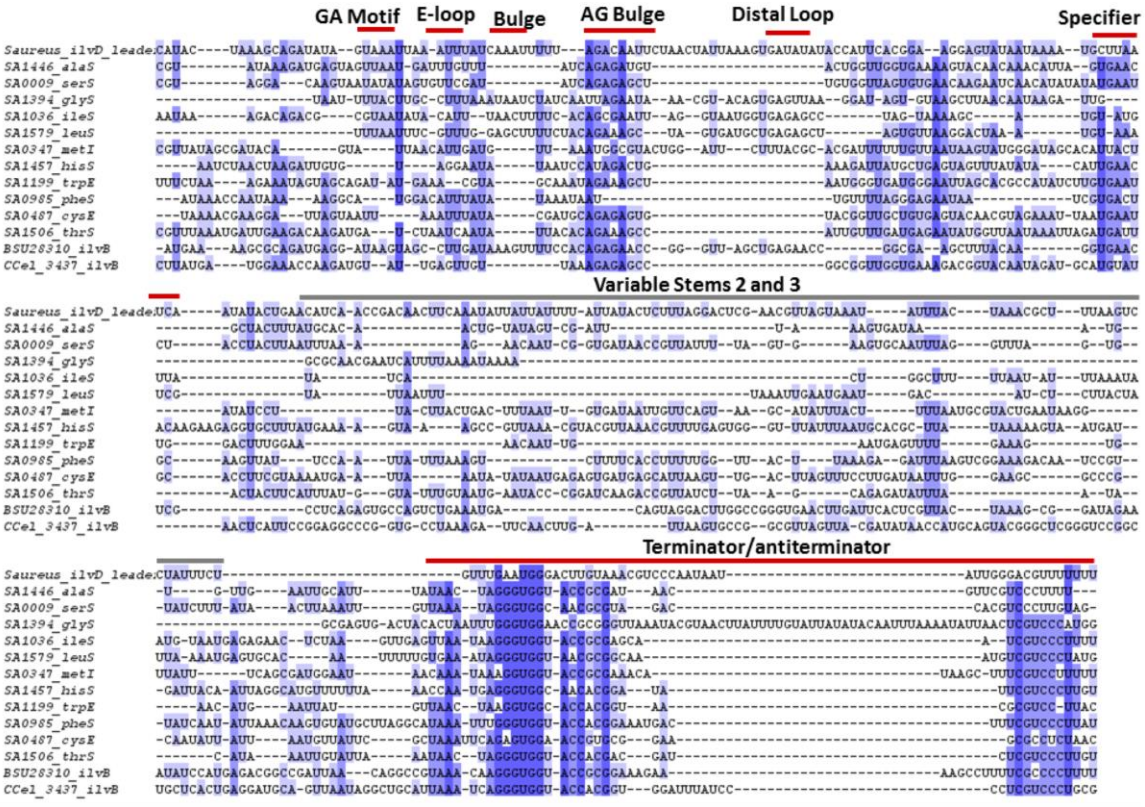


Figure 4.9 Nucleotide sequence alignment of *ilvD* 5'UTR with known T-box riboswitches

Sequences of all annotated *S. aureus* T-boxes (based on the *S. aureus* subsp. *aureus* N315 genome NC_002745.2), the *B. subtilis ilvB* T-box (NC_000964.3) and predicted *Clostridium* spp. *ilvB* T-box (NC_001898.1), labelled by regulated gene, were analyzed. Key features analyzed include the GA Motif (GA); E-loop (AGUA-box), the AG Bulge (AGVGA-box), Distal Loop (GNUG-box); and the Specifier Loop (GAA...XXXA) where XXX represents the tRNA codon.

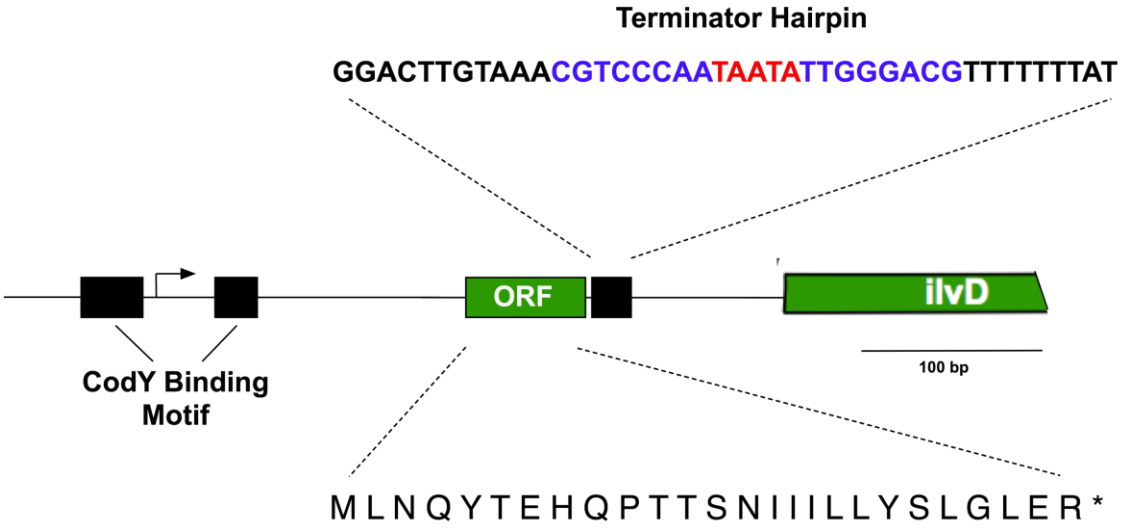


Figure 4.10 Attenuator features in the 5'UTR of *ilvD*

Sequence analysis of the 5'UTR of *ilvD* revealed a sequence consistent with a terminator hairpin i.e. stem (highlighted in blue), loop (highlighted in red), followed by a poly-U tail. A putative open reading frame (ORF) containing multiple Ile and Leu codons was also identified. Both features occur downstream of the transcriptional start site (arrow).

Table 4.5 Effects of mutations on 5'UTR features

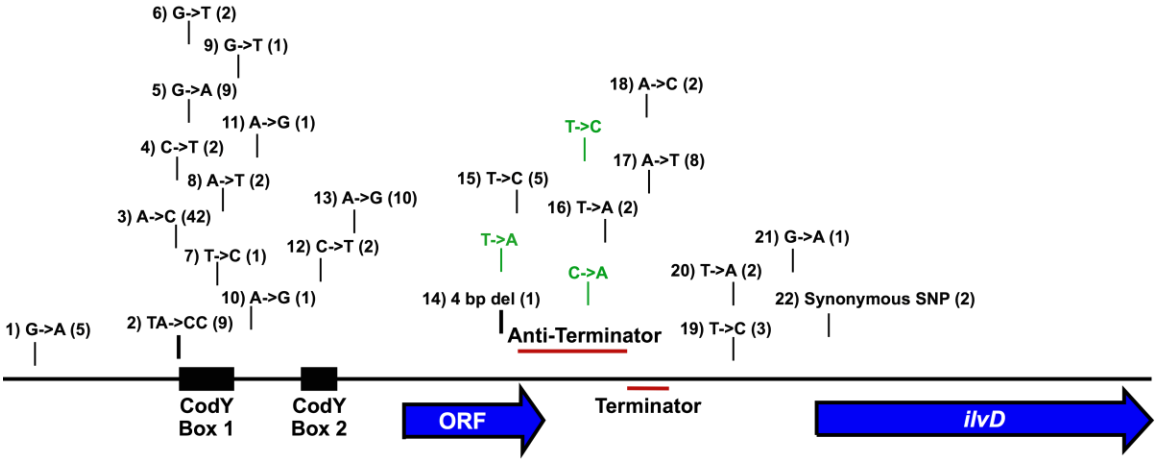
5' UTR Feature	
Leader peptide: WT	MLNQYTEHQPTTSNIIILLYSLGLER*
Leader peptide: Val^S-1	MLNQYTEHQPTTSNIIIL*
Terminator hairpin: WT	CGTCCCAATAATATTGGGACGTTTTTTTAT
Terminator hairpin: Val^S-5/6/11	----------TTTTTTTAT

We were curious to investigate whether the features in the *ilvD* 5'UTR are conserved across *S. aureus* strains, and therefore compared the USA300 FPR3757 sequence to all complete genome sequences of *S. aureus* (168). Overall, there was high sequence conservation (Appendix A), however, several variants were identified in the putative regulatory regions (Figure 4.11). Intriguingly, two variants occur in the putative ORF and both alter the number of Leu codons in the peptide; mutation 14 results in a frame shift that deletes one Leu, and mutation 15 changes a Leu codon to a Ser (Figure 4.11). Several sequence variants also occur in the first CodY binding motif (CodY Box 1) (Figure 4.11). In addition, two strains, HC1340 and Gv69, had a 1.8 kb transposase gene inserted between the 5'UTR and the start codon of *ilvD*, and the terminator is deleted because of the insertion (Appendix A). It would be of interest to investigate the consequence of these mutations on the level *ilv-leu* expression and subsequent BCAA biosynthesis.

4.3.6 Absence of exogenous Ile restores growth in media lacking Leu, but not Val

Altogether, our data point towards a model where both Ile and Leu contribute to *ilv-leu* regulation, with Ile regulating the CodY-dependent mechanism, and Leu (and possibly Ile) regulating attenuation (Figure 4.12). With this information in hand, we returned to our growth assay where we grow *S. aureus* in the absence of exogenous BCAAs to test whether we could induce synthesis by modulating Ile and Leu availability. We hypothesized that omission of Ile from the growth medium would result in CodY-dependent de-repression of the *ilv-leu* operon, and therefore would rescue the growth lag of *S. aureus* in media lacking Leu or Val. Indeed, we found that the growth of *S. aureus* in CDM lacking Ile and Leu initiated more rapidly than growth in CDM lacking Leu alone, suggesting that depletion of Ile relieves *ilv-leu* repression (Figure 4.13A). Unexpectedly, *S. aureus* grown in CDM lacking Ile and Val resembled growth of *S. aureus* in media lacking Val alone (Figure

4.13B). Since the presence of Leu also contributes to repression of the operon, we further examined growth of *S. aureus* in CDM lacking all three BCAAs, however the growth of *S. aureus* remained attenuated, with no observable growth until a prolonged period of ~ 16 hr (Figure 4.13C). Our promoter:reporter data suggest that *ilvD* expression is up-regulated in media limited for all three BCAAs (Figure 4.7A,B), therefore we postulate that posttranslational regulation of the *ilv-leu* operon controls BCAA synthesis under these conditions.



	Strains
1	GR2; NCTC13435; 11819-97; JKD6159; ILRI_Eymole1/1
2	08S00974; NZ15MR0322; RIVM3897; RIVM1607; 08BA02176; ST398; LA-MRSA ST398; RIVM1295; 71193
3	ST20130943; FORC_026; 08-02119; ST20130939; ST20130938; ST20130941; ST20130940; XQ; 93b_59; ED133; MW2; SJTUF_J27; MCRF184; JS395; ATCC 25923; 6850; 55/2053; CA-347; LGA251; TCH60; MSSA476; 71A_S11; FORC_001; ILRI_Eymole1/1; SA40TW; SA268; H-EMRSA-15; SA40; SA957; HO 5096 0412; M013; MRSA252; 08500974; NZ15MR0322; Tager 104; RIVM3897; RIVM1607; 08BA02176; ST398; LA-MRSA ST398; RIVM1295; 71193; BB155; SA17_S6; RF122;
4	RKI4; MSSA476
5	08S00974; NZ15MR0322; RIVM3897; RIVM1607; 08BA02176; ST398; LA-MRSA ST398; RIVM1295; 71193
6	BB155; SA17_S6
7	71193
8	BB155; SA17_S6;
9	08-02300
10	MRSA252
11	BB155
12	RKI4; MSSA476
13	FORC_001; 08S00974; NZ15MR0322; RIVM3897; RIVM1607; 08BA02176; ST398; LA-MRSA ST398; RIVM1295; 71193
14	RF122
15	SA40TW; SA957; M013; SA268; SA40
16	BB155; SA17_S6
17	ST20130939; ST20130938; ST20130941; ST20130940; MW2; MSSA476; FORC_026; 08-02119
18	H-EMRSA-15; HO 5096 0412
19	JS395; BB155; SA17_S6
20	BB155; SA17_S6
21	RKI4
22	ATCC6538; FDA209P

Figure 4.11 Mutations identified in the 5'UTR of *ilvD*

The *ilvD* 5'UTR was aligned across 168 complete *S. aureus* genomes. The location and nature of the mutations identified are indicated, with the frequency in brackets. Mutations in green are the SNPs identified in this study.

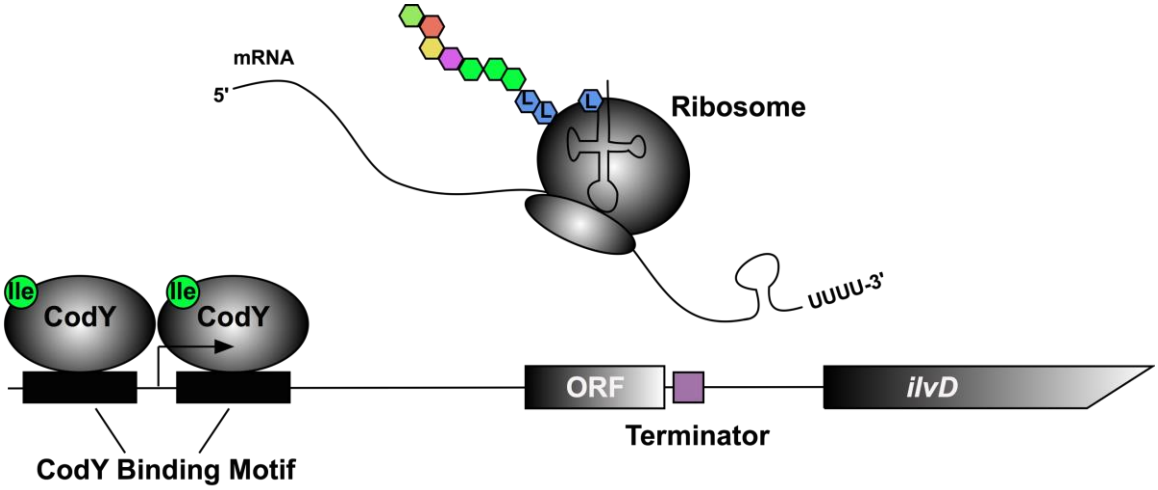


Figure 4.12 Model of mechanisms regulating *ilv-leu* expression

Transcription of the *ilv-leu* operon is repressed by CodY. In the presence of Ile, CodY binds and represses transcription. As Ile is depleted, CodY becomes inactive and expression of the operon is induced. As the operon is transcribed, the ribosome beings translating the open reading frame (ORF) upstream of *ilvD*. The ORF is rich in Ile and Leu and will stall if cells are depleted of either tRNA. Stalling of the ribosome prevents formation of the terminator hairpin, allowing transcription of the operon to proceed. When there is sufficient Ile/Leu tRNA, the ORF is translated and the terminator hairpin forms, terminating transcription.

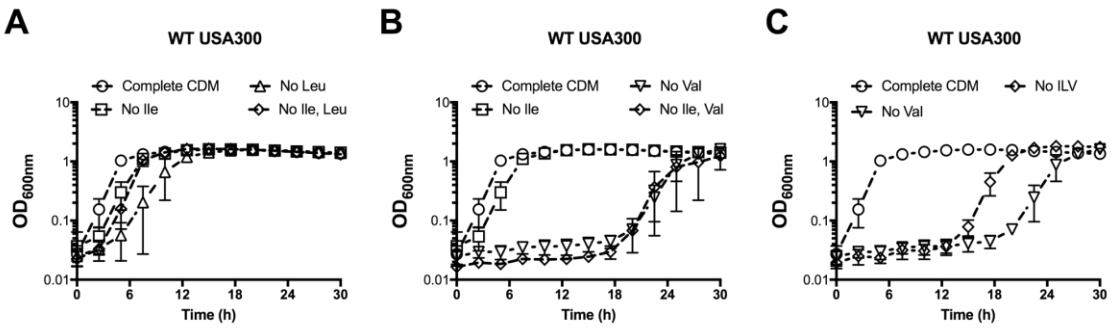


Figure 4.13 Omission of Ile from CDM restores growth in media lacking Leu, but not Val

A-C) Strains were pre-grown in complete CDM to mid-exponential phase and then sub-cultured into either complete CDM or CDM with amino acids omitted, as indicated. Data are the mean +/- SD of three biological replicates.

4.4 Discussion

In this study, we selected for genetic mutations that permit growth in media lacking Val to identify the mechanisms regulating BCAA biosynthesis during growth of *S. aureus* in a chemically-defined medium. Using this approach, we a *trans*-activing mechanism of repression involving CodY and a *cis*-acting mechanim of repression involving a putative attenuator peptide. We demonstrate that CodY-dependent repression responds to Ile deprivation and that BrnQ2 is the predominant transporter to link exogenous Ile with CodY activity. We postulate that the 5'UTR region of *ilvD* encodes a leader peptide that regulates transcription by attenuation and responds to Leu deprivation and possibly Ile deprivation, which are supplied by BrnQ1 and BcaP. Therefore, expression of the *ilv-leu* operon occurs upon depletion of both Ile and Leu.

Attenuation regulates BCAA biosynthesis in *E. coli* and *S. typhimurium* (29–31), and predicted attenuators can be found in various Gram-negative and Gram-positive bacteria (55–57, 59). In support of attenuation regulation of *ilv-leu* in *S. aureus*, one of the mutations we identified in our study occurs in the predicted leader peptide and changes a Leu codon to a stop codon, which we predict would reduce Leu-dependent repression. The leader peptide also contains three Ile, suggesting that the level of uncharged ^{Ile}tRNA also regulates *ilv-leu* expression. In support of this, expression of the *ilv-leu* operon is increased upon exposure to mupirocin, an antibiotic that binds to isoleucyl-tRNA synthetase and blocks the charging of ^{Ile}tRNA (60). It appears that CodY is the more dominant mechanism of repression, since *S. aureus* exhibits a growth delay in media lacking Leu, but not Ile, suggesting that Leu deprivation alone is not sufficient to relieve repression. Two additional CodY binding motifs are present in the *ilv-leu* operon (18), and therefore transcription of downstream genes in the operon would be expressed in a *codY* mutant, bypassing transcriptional termination at the *ilvD* leader. Notably, these conditions did not select for mutations in other previously described regulators of the *ilv-leu* operon, such as Gcp and YeaZ (41, 42). Since Gcp and YeaZ are essential genes in *S. aureus*, we did not expect to isolate mutations in these genes, however, we cannot rule out the possibility that the mutations in the 5' UTR of *ilvD* reduce binding of YeaZ to the promoter region (42).

The fact that mutations in CodY are selected for when *S. aureus* is grown in the absence of exogenous Val supports the notion that Val is not a relevant co-factor of CodY during growth of *S. aureus*. This contradicts *in vitro* DNA-binding data, which suggest that all three BCAAs bind to CodY and increase its DNA-binding activity to target gene promoters (21, 52, 53). However, Ile has previously been suggested to act as the predominant BCAA to regulate CodY activity during growth of *S. aureus* (17), *B. subtilis* (21), *L. lactis* (52), and *L. monocytogenes* (61), and our data corroborate these observations in *S. aureus* strain USA300. Further, we show that exogenous Ile is linked to CodY activity via the BrnQ2 transporter. This is intriguing, as BrnQ1 and BcaP also transport Ile, warranting further investigation into the mechanism that links BrnQ2 to CodY activity. BCAA transporters exist in other organisms, yet none function, as BrnQ2 does, as a dedicated Ile-transporter (62–64). This suggests that BrnQ2 might provide an advantage to the adaptation of *S. aureus* to Ile-depleted environments. It is surprising that Leu and Val did not have even an indirect effect on CodY-regulation of the *ilvD* promoter region, since Leu/Val deprivation has been shown to trigger up-regulation of CodY target genes in a stringent response-dependent (i.e. GTP depletion) manner in *S. aureus* (65). One might have predicted that if depletion of GTP was required for de-repression of the *ilv-leu* operon, growth of *S. aureus* in the absence of Val would select for mutations in genes involved in GTP synthesis (i.e. *guaA* or *guaB*), as has been observed in *B. subtilis* (66). This might be related to strain differences in these studies (Newman vs USA300) or slight differences in CDM composition (17, 65). Further investigations into the connection between the stringent response and CodY activity as it relates to *ilvD* expression in USA300 are required.

The *codY* mutations isolated in this study occur in the linker region of the protein. We hypothesize that this might affect dimer formation and, consequently, DNA binding activity (50, 67). Interestingly, the non-synonymous point mutation in Val^{S}-10 did not result in a *codY* mutant phenotype. This mutation might affect the binding affinity of CodY to its target sequence, such that it no longer binds to target sequences upstream of the *ilv-leu* operon, but retains its ability to bind to target sequences of other target genes, as CodY binding affinity dictates the hierarchal expression of its target genes (16, 68). Genome sequencing of this strain revealed a second point mutation in ribosome-binding factor A, however, we could

complement this strain with *codY in trans,* supporting the conclusion that the *codY* mutation is responsible for its growth phenotype.

We observed a disconnect between our model of transcriptional regulation and the growth phenotype of *S. aureus* in media lacking all three BCAAs. Our model predicts that expression of the *ilv-leu* operon would be de-repressed upon Ile and Leu deprivation, yet *S. aureus* exhibits a significant growth lag in the absence of all three BCAAs. One possible explanation for this observation is potential allosteric regulation of the BCAA biosynthetic enzymes. The last gene in the *ilv-leu* operon, *ilvA*, encodes a threonine deaminase (TD), which catalyzes the first step in Ile synthesis by converting threonine to α-ketobuytrate. TD is a pyridoxal phosphate (PLP)-dependent enzyme that contains an N-terminal catalytic domain and a C-terminal regulatory domain. The C-terminal regulatory domain contains one or two ACT-like sub-domains. The *E. coli* TD enzyme, which contains two ACT-like sub-domains, is inhibited by Ile and activated by Val (69–71). The *B. subtilis* TD enzyme, which contains one ACT-like sub-domain, is inhibited by Ile and it is proposed that Val activates TD in the presence of Ile and inhibits TD at high concentrations (72). If TD activity in *S. aureus* is most efficient in the presence of Val, it follows that Ile synthesis would be reduced in the absence of Val. This could explain the absence of growth in media lacking Ile and Val. It would be of interest to investigate whether TD in *S. aureus* is similarly subject to allosteric regulation. Whether this is indeed the mechanism, the fact that *S. aureus* is unable to support growth in conditions where Val is absent poses a challenge to *S. aureus* if it encounters this environment *in vivo* and suggests that Val transport is critical for its growth. Indeed, we have previously shown that BCAA transporters BrnQ1 and BcaP are required for *S. aureus* growth *in vivo* (Chapters 2 and 3). Studies are ongoing to further understand the requirement of Val acquisition for the growth of *S. aureus* in models of infection.

Altogether, this study details the molecular mechanisms regulating BCAA biosynthesis in *S. aureus* and uncovers environments where *S. aureus* engages in BCAA biosynthesis. In doing so, we reveal a predominant role for Ile in regulating CodY binding activity on the *ilvD* and *brnQ1* target sequences. Given the role of CodY in additionally regulating virulence genes, these data suggest that environmental availability of Ile, sensed by BrnQ2, is an important regulatory cue for *S. aureus* adaptation to nutrient limitation and virulence gene expression.

4.5 References

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Chapter 5

5 Concluding remarks and future directions

5.1 BCAA source preference in *S. aureus*

In the studies presented, we characterize the mechanisms *S. aureus* uses to maintain intracellular levels of BCAAs and demonstrate the consequence of perturbing these mechanisms on *S. aureus* physiology and virulence. We demonstrate that *S. aureus* represses BCAA biosynthesis when an exogenous source of BCAAs is available and instead acquires BCAAs through three dedicated transporters; BrnQ1, BrnQ2 and BcaP. The transporters are required for growth in media where the BCAA source is free amino acids, but are not required when a peptide source is added to the growth medium (Figure 3.7). It therefore appears that when obtaining BCAAs, *S. aureus* prefers transport of BCAA-containing peptides by oligopeptide or di/tri-peptide transporters to transport of free BCAAs, and reserves synthesis for environments depleted of both sources. This contrasts other Gram-positive organisms, like *B. subtilis*, where BCAAs are synthesized even in the presence of an exogenous source (1). Although it appears that BCAA transporters are not required when a peptide source is present, it cannot be ruled out that peptide transporters are up-regulated to overcompensate for the loss of the permeases. Data from our laboratory and other research groups indicate that acquisition of oligopeptides and di/tri-peptides in *S. aureus* is driven by Opp3 and DtpT, respectively (Sheldon *et al*., unpublished data, (2)). Therefore, to fully detail the relative importance of peptide transport vs BCAA transport, *S. aureus* strains deficient in both peptide transport and BCAA transport will need to be characterized.

Our studies confirm that CodY regulates both BCAA transport and synthesis in *S. aureus* (3–5). Given this, how does *S. aureus* achieve BCAA source preference? Waters *et al*., have demonstrated that in the hierarchy of CodY regulation, BCAA transporters are de-repressed upon moderate reductions in CodY activity, whereas de-repression of BCAA biosynthesis genes requires more significant reductions in CodY activity (5). Since the extent of CodY activity relates to availability of its ligands (GTP and BCAAs; of which we show Ile is the most important (Figure 4.7)), it is evident that availability of exogenous Ile is an important signal for the regulation of transport versus synthesis. In addition, we described a second

mechanism governing BCAA synthesis that responds to Leu. This suggests that not only is severe Ile depletion necessary for induction of synthesis, but also a simultaneous depletion of Leu.

How does *S. aureus* cope if it encounters an environment depleted of only Leu or Val? A Val limited environment in particular could prove challenging for *S. aureus* growth, since our data indicate that a mutation is required to relieve repression of synthesis in this condition. Leu and Val have both been shown to induce the stringent response, which depletes the other CodY effector GTP, and consequently leads to CodY target gene de-repression in *S. aureus* (6), however, we did not detect evidence of a stringent response-dependent de-repression of the BCAA biosynthetic operon in our studies (i.e. the extended growth lags observed in the absence of exogenous Leu or Val). One possible explanation for this difference might be related to fact that the CDM used in our studies is more limited for amino acids than the CDM used in the other studies (4, 6). As a result, the stringent response might be more active in our conditions, causing CodY regulatory activity to primarily respond to Ile concentrations. The differences could also be related to the different strains used in these studies (Newman vs USA300). Nonetheless, the role of GTP in regulating BCAA transport and synthesis via CodY requires further investigation. Notably, comparison of the *ilvD* 5'UTR from complete *S. aureus* genomes revealed mutations in the CodY binding motif and the attenuator (Figure 4.11). It might prove interesting to investigate whether these mutations affect the regulation of the *ilv-leu* operon. If these mutations help relieve repression of synthesis, it could indicate that *S. aureus* does indeed encounter this conundrum in its natural environment.

Additional studies are required to fully uncover the contribution of environmental and synthesized BCAAs to *S. aureus* growth *in vivo*. In general, there is limited knowledge of the BCAA requirement of pathogens during infection and the effect that perturbing these mechanisms has on infection outcomes, and our studies provide the foundation for resolving this. Our *in vivo* studies indicate that *S. aureus* encounters BCAA limitation in certain organs (i.e. the heart (Figure 3.9)) and the nasal cavity (Figure 3.10), however *S. aureus* virulence is only moderately attenuated, suggesting that peptide transport and/or synthesis also contribute to growth at these infection sites. As eluded to in Chapter 3, we hypothesize that BCAA synthesis is important for growth during murine nasal colonization. Human nasal secretions

are limited for BCAAs, especially Ile (7), and peptides are also presumably limited because *S. aureus* relies on methionine biosynthesis for growth in this niche (7). This hypothesis can be tested by mutating a gene required for BCAA biosynthesis (i.e. *ilvD*) in a *brnQ1bcaP* mutant. Strains with mutations in both synthesis and scavenging pathways would also be of use in other animal models, such as our systemic infection model, given that the biosynthetic operon is significantly induced during *S. aureus* growth in human serum and blood (8). Our laboratory is also poised to examine the role of peptide transport in supplying BCAAs, as we have characterized the phenotypes associated with mutations in the oligopeptide transporter (*opp3*) and the di/tri-peptide transporter (*dtpT*). These mutations, coupled with either BCAA transporters or synthesis, would allow for a comprehensive study of the relative contribution of BCAA scavenging vs synthesis systems in different infection models, including a wound model, pneumonia, sepsis and skin abscess. One limitation of using single gene mutations to dissect this problem is that the strategies *S. aureus* employs to obtain BCAAs *in vivo* are likely dynamic. It is not fully understood what threshold concentrations of Ile or Leu are required for repression of synthesis via the mechanisms we identified, and it is likely that there are waves of synthesis that correlate with fluctuations in the extracellular availability of BCAAs. To fully appreciate the adaptability of *S. aureus* as it encounters changing environments, an ideal approach moving forward would be to monitor the infectious process in real-time using *in vivo* imaging technologies and to measure the kinetics of gene-expression using promoter:reporter systems. These studies would be insightful towards understanding the environments where pathogens encounter nutrient limitation and how they overcome this, which might inform efforts to develop novel therapeutics.

5.2 Does transporter specificity provide an adaptive advantage in *S. aureus*?

An intriguing finding of our studies is the specificity of a given transporter for supporting a certain physiological role of BCAAs. This is best exemplified by the results of the fatty acid membrane analysis of each of the transporter mutants. BcaP functions as a Leu and Val transporter as evidenced by the C^{14}-uptake and growth assays (Figure 3.2 and Figure 3.5), and yet BcaP-dependent Leu and Val transport does not contribute to the synthesis of Leu- and Val-derived BCFAs. Therefore, it remains to be determined how *S. aureus* differentiates between Leu and Val transported by BrnQ1 versus BcaP. A similar phenomenon is observed

with CodY regulation; although all three transporters contribute to Ile uptake, CodY activity is most effected in response to BrnQ2-dependent Ile depletion. Such detailed analysis of transporter function as it relates to bacterial physiology (aside from growth) are limited, if not non-existent. Of closest comparison is a recent study in *B. subtilis*, that demonstrated that CodY activity is diminished in a strain lacking all three BCAA transporters identified in this species to date (1). All three transporters are capable of Ile transport, the predominant BCAA to regulate CodY activity in this species as well (9), thus it might prove interesting to investigate the effect of each on CodY activity individually. Studies investigating the localization of transporters in the membrane or assessing protein-protein interactions might help explain the link between an individual transporter and the physiological role of BCAAs.

Whatever the mechanism may be, the data support a clear distinction between transporters supporting nutritional needs (BrnQ1 and BcaP) and transporters supporting regulatory needs (BrnQ2). It is tempting to speculate that this distinction, accomplished by the evolution of a dedicated Ile transporter, might confer a fitness advantage, whereby BrnQ2 provides a direct connection between exogenous availability of the most predominant CodY effector, Ile, to endogenous CodY activity. No other BrnQ or BcaP homologs described to date exhibit such substrate specificity (Table 1.2, Table 1.3). To fully appreciate the link between BrnQ2 and CodY-dependent virulence gene regulation, it would be beneficial to compare the transcriptomes of a *brnQ2* mutant to that of a *codY* mutant. Ile has previously been shown to relieve CodY-dependent repression of the hyaluronidase virulence factor in *S. aureus* (10) and describing the exact mechanism governing this would prove novel towards understanding the role of exogenous BCAAs in regulating virulence gene expression. It is also of interest to evaluate the virulence of the *brnQ2* mutant in various animal models to test the hypothesis that levels of Ile in host environments regulate *S. aureus* virulence. Mouse models of skin infection and necrotizing pneumonia would be of priority, since decreased CodY activity in these environments has drastic consequences on *S. aureus* virulence (11). If a *brnQ2* mutant mimics a *codY* mutant in these models, it would indicate that the murine skin and lung environments are high in Ile and CodY is typically active. Another interesting environment to explore is the intracellular environment. Host cytosolic Ile is an important metabolic cue for intracellular infection of pathogen *L. monocytogenes* in a CodY-dependent manner (12), and *S. aureus* can survive and replicate within the macrophage phagolysosome

(13). It would be of interest to investigate whether Ile similarly acts as a metabolic signal for *S. aureus*. One of the main challenges limiting the study of CodY regulation *in vivo* is that it functions primarily a repressor in *S. aureus*; that is to say, a WT and *codY* mutant strain would behave the same in any environment where low CodY activity is required for replication and virulence. Although variants of CodY with reduced regulatory activity have been generated, the opposite, variants with increased regulatory activity, have proved difficult to generate (14). A full appreciation for CodY regulation will therefore likely require real-time monitoring of CodY target gene expression *in vivo* using luminescent or fluorescent promoter:reporter strains. With this system, it would be of high interest to investigate CodY regulation in the nasal cavity. Human nasal secretions are nutrient limited, especially for Ile and, as a consequence, *S. aureus* up-regulates metabolic genes, including amino acid synthesis genes, when grown in a synthetic nasal medium (7). The low Ile availability might be key to regulating its adaptation to this host environment.

5.3 Conclusion

The survival of *S. aureus* in diverse host environments reflects its ability to adapt to physical and nutritional changes, yet the molecular mechanisms that facilitate this adaptation have remained incompletely understood. This thesis investigated unanswered questions related the initial stages of this adaptation – including what strategies does *S. aureus* have available to acquire metabolites in nutrient deprived environments, how does it regulate which strategy it employs, and how does nutrient availability contribute to virulence gene regulation to promote *S. aureus* survival. Specifically, this research focused on the BCAAs given their importance for *S. aureus* growth (i.e. protein synthesis and BCFA synthesis) and environmental adaptation via CodY.

This work provides the first detailed description of how *S. aureus* obtains BCAAs and discerns what mechanisms best serve the various physiological roles of BCAAs in the cells. The implications of this are two-fold; first, the identification and characterization of BrnQ1 and BcaP implicates these transporters as important nutrient scavenging systems for protein and BCFA synthesis. Their requirement for *S. aureus* infection and colonization indicates that *S. aureus* does indeed encounter BCAA limitation *in vivo* and that these transporters are necessary for exploitation of host sources of BCAAs. These advancements lay the foundation

for future studies to continue to investigate the contribution of these transporters in additional host environments, with the potential of revealing infection sites where therapeutics targeting BCAA metabolism could be used to reduce *S. aureus* replication. Second, the elucidation of the mechanisms regulating BCAA biosynthesis reveal a novel regulatory pathway involving the Ile-specific transporter BrnQ2 that links exogenous Ile availability to CodY activity. These data suggest that Ile is an important cue for the adaptation of *S. aureus* to nutrient limitation, and might also potentially serve as an important cue for virulence gene regulation.

Altogether this work advances the knowledge of *S. aureus* basic biology, revealing important growth and adaptation strategies necessary for survival in the human host.

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Appendix

Appendix A: BLAST sequence alignment of *S. aureus ilvD* 5'UTR

Strain[a]	% Identity
Staphylococcus aureus strain USA300-SUR24	719/719 (100%)
Staphylococcus aureus strain USA300-SUR23	719/719 (100%)
Staphylococcus aureus strain USA300-SUR22	719/719 (100%)
Staphylococcus aureus strain USA300-SUR21	719/719 (100%)
Staphylococcus aureus strain USA300-SUR20	719/719 (100%)
Staphylococcus aureus strain USA300-SUR19	719/719 (100%)
Staphylococcus aureus strain USA300-SUR18	719/719 (100%)
Staphylococcus aureus strain USA300-SUR17	719/719 (100%)
Staphylococcus aureus strain USA300-SUR16	719/719 (100%)
Staphylococcus aureus strain USA300-SUR15	719/719 (100%)
Staphylococcus aureus strain USA300-SUR14	719/719 (100%)
Staphylococcus aureus strain USA300-SUR13	719/719 (100%)
Staphylococcus aureus strain USA300-SUR12	719/719 (100%)
Staphylococcus aureus strain USA300-SUR11	719/719 (100%)
Staphylococcus aureus strain USA300-SUR10	719/719 (100%)
Staphylococcus aureus strain USA300-SUR9	719/719 (100%)
Staphylococcus aureus strain USA300-SUR8	719/719 (100%)
Staphylococcus aureus strain USA300-SUR7	719/719 (100%)
Staphylococcus aureus strain USA300-SUR6	719/719 (100%)
Staphylococcus aureus strain USA300-SUR4	719/719 (100%)
Staphylococcus aureus strain USA300-SUR3	719/719 (100%)
Staphylococcus aureus strain USA300-SUR2	719/719 (100%)
Staphylococcus aureus strain USA300-SUR1	719/719 (100%)
Staphylococcus aureus strain USA300-SUR5	719/719 (100%)
Staphylococcus aureus strain SR434	719/719 (100%)
Staphylococcus aureus subsp. aureus strain USA300_SUR1	719/719 (100%)
Staphylococcus aureus subsp. aureus isolate Clinical isolate	719/719 (100%)
Staphylococcus aureus subsp. aureus strain HG001	719/719 (100%)
Staphylococcus aureus DNA, complete genome, strain: OC8	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 2148.CO1	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 5118.N	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 1625.CO1	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 1969.N	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 1971.CO1	719/719 (100%)
Staphylococcus aureus subsp. aureus strain 2148.N	719/719 (100%)

Staphylococcus aureus isolate Sa_Newman_UoM	719/719 (100%)
Staphylococcus aureus strain V605	719/719 (100%)
Staphylococcus aureus strain V521	719/719 (100%)
Staphylococcus aureus strain FORC_012	719/719 (100%)
Staphylococcus aureus strain UTSW MRSA 55	719/719 (100%)
Staphylococcus aureus subsp. aureus strain Gv88	719/719 (100%)
Staphylococcus aureus subsp. aureus strain Gv51	719/719 (100%)
Staphylococcus aureus subsp. aureus strain Be62	719/719 (100%)
Staphylococcus aureus subsp. aureus strain HC1335	719/719 (100%)
Staphylococcus aureus strain HUV05	719/719 (100%)
Staphylococcus aureus strain CA12	719/719 (100%)
Staphylococcus aureus strain V2200	719/719 (100%)
Staphylococcus aureus subsp. aureus strain USA300_2014.C01	719/719 (100%)
Staphylococcus aureus subsp. aureus strain USA300_2014.C02	719/719 (100%)
Staphylococcus aureus strain CA15	719/719 (100%)
Staphylococcus aureus strain M121	719/719 (100%)
Staphylococcus aureus subsp. aureus DSM 20231	719/719 (100%)
Staphylococcus aureus subsp. aureus DNA	719/719 (100%)
Staphylococcus aureus subsp. aureus DNA	719/719 (100%)
Staphylococcus aureus strain 27b_MRSA	719/719 (100%)
Staphylococcus aureus strain 25b_MRSA	719/719 (100%)
Staphylococcus aureus strain 26b_MRSA	719/719 (100%)
Staphylococcus aureus strain 33b	719/719 (100%)
Staphylococcus aureus strain 31b_MRSA	719/719 (100%)
Staphylococcus aureus strain 29b_MRSA	719/719 (100%)
Staphylococcus aureus strain 2395 USA500	719/719 (100%)
Staphylococcus aureus strain XN108	719/719 (100%)
Staphylococcus aureus strain UA-S391_USA300	719/719 (100%)
Staphylococcus aureus strain NRS 100	719/719 (100%)
Staphylococcus aureus USA300-ISMMS1	719/719 (100%)
Staphylococcus aureus subsp. aureus Z172	719/719 (100%)
Staphylococcus aureus subsp. aureus CN1	719/719 (100%)
Staphylococcus aureus Bmb9393	719/719 (100%)
Staphylococcus aureus M1	719/719 (100%)
Staphylococcus aureus subsp. aureus VC40	719/719 (100%)
Staphylococcus aureus subsp. aureus T0131	719/719 (100%)
Staphylococcus aureus subsp. aureus str. JKD6008	719/719 (100%)
Staphylococcus aureus subsp. aureus TW20	719/719 (100%)
Staphylococcus aureus subsp. aureus USA300_TCH1516	719/719 (100%)
Staphylococcus aureus subsp. aureus str. Newman DNA	719/719 (100%)

Staphylococcus aureus subsp. aureus COL	719/719 (100%)
Staphylococcus aureus subsp. aureus NCTC 8325	719/719 (100%)
Staphylococcus aureus subsp. aureus USA300_FPR3757	719/719 (100%)
Staphylococcus aureus subsp. aureus strain ATCC 6538	718/719 (99%),
Staphylococcus aureus subsp. aureus strain UCI 28	718/719 (99%),
Staphylococcus aureus subsp. aureus strain UCI62	718/719 (99%),
Staphylococcus aureus strain AUS0325	718/719 (99%),
Staphylococcus aureus strain FORC_027	718/719 (99%),
Staphylococcus aureus strain 08-02300	718/719 (99%),
Staphylococcus aureus strain NCCP14562	718/719 (99%),
Staphylococcus aureus strain NCCP14558	718/719 (99%),
Staphylococcus aureus strain ZJ5499	718/719 (99%),
Staphylococcus aureus strain RIVM6519	718/719 (99%),
Staphylococcus aureus strain ST20130943	718/719 (99%),
Staphylococcus aureus strain ST20130942	718/719 (99%),
Staphylococcus aureus strain FDAARGOS_159	718/719 (99%),
Staphylococcus aureus strain NZAK3	718/719 (99%),
Staphylococcus aureus DNA, strain: MI	718/719 (99%),
Staphylococcus aureus DNA, strain: FDA209P	718/719 (99%),
Staphylococcus aureus subsp. aureus strain GR2	718/719 (99%),
Staphylococcus aureus strain SA564	718/719 (99%),
Staphylococcus aureus strain HOU1444-VR	718/719 (99%),
Staphylococcus aureus strain FCFHV36	718/719 (99%),
Staphylococcus aureus genome assembly NCTC13435	718/719 (99%),
Staphylococcus aureus strain 502A	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus ST228, isolate	718/719 (99%),
Staphylococcus aureus subsp. aureus 11819-97	718/719 (99%),
Staphylococcus aureus subsp. aureus ECT-R 2	718/719 (99%),
Staphylococcus aureus 04-02981	718/719 (99%),
Staphylococcus aureus subsp. aureus Mu3 DNA	718/719 (99%),
Staphylococcus aureus subsp. aureus JH1	718/719 (99%),
Staphylococcus aureus subsp. aureus JH9	718/719 (99%),
Staphylococcus aureus subsp. aureus Mu50 DNA	718/719 (99%),

Staphylococcus aureus subsp. aureus N315 DNA	718/719 (99%),
Staphylococcus aureus strain FORC_026	717/719 (99%),
Staphylococcus aureus strain 08-02119	717/719 (99%),
Staphylococcus aureus strain ST20130939	717/719 (99%),
Staphylococcus aureus strain ST20130938	717/719 (99%),
Staphylococcus aureus strain ST20130941	717/719 (99%),
Staphylococcus aureus strain ST20130940	717/719 (99%),
Staphylococcus aureus strain XQ	717/719 (99%),
Staphylococcus aureus strain RKI4	717/719 (99%),
Staphylococcus aureus strain 93b_S9	717/719 (99%),
Staphylococcus aureus subsp. aureus JKD6159	717/719 (99%),
Staphylococcus aureus subsp. aureus ED133	717/719 (99%),
Staphylococcus aureus subsp. aureus ED98	717/719 (99%),
Staphylococcus aureus subsp. aureus MW2 DNA	717/719 (99%),
Staphylococcus aureus strain SJTUF_J27	716/719 (99%),
Staphylococcus aureus strain MCRF184	716/719 (99%),
Staphylococcus aureus subsp. aureus strain JS395	716/719 (99%),
Staphylococcus aureus strain 79_S10	717/720 (99%),
Staphylococcus aureus strain 144_S7	717/720 (99%),
Staphylococcus aureus subsp. aureus strain ATCC 25923	716/719 (99%),
Staphylococcus aureus subsp. aureus 6850	716/719 (99%),
Staphylococcus aureus subsp. aureus 55/2053	716/719 (99%),
Staphylococcus aureus CA-347	716/719 (99%),
Staphylococcus aureus subsp. aureus LGA251	716/719 (99%),
Staphylococcus aureus subsp. aureus TCH60	716/719 (99%),
Staphylococcus aureus strain MSSA476	716/719 (99%),
Staphylococcus aureus strain 71A_S11	715/719 (99%),
Staphylococcus aureus subsp. aureus ST772-MRSA-V strain DAR4145	716/720 (99%),
Staphylococcus aureus subsp. aureus strain FORC_001	715/719 (99%),
Staphylococcus aureus genome assembly Staphylococcus_aureus_ILR	715/719 (99%),
Staphylococcus aureus strain SA40TW	714/719 (99%),
Staphylococcus aureus subsp. aureus SA268	714/719 (99%),
Staphylococcus aureus subsp. aureus strain H-EMRSA-15	714/719 (99%),
Staphylococcus aureus subsp. aureus SA40	714/719 (99%),
Staphylococcus aureus subsp. aureus SA957	714/719 (99%),
Staphylococcus aureus subsp. aureus HO 5096 0412	714/719 (99%),
Staphylococcus aureus subsp. aureus M013	714/719 (99%),
Staphylococcus aureus subsp. aureus strain MRSA252	714/719 (99%),
Staphylococcus aureus strain 08S00974	713/719 (99%),
Staphylococcus aureus strain NZ15MR0322	713/719 (99%),

Staphylococcus aureus subsp. aureus Tager 104	713/719 (99%),
Staphylococcus aureus strain RIVM3897	713/719 (99%),
Staphylococcus aureus strain RIVM1607	713/719 (99%),
Staphylococcus aureus 08BA02176	713/719 (99%),
Staphylococcus aureus subsp. aureus ST398	713/719 (99%),
Staphylococcus aureus subsp. aureus strain LA-MRSA ST398 isolate	712/719 (99%),
Staphylococcus aureus strain RIVM1295	713/720 (99%),
Staphylococcus aureus subsp. aureus 71193	712/719 (99%),
Staphylococcus aureus strain BB155	711/719 (99%),
Staphylococcus aureus strain SA17_S6	710/719 (99%),
Staphylococcus aureus RF122	710/719 (99%),
Staphylococcus aureus subsp. aureus strain HC1340	562/562 (100%)
Staphylococcus aureus subsp. aureus strain HC1340	132/132 (100%)
Staphylococcus aureus subsp. aureus strain Gv69	562/562 (100%)
Staphylococcus aureus subsp. aureus strain Gv69	132/132 (100%)

[a] The 5'UTR sequence of *ilvD* from USA300 FPR3757 (719 bp) was aligned to all *S. aureus* complete assembled genomes (168 as of May 7th 2017) using BLAST.

Curriculum Vitae

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Post-secondary Education and Degrees:

University of Western Ontario
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McMaster University
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2011-2013 M.Sc.

McGill University
Montreal, Quebec, Canada
2007-2011 B.Sc.

Scholarships:

RGE Murray Scholarship, $20,000
2016-2017
Ontario Graduate Scholarship, Ph.D. (Declined), $15,000
2016-2017
Canada Graduate Scholarship Doctoral Award, CIHR, $30,000/yr
2013-2016
Frederick Banting and Charles Best Canada Graduate Scholarship, Master's Award, CIHR, $17,500
2012-2013
Ontario Graduate Scholarship, M.Sc. (Declined), $15,000
2012-2013
Ontario Graduate Scholarship, M.Sc., $15,000
2011-2012

Honours and Awards:

John A. Thomas Award, Department of Microbiology and Immunology, University of Western Ontario
2017
Oral Presentation Award, Third Place, Infection and Immunity Research Forum, University of Western Ontario
2015
Dr. Frederick W. Luney Graduate Entrance Award, Microbiology and Immunology, University of Western Ontario
2014
Graduate Program Excellence Award, Faculty of Health Science, McMaster University
2013
Excellence in Oral Presentations, Faculty of Health Science Research Plenary, McMaster University
2013

Institute for Infectious Disease Research Award of Excellence M.Sc. Poster Award, McMaster University
2012
Microbiology Honours Student Seminar Award, McGill University
2011
Entrance Scholarship, McMaster University
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Dean's Honour List, McGill University
2009, 2011
Faculty of Science Scholarship, McGill University
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Related Work Experience

Teaching Assistant
University of Western Ontario, London ON, Canada
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Research Assistant
McMaster University, Hamilton ON, Canada
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Research Assistant
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Publications:

Kaiser JC, Sen S, Sinha A, Wilkinson BJ, Heinrichs DE. The role of two branched-chain amino acid transporters in *Staphylococcus aureus* growth, membrane fatty acid composition and virulence. *Mol Micro*. 2016 Dec 2015; 102(5):850-864

Alnaseri H, Arsic B, Schneider JE, **Kaiser JC**, Scinocca ZC, Heinrichs DE, McGavin MJ. Inducible expression of a resistance-nodulation-division-type efflux pump in *Staphylococcus aureus* provides resistance to linoleic and arachidonic acids. *J Bacteriol*. 2015 Jun;197(11):1893-905

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Oral Presentations:

Defining the role of branched-chain amino acid transporters in *Staphylococcus aureus* growth and virulence
Infection and Immunity Research Forum, 2015
University of Western Ontario, London ON, CA

Cytokine profiles define the immunogenic spectrum of the *Streptococcus* Milleri Group
Faculty of Health Sciences Research Plenary, 2013
McMaster University, Hamilton ON, CA

Poster Presentations:

The role of two branched-chain amino acid transporters in *Staphylococcus aureus* growth, membrane fatty acid content, and virulence. 2016, Kaiser J, Sen S, Sinha A, Wilkinson BJ, Heinrichs DE
International Conference on the Pathophysiology of Staphylococci, Tübingen, Germany

The role of two branched-chain amino acid transporters in *Staphylococcus aureus* growth, membrane fatty acid content, and virulence. 2016, Kaiser J, Sen S, Sinha A, Wilkinson BJ, Heinrichs DE
American Society of Microbiology, Boston MA, USA

The role of two branched-chain amino acid transporters in *Staphylococcus aureus* growth, membrane fatty acid content, and virulence. 2016, Kaiser J, Sen S, Sinha A, Wilkinson BJ, Heinrichs DE
Canadian Society of Microbiologists, Toronto ON, CA

The role of branched-chain amino acid transporters in nutrient acquisition and virulence of *Staphylococcus aureus*. 2015, Kaiser J, Omer S, Sheldon JR, Welch I, Heinrichs DE
American Society of Microbiology, New Orleans LA, USA

Role of CodY-regulated BrnQ1 and BrnQ2 in uptake of branched-chain amino acids and virulence in *Staphylococcus aureus*. 2015, Kaiser J, Omer S, Sheldon JR, Welch I, Heinrichs DE
London Health Research Day, University of Western Ontario, London ON, CA

Role of CodY-regulated BrnQ1 and BrnQ2 in uptake of branched-chain amino acids and virulence in *Staphylococcus aureus*. 2014, Kaiser J, Omer S, Sheldon JR, Welch I, Heinrichs DE

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International Symposium on Staphylococci and Staphylococcal Infections, Chicago IL, USA

Host-Microbe Interactions: Survey of strain variability in the *Streptococcus* Milleri Group. 2012, Kaiser J, Verschoor C., Bowdish DM, Surette MG
Infection and Immunity Research Forum, University of Western Ontario, London ON, CA

Exploring the mechanisms of pathogenesis of the *Streptococcus* Milleri Group. 2012, Kaiser J, Pinto M, Jayanth P, Stearns J, Pelka P, Bowdish DM, Surette MG
Buffalo Immunology Conference, Ellicottville NY, USA

Exploring the mechanisms of pathogenesis of the *Streptococcus* Milleri Group. 2012, Kaiser J, Pinto M, Jayanth P, Stearns J, Pelka P, Bowdish DM, Surette MG
Institute for Infectious Disease Research Trainee Day, McMaster University, Hamilton ON, CA

Professional Activities:

Student Representative to the Graduate Studies Committee
University of Western Ontario, London ON
2013-2017

Organizing Committee, Infection and Immunity Research Forum
University of Western Ontario, London ON
2013-2016

Judge, Thames Valley Science & Engineering Fair
London, ON
2014, 2015

Contributing author, Let's Talk Science Challenge Handbook
2014

Organizing Committee, Women in Science and Technology Panel Discussion, Canadian Science Policy Centre
2013

Judge, Bay Area Science & Engineering Fair
Hamilton, ON
2013

Graduate Student Mentor, McMaster International Genetically Engineered Machine (iGEM) Team
McMaster University, Hamilton ON
2012-2013

Executive Member, Human Microbiome Journal Club (HMjc)
McMaster University, Hamilton ON
2011-2013