

Characterization of the branched chain amino acid metabolic pathway and its
regulators in *Aspergillus nidulans*

by

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BA (Honors), William Jewell College, 2016

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Plant Pathology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

The branched chain amino acids (BCAAs) isoleucine, leucine, and valine are essential amino acids because they are not synthesized by mammals. Many fungal species, including *Aspergillus*, *Magnaporthe*, *Fusarium*, and *Candida* are capable of de novo BCAAs synthesis. Crop and human pathogens, such as *Magnaporthe oryzae* and *Aspergillus fumigatus*, respectively, require BCAAs for full pathogenicity. BCAA pathway enzymes and transcriptional regulators are potential targets for anti-fungal treatments as treatments can target the pathogen without harming its host. In this dissertation, the model eukaryotic filamentous fungus *Aspergillus nidulans* is used to complete the BCAA biosynthetic pathway and characterize transcription factors that regulate the BCAA pathway. The first part of this dissertation focuses on analysis of the full BCAA biosynthesis pathway in *A. nidulans*. The final step in BCAA biosynthesis and first step in BCAA catabolism is catalyzed by branched chain amino acid aminotransferase (BAT) enzymes. *A. nidulans* encodes six putative *bat* genes. Separate deletion of each *bat* gene indicated that each is dispensable. Two of the *bat* genes play a role in secondary metabolite production so the roles of the remaining four *bat* genes were the focus of this work. Double, triple, and quadruple deletion mutants of the four *bat* genes were constructed. Deletion of both *batA* and *batB* in the double, triple, or quadruple deletion mutants confers tight BCAA auxotrophy, revealing that both genes act in BCAA biosynthesis. The *batA* and *batB* genes were also the two most highly expressed *bat* genes. Furthermore, the biosynthetic and catabolic profiles for each enzyme were characterized, *batA* primarily being biosynthetic and *batB* primarily catabolic. During this research, spontaneous suppressor mutants of the *batA batB* double deletion mutant arose and these new mutants were BCAA prototrophs. To determine the genetic mutation that suppressed the BCAA auxotrophy, Whole Genome Sequencing was performed on ten independent spontaneous suppressor mutants. Single Nucleotide Polymorphism (SNP) analysis revealed that all ten spontaneous suppressor mutants had mutations in six genes. One of these genes, which is currently uncharacterized in *A. nidulans*, contains a SPT2 domain sequence. SPT2 domains are associated with transcriptional regulation

in hypoxic conditions. Disruption of the SPT2 domain may de-repress expression of genes that are normally repressed during oxygen-replete conditions, and de-repression of an unknown gene may overcome the BCAA auxotrophy of the *batA batB* double mutant. The second part of this research focused on understanding the transcriptional regulation of the BCAA pathway genes. In *A. nidulans*, the transcription factor LeuB regulates leucine biosynthesis genes and the major nitrogen assimilation gene, *gdhA*. The *leuB* deletion mutant is a partial leucine auxotroph, meaning it grows poorly without exogenous leucine. One explanation is that another transcription factor may also activate leucine biosynthesis genes. *A. nidulans* encodes two additional transcription factors with sequence similarity to LeuB. To further understand the relationship between LeuB and its paralogs, phylogenetic analysis was performed. LeuB is more broadly conserved than the other two transcription factors. The more closely related transcription factor was named LeuR and it was hypothesized that LeuR regulation accounted for the partial leucine auxotrophy observed in the *leuB* deletion mutants. The *leuR* deletion mutant is a leucine prototroph. However, the *leuB leuR* double deletion mutant shows tight leucine auxotrophy indicating a role for LeuR in regulation of leucine biosynthesis. To characterize the function of LeuR, reporter gene assays were utilized to determine if LeuR also regulates transcription of the important nitrogen assimilation gene *gdhA*, which encodes NADP-glutamate dehydrogenase. *gdhA-lacZ* reporter gene assays showed that LeuR, in the absence of LeuB, regulates *gdhA*. Using promoter-deletion strains, the regions where LeuR acts were narrowed to two sites. These sites of action are also shared by LeuB, indicating conservation of sites of action between the two transcription factors. Additionally, RNA-Seq was used to characterize the genome-wide regulation of *A. nidulans* by LeuB and LeuR to determine which gene targets they regulate. The research detailed in this dissertation builds the framework of the branched chain amino acid biosynthetic pathway and the major regulators of the pathway, showcasing which enzymes and transcription factors are the most prudent potential antifungal targets. This research highlights the need for in-depth analysis to reveal the roles of paralogous genes as we show that loss of one functional gene does not cause BCAA auxotrophy. Therefore, incomplete disruption of the pathway or its regulation by anti-

fungal treatments may cause adaptation and resistance rendering such anti-fungal agents ineffective.

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Approved by:

Major Professor
Dr. Richard B. Todd

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Thank you all, from the bottom of my heart.

Dedication

"Live now; make now always the most precious time.

Now will never come again."

– Captain Jean-Luc Picard, Star Trek: The Next Generation

This is dedicated to everyone that made each moment
throughout my Ph.D. a memory to cherish.

My family and my friends, both near and far.

To the joy we created.

To the laughter we made.

To the love we share.

Thank you.

Chapter 1 - Introduction and Objectives

1.1 Introduction

1.1.1 Importance of Branched Chain Amino Acids in Fungal Growth

Fungi occupy a myriad of diverse niches in the environment, spanning biomes and ecological strategies from heterotrophs, necrotrophs, to saprotrophs. Fungi also live with and inside of plants and animals, some as symbionts and others as pathogens. Many aspects, such as timing, weather, and nutrient availability, can affect the ability of a fungal pathogen to infect a host. The branched chain amino acids (BCAAs) isoleucine, leucine, and valine are especially important as they are not produced in mammalian hosts and can be a limiting nutrient factor in hosts during infection. BCAAs are needed as precursors in protein synthesis, are involved in secondary metabolite production, and affect the virulence of human and plant fungal pathogens. BCAA mutants of human fungal pathogens such as *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus* show decreased pathogenicity in comparison to wild type fungal pathogens (Do et al., 2015; Garcia et al., 2018; Kingsbury and McCusker, 2010; Oliver et al., 2012; Orasch et al., 2019; Richie et al., 2013). Plant pathogens such as *Magnaporthe oryzae* and *Fusarium graminearum* require BCAA biosynthesis for full virulence (Du et al., 2013; Du et al., 2014; Liu et al., 2014; Liu et al., 2015; Liu et al., 2016; Que et al., 2020). By producing BCAAs de novo, fungal pathogens do not have to outcompete host cells for the limited availability of BCAAs.

The BCAA biosynthesis pathway is well-characterized in the yeast *Saccharomyces cerevisiae* (Kohlhaw, 2003). Isoleucine, leucine, and valine share the early steps of the biosynthesis pathway before diverging into amino acid specific steps. While the steps are similar in filamentous fungi, the number of genes encoding the enzymes for each step varies greatly. Paralogous genes can provide genome robustness in terms of environmental responses as loss of one gene or functional protein can be compensated by the other (Escalera-Fanjul et al., 2019; Wapinski et al., 2007). Gene duplications can arise from whole-genome duplication events, as in

the *S. cerevisiae* lineage, or by other genomic events such as unequal crossover during sexual reproduction in the *Aspergillus* lineage (Galagan et al., 2005; Escalera-Fanjul et al., 2019; Wapinski et al., 2007; Montalvo-Arredondo et al., 2015).

The model eukaryotic filamentous fungus *Aspergillus nidulans* is a non-pathogenic filamentous fungus that is closely related to other ascomycete fungal pathogens. *A. nidulans* has a fully sequenced genome and many areas of research have benefitted from the ease of genetic manipulation that *A. nidulans* can provide (Galagan et al., 2005; Nayak et al., 2006; Pontecorvo et al., 1953; Todd et al., 2007a; Todd et al., 2007b). The understanding and manipulation of industrially relevant microorganisms as well as characterization of important human and plant fungal pathogens has benefitted from work done in *A. nidulans* due to its genetic techniques, secondary metabolite biosynthetic gene clusters, and ease of laboratory culture methods (Osmani et al., 2006; Davis et al., 1988; MacCabe et al., 2001; Chiang et al., 2008; Chiang et al., 2016).

The leucine biosynthesis pathway was not fully characterized in *A. nidulans* at the outset of this work. The first two steps, α -isopropylmalate (α -IPM) synthesis by α -IPM synthetase and conversion of α -IPM to β -IPM by isopropylmalate isomerase, have been characterized (Downes et al., 2013; Polotnianka et al., 2004). The final two steps, catalyzed by the β -IPM dehydrogenase and the branched chain amino acid aminotransferase (BAT), are characterized in this dissertation. This is a significant gap in knowledge and determining the genes involved would be beneficial to both understanding fungal pathogens better and generating more anti-fungal treatment targets.

1.1.2 Spontaneous Mutants and Genome Evolution

DNA is replicated continuously by DNA polymerases, and most of the time they have an extremely high-fidelity rate. When DNA polymerases do make a mistake in replication, that new base pair is incorporated as part of the DNA template and propagated the next time DNA is replicated (Falaschi and Kornberg, 1966). This base pair change in the DNA is called a single nucleotide polymorphism (SNP). SNPs can arise in any region of the genome and depending on the location of the change, can

either be detrimental or harmless. SNPs are classified based on what kind of change it causes. If a SNP occurring in a coding region would change the amino acid encoded during translation, it is a nonsynonymous substitution. Nonsynonymous substitutions can also cause premature termination of the protein product if the change generates a stop codon. SNPs in coding regions that do not change the amino acid encoded are considered synonymous substitutions. Faulty replication of the DNA is not the only way SNPs can occur, as mutagenesis with toxic compounds, UV, and extreme conditions can cause widespread mutations in a genome (Boeke et al., 1987; Downes et al., 2014a; Scott and Maley, 1981; Álvarez-Escribano et al., 2019).

A majority of SNPs have no phenotypic effect as they occur outside of protein coding regions or promoter regions. Some SNPs do happen in protein coding regions however, altering the functionality of the protein. These changes can be loss-of-function mutations, in which the protein has lost some functional aspect that it would normally have, or gain-of-function, where a protein has a new function that it normally does not. Other classifications of SNPs include suppressor mutants. This is the case when a SNP or set of SNPs suppresses a mutant phenotype, such as lack of colony growth under a certain set of conditions (Prelich, 1999). Suppressors can occur under numerous conditions, such as alternative nitrogen and carbon metabolism, temperature sensitivity, sexual reproduction and conidiation (Seo et al., 2003; Arst and Cove, 1973; Mooney et al., 1990; Jarvik and Botstein, 1975; Roberts et al., 1979).

SNPs are important in genetics as they can provide insight into the residues that a protein needs to function, helping to determine the catalytic domains and functions of those proteins. Additionally, SNPs can inform additional enzymatic or regulatory genes that interact in a metabolic pathway that were previously unknown to function in that pathway. This provides a more robust understanding of metabolic pathways and the additional measures that the organism can implement to overcome a genetic obstacle.

1.1.3 Transcriptional Regulation of Branched Chain Amino Acid

Metabolism

The regulation of the BCAA pathway occurs primarily through the pathway-specific transcription factor Leu3p in *S. cerevisiae* and the orthologous transcription factor LeuB in both *Aspergillus nidulans* and *Aspergillus fumigatus* (Kohlhaw, 2003; Polotnianka et al., 2004; Steyer et al., 2021; Downes et al., 2013; Long et al., 2018). Both are involved in a negative feedback loop that is regulated by leucine concentrations, modulating the expression of leucine biosynthesis pathway genes (Brisco and Kohlhaw, 1990; Downes et al., 2013; Polotnianka et al., 2004). Feedback inhibition occurs in other metabolic processes to regulate end product production (Scott and Spencer, 1968; Borgia, 1992). The *A. nidulans leuB* Δ deletion mutant is a leaky leucine auxotroph, meaning that there is some growth without exogenous leucine (Downes et al., 2013).

The transcription factors Leu3p and LeuB have also been shown to regulate their respective NADP-dependent glutamate dehydrogenase genes, *GDH1* and *gdhA* (Downes et al., 2013; Hu et al., 1995; Long et al., 2018; Polotnianka et al., 2004). In addition to leucine and nitrogen metabolism regulation, the LeuB ortholog in *A. fumigatus* also regulates iron metabolism and iron acquisition genes (Long et al., 2018; Orasch et al., 2019). Iron is an essential nutrient for all eukaryotes and has low bioavailability due to its ability to produce reactive oxygen species (Kaplan and Kaplan, 2009; Halliwell and Gutteridge, 1984). Iron is an important cofactor for certain enzymes, including the NAD-dependent glutamate synthase (GOGAT) and the leucine biosynthesis enzymes dihydroxy acid dehydratase and α -IPM isomerase LuA (Kohlhaw, 2003; Schrettl et al., 2010; Shakoury-Elizeh et al., 2004). Iron is necessary for effective virulence for fungal pathogens and many pathogens have developed ways to acquire bioavailable iron from their hosts before the hosts sequesters it (Haas et al., 2008; Long et al., 2018; Schrettl et al., 2004; Schrettl et al., 2007; Schrettl et al., 2010).

Overall, the BCAA pathway and the transcription factor LeuB represent potential anti-fungal treatment targets during fungal infection. The BCAA biosynthesis pathway

enzymes are found in fungi and not in mammals, making them potential targets for treatments that can be targeted to the pathogen without affecting the host. The transcription factors, which belong to the Zn(II)₂Cys₆ zinc binuclear cluster DNA-binding domain family only found in fungi (Todd and Andrianopoulos, 1997), are also potential anti-fungal targets as they regulate two important metabolic processes shown to be involved in infection and pathogenicity, specifically BCAA biosynthesis and iron metabolism. Understanding the genes involved in the metabolic pathway is vitally important as many of the steps contain paralogous genes that can overcome or compensate for the loss of one of the genes. To generate effective and comprehensive treatment options, the full array of genes involved in the metabolic pathways should be considered. Incomplete anti-fungal treatments can introduce selective pressure on the pathogen, potentially causing the pathogen to overcome and become resistant.

In this study, the final step of the BCAA biosynthesis pathway is characterized, determining that *batA* and *batB* encode the primary BAT enzymes for both BCAA biosynthesis and catabolism, respectively. Genome analysis revealed that there were two other transcription factors, AN4744 and AN7596, with sequence similarity to *leuB*. The transcription factor LeuR, encoded by AN4744, is shown to be redundant in regulating leucine biosynthesis, *gdhA* expression, and iron metabolic genes. LeuB and LeuR diverge in the number of overall genes they regulate as determined by RNA-Seq.

1.2 Objectives

The overall objectives of this research were to improve our understanding of BCAA metabolism and its regulation in *Aspergillus nidulans*. The major goals were to: (i) characterize the molecular genetics underlying the penultimate step in leucine biosynthesis and the final step in the branched chain amino acid metabolic pathway, (ii) characterize spontaneous suppressor mutants of the BCAA auxotroph *batA*Δ *batB*Δ double mutant, and (iii) understand the regulatory roles of the paralogous transcription factors LeuB, LeuR, and AN7596 in leucine biosynthesis, nitrogen assimilation, and iron metabolism.

Chapter 2 - Experimental Procedures

2.1 Strains

2.1.1 *Aspergillus nidulans* strains

Aspergillus nidulans strains used in this research constructed in the Hynes-Davis lab at the University of Melbourne, Australia, are listed in Table 2.1 using the conventional annotation (Clutterbuck, 1973). Strains constructed in the Todd Lab at Kansas State University by others (Table 2.2) or by J.T. Steyer (Table 2.3) are listed using the conventional annotation. All strains contain the *veA1* mutation. More detailed descriptions of strain construction and usage are specified in the appropriate chapter.

Table 2.1 “Michael Hynes” *Aspergillus nidulans* strains.

Strain	Genotype
MH1	<i>biA1</i>
MH10217	<i>biA1 pyroA4 riboB2 nkuAΔ::Bar leuB</i>
MH10523	<i>biA1 luA1 riboB2</i>
MH10865	<i>yA1 pabaA1 pyrG89 areAΔ::riboB(at 5') fmdS-lacZ</i>
MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar amdA7</i>
MH11094	<i>amdS::lacZ pyroA4 niiA4 nkuAΔ::Bar</i>
MH11324	<i>biA1 pyroA4 tamAΔ leuB::riboB</i>
MH12101	<i>AfpyroA-gdhA(-753 bp)-lacZ@amdS-lacZ pyroA4 nkuAΔ::Bar niiA4</i>
MH12181	<i>biA1 leuBΔ::riboB A.f.pyroA-gdhA(-753 bp)-lacZ@amdS-lacZ pyroA4 niiA4</i>
MH12609	<i>yA1 leuBΔ::riboB nkuAΔ::Bar niiA4 pyroA4 pabaA1</i>

Table 2.2 “Richard Todd” *Aspergillus nidulans* strains constructed by others.

Strain	Origins	Genotype
RT152	Transformant of MBLACB4	<i>niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT155	Transformant of MBLACB4	<i>niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT156	Transformant of MBLACB4	<i>niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT157	Transformant of MBLACB4	<i>niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT182	MH12101 X MH10523	<i>biA1 niiA4 luA1 nkuA::Bar pyroA4 AfpyroA-800bp_{gdhAp-lacZ@amdS-lacZ}</i>
RT195	MH11324 X RT152	<i>leuBΔ biA1niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>

Strain	Origins	Genotype
RT198	MH11324 X RT155	<i>leuBΔ biA1 niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT201	MH11324 X RT156	<i>leuBΔ biA1 niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT204	MH11324 X RT157	<i>leuBΔ biA1 niiA4 nkuA::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT219	Transformant of MH11036	<i>pyroA4 riboB2 leuCΔ::A.f-pyroA nkuA::argB+</i>
RT220	RT219 X MH12102	<i>pyroA4 niiA4 leuCΔ::A.f-pyroA riboB2 AfpyroA-753bp gdhAp- lacZ@amdS-lacZ nkuA::argB</i>
RT349	Transformant of MH11094	<i>AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp- lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT350	RT349 X MH11324	<i>biA1 leuBΔ AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to - 241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT352	Transformant of MH11094	<i>AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT353	RT352 X MH11324	<i>biA1 leuBΔ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT356	Transformant of MH11094	<i>AfpyroA-240bp gdhAp-lacZ@amdS-lacZ mus51Bar pyroA4 niiA4</i>
RT357	RT356 X MH11324	<i>biA1 leuBΔ AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT411	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar ΔAN0912(leuD)::AfpyrG+</i>
RT412	RT411 X MH10865	<i>yA1 pyrG89 nkuAΔ::Bar pabaA1 leuDΔ::AfpyrG+</i>
RT413	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar ΔAN2793(leuE)::AfpyrG+</i>
RT414	RT413 X MH10865	<i>yA1 pyrG89 nkuAΔ::Bar pabaA1 ΔAN2793::AfpyrG+</i>
RT415	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar ΔAN4323(batAΔ)::AfpyrG+</i>
RT416	RT415 X MH10865	<i>yA1 pyrG89 pabaA1 nkuAΔ::Bar batAΔ::AfpyrG+</i>
RT417	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar ΔAN0385::AfpyrG+</i>
RT418	RT417 X MH10865	<i>yA1 pyrG89 pabaA1 nkuAΔ::Bar ΔAN0385::AfpyrG+</i>
RT419	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar ΔAN7876::AfpyrG+</i>
RT440	Transformant of MH11068	<i>pyrG89, pyroA4, nkuAΔ::Bar AN5957Δ(batBΔ)::AfpyrG+</i>
RT441	Transformant of MH11068	<i>pyrG89, pyroA4, nkuAΔ::Bar AN8511Δ(batF)::AfpyrG</i>
RT444	RT411 X RT414	<i>pyrG89 pyroA4 nkuAΔ::Bar leuDΔ leuEΔ</i>
RT453	Cross of MH12181 by MH11068	<i>niiA4 pyroA4 pyrG89 leuBΔ::riboB AfpyroA-800bp gdhAp- lacZ@amdS-lacZ nkuAΔ::Bar</i>

Strain	Origins	Genotype
RT454	Cross of RT418 and RT419	<i>yA1 pyrG89 nkuAΔ::Bar pabaA1 ΔAN0385::AfpYrG+ ΔAN7876::AfpYrG+</i>
RT456	Cross of RT416 and RT440	<i>yA1 pyrG89 nkuAΔ::Bar pabaA1 batAΔ::AfpYrG+ batBΔ::AfpYrG+</i>
RT457	Cross of RT416 and RT440	<i>pyrG89 pyroA4 nkuAΔ::Bar batAΔ::AfpYrG+ batBΔ::AfpYrG+</i>
RT458	RT412 X RT453	<i>yA1 pabaA1 pyrG89 amdS::AfpYrA-gdhA(-753 bp)-lacZ leuDΔ::AfpYrG+</i>
RT466	Cross of RT441 and RT454	<i>pabaA1 pyrG89 AN0385Δ::AfpYrG+AN8511Δ::AfpYrG+ nkuAΔ::Bar</i>
RT475	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar AN7878Δ::AfpYrG+</i>
RT478	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuRΔ::AfpYrG</i>
RT499	RT453 X RT478	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpYrG+ leuBΔ::riboB+ niiA4 gdhA-lacZ-AfpYrA</i>
RT500	RT453 X RT478	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpYrG+ leuBΔ::riboB+</i>
RT503	RT453 X RT478	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpYrG+ niiA4 gdhA-lacZ</i>

Table 2.3 “Richard Todd” *Aspergillus nidulans* strains constructed by Steyer.

Strain	Origins	Genotype
RT520	RT457 X RT466	<i>batAΔ::AfpYrG+ batBΔ::AfpYrG+ batEΔ::AfpYrG+ nkuAΔ::Bar</i>
RT523	RT457 X RT466	<i>batAΔ::AfpYrG+ batBΔ::AfpYrG+ batFΔ::AfpYrG+ pabaA1 nkuAΔ::Bar</i>
RT524	RT457 X RT466	<i>batAΔ::AfpYrG+ batBΔ::AfpYrG+ batEΔ::AfpYrG+ pabaA1 nkuAΔ::Bar</i>
RT525	RT457 X RT466	<i>batBΔ::AfpYrG+ batEΔ::AfpYrG+ batFΔ::AfpYrG+ pyroA4 nkuAΔ::Bar</i>
RT526	RT457 X RT466	<i>batBΔ::AfpYrG+ batFΔ::AfpYrG+ nkuAΔ::Bar</i>
RT531	RT457 X RT466	<i>batBΔ::AfpYrG+ batEΔ::AfpYrG+ batFΔ::AfpYrG+ nkuAΔ::Bar</i>
RT539	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT540	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT541	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT542	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT543	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT544	Transformant of RT444	<i>pyrG89 pyroA4 [leuE::repaired] leuDΔ::AfpYrG</i>
RT636	B2 X A23	<i>batBΔ::AfpYrG+ batEΔ::AfpYrG+ nkuAΔ::Bar</i>
RT642	B2 X A17	<i>pyroA4 nkuAΔ::Bar batAΔ::AfpYrG+ batBΔ::AfpYrG+ batEΔ::AfpYrG+ batFΔ::AfpYrG+</i>
RT645	RT415 X RT 466	<i>pyroA4 nkuAΔ::Bar pabaA1 batAΔ::AfpYrG+ batFΔ::AfpYrG+</i>
RT647	RT415 X RT 466	<i>pabaA1 nkuAΔ::Bar batAΔ::AfpYrG+ batEΔ::AfpYrG+ batFΔ::AfpYrG+</i>
RT648	RT415 X RT 466	<i>pyroA4 nkuAΔ::Bar batAΔ::AfpYrG+ batEΔ::AfpYrG+</i>

Strain	Origins	Genotype
RT649	RT415 X RT 466	<i>pyroA4 nkuAΔ::Bar batAΔ::AfpyrG+ batEΔ::AfpyrG+</i>
RT651	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT652	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT653	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT654	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT655	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT656	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT657	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT658	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT659	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT660	RT457 sector	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT678	Transformant of RT457	<i>pyrG89 nkuAΔ::Bar pyroA4 batAΔ::AfpyrG+ [batB::repaired]</i>
RT681	Transformant of RT457	<i>pyrG89 nkuAΔ::Bar pyroA4 [batA::repaired] batBΔ::AfpyrG+</i>
RT687	RT478 X RT198	<i>leuRΔ::AfpyrG+ niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT689	RT478 X RT195	<i>leuRΔ::AfpyrG+ niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT691	RT478 X RT204	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT692	RT478 X RT204	<i>leuRΔ::AfpyrG+ pyrG89 biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT693	RT478 X RT350	<i>biA1 leuBΔ::riboB+ leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT695	RT478 X RT353	<i>leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT696	RT478 X RT353	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4 biA pyrG89</i>
RT705	RT478 X RT198	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT750	RT478 X RT201	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT751	RT478 X RT201	<i>leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT752	RT478 X RT357	<i>pyroA4 AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar</i>
RT753	RT478 X RT357	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 pyroA4 AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar</i>
RT760	RT478 X RT357	<i>leuRΔ::AfpyrG+ pyroA4 AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar</i>
RT769	RT411 Transformant	<i>pyroA4 pyrG89 nkuAΔ::Bar [AN0912(leuD)::repair]</i>
RT795	RT415 X RT453	<i>leuBΔ::riboB+ batAΔ::AfpyrG pyroA4 pyrG89</i>
RT796	RT440 X RT453	<i>leuBΔ::riboB+ batBΔ::AfpyrG pyroA4 pyrG89</i>
RT808	RT456 X RT478	<i>yA1 pabaA1 leuRΔ::AfpyrG+ batAΔ::AfpyrG+</i>

Strain	Origins	Genotype
RT809	RT456 X RT478	<i>yA1 pyroA4 leuRΔ::AfpyrG+ batbΔ::AfpyrG+</i>
RT814	Transformant of RT500	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpyrG+ leuB::[repaired by linear DNA]</i>
RT816	Transformant of RT500	<i>pyroA4 pyrG89 nkuAΔ::Bar leuR::[repaired by linear DNA] leuBΔ::riboB+</i>
RT837	RT478 X RT195	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ pyrG89 biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT840	RT478 X RT350	<i>biA1 leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT873	Transformant of MH11068	<i>veA1 pyrG89 pyroA4 nkuAΔ::Bar amdA7 AN7596Δ::AfpyrG+</i>
RT877	RT503 X RT182	<i>biA1 niiA4 pyroA4 pyrG89 luA1 leuRΔ::AfpyrG+ AfpyroA-800bpgdhAp-lacZ@amdS-lacZ</i>
RT880	MH12181 X RT182	<i>niiA4 pyroA4 luA1 leuBΔ::riboB+ AfpyroA-800bpgdhAp-lacZ@amdS-lacZ</i>
RT884	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuBΔ::riboB+ AN7596Δ::AfpyrG+</i>
RT885	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuRΔ::AfpyrG+ AN7596Δ::AfpyrG+</i>
RT888	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuBΔ::riboB+ leuRΔ::AfpyrG+ AN7596Δ::AfpyrG+</i>
RT895	RT220 X RT452	<i>pyroA4 leuCΔ::AfpyroA leuBΔ::riboB+ AfpyroA-753 gdhA(p)-lacZ@amdS-lacZ</i>
RT899	RT220 X RT478	<i>pyroA4 niiA4 leuCΔ::AfpyroA leuRΔ::AfpyrG+ AfpyroA-753 gdhA(p)-lacZ@amdS-lacZ</i>

2.1.2 *Escherichia coli* strains

Plasmid construction and manipulation used the *Escherichia coli* strain NM522 [F' *proA⁺B⁺ lacI^q Δ(lacZ)M15/Δ(lac-proAB) glnV thi-1 Δ(hsdS-mcrB)5*] (Gough and Murray, 1983). Details of plasmid construction are provided in each chapter.

2.2 Media and Growth Conditions

2.2.1 *Aspergillus nidulans* media and growth conditions

A. nidulans growth conditions and media were made as dictated by Cove (1966). Aspergillus Nitrogen-free Minimal media (ANM) was adjusted to pH 6.5 and supplemented for nutrient auxotrophies (Todd et al., 2007a; Todd et al., 2007b). Glucose was the sole carbon source at 1% (w/v), nitrogen sources were added to a final concentration of 10mM, and branched chain amino acids (isoleucine, leucine,

and valine) were added to supplement auxotrophies at a final concentration 2mM, unless otherwise stated in the text.

2.2.2 *Escherichia coli* media and growth conditions

E. coli strains were grown on Luria Broth (LB) supplemented with 50 µg/ml ampicillin (LBA) to select for ampicillin resistance plasmids. When using a blue-white colony screening for integration, 0.04 mM IPTG (isopropyl-β-D-thiogalactopyranoside) with 0.005% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) were used as needed (Sambrook et al., 1989)

2.3 Chemical Reagents and Solutions

Lysis Buffer

50 mM Tris-HCl pH 7.2
50 mM EDTA
3% SDS
1% β-Mercaptoethanol

MOPS (1X)

20 mM MOPS pH 7.0
5 mM Sodium acetate
1 mM EDTA

Phosphate Buffer (0.2 M)

78 mM Na₂HPO₄
122 mM NaH₂PO₄

RNA Extraction Buffer

7 M Urea
100 mM Tris-HCl pH 8.0
10 mM EDTA
1% Sodium dodecyl sulfate

TAE (50X Stock Solution)

2 M Tris-Base
1 M Glacial Acetic Acid
50 mM 0.5 EDTA Solution pH 8.0

O-CAS Solutions

Solution 1: 1mM FeCl₃*6 H₂O in 10mM HCl

Solution 2: 2mM Chrome Azurol-S (CAS), 0.5mM FeCl₃*6 H₂O – HCl solution

Solution 3: 5mM Hexadecyl trimethyl ammonium bromide

PIPES Solution: 1.3 M PIPES, 2 M NaOH pH 6.8

2.4 Standard Molecular and Microbiology Techniques

2.4.1 DNA Isolation

Plasmid DNA was isolated from *E. coli* colonies using the Wizard Plus SV Miniprep DNA purification kit (Promega) following the manufacturer's instructions. *A. nidulans* genomic DNA was isolated according to Lee and Taylor (1990). DNA generated by PCR and DNA fragments from restriction enzyme digest were either isolated from agarose gels or PCR reaction tubes by the Wizard SV Gel and PCR Clean Up System (Promega) following the manufacturer's instructions. Restriction enzyme digest (Promega; New England Biolabs) and ligations using the T4 DNA ligase (Promega) were performed to manufacturer's instructions. DNA separation for size determination by electrophoresis was performed with a 1-2% agarose gel in 1x TAE Buffer. DNA quantification was performed with a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

2.4.2 Competent Bacterial Cell Generation

Competent bacterial cells were made with calcium chloride treatment (Sambrook et al., 1989) and stored at -80°C until needed. Minimum 100 µL of competent cells and desired DNA fragment were incubated on ice for 10 minutes, followed by a 1-minute heat-shock at 37°C, and returned to ice for 1-2 minutes before plating on LBA media.

2.4.3 RNA Isolation

Total RNA was isolated by grinding 0.2 g of mycelia under liquid nitrogen until ground into a fine powder. TRIzol Reagent (ThermoFisher) was added to the ground mycelia and mixed before being added to RNA extraction buffer. Resuspended mycelia were separated with a second phenol-chloroform-isoamyl alcohol extraction

and a chloroform purification. RNA was precipitated first with 2M ammonium acetate and 50% isopropanol and incubated overnight at -20°C. A second precipitation was performed with DEPC-H₂O and 4M lithium chloride, also overnight at -20°C, before being resuspended in DEPC-H₂O and stored at -80°C (Sambrook and Russell, 2001). RNA quality was assessed by visualization of ribosomal RNA bands. RNA bands were separated by electrophoresis in a 1.2% agarose gel containing 1.1% formaldehyde in a 1x MOPS buffer. RNA concentration was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

2.4.4 Polymerase Chain Reaction

Polymerase Chain Reactions (PCR) were carried out using Ex-Taq (TaKaRa), Phusion (Finnzymes), or Bullseye (MidSci) DNA polymerases according to manufacturers' instructions. Template DNA varied between DNA source, approximately 1 ng for DNA isolated from plasmids and 100 ng for genomic DNA isolated from mycelia. All reactions followed the recommended denaturing, annealing, and extension condition with 35 amplification cycles, unless otherwise specified. Oligonucleotide primers (IDT) are specified in each relevant chapter.

2.4.5 Southern Blot

DNA samples were prepared by electrophoresis on 1% agarose gels before being treated with 0.25M HCl for 10 minutes for depurination. Transfer of DNA to either Hybond N+ or XL positively charged membranes (Amersham) used capillary transfer with 0.4M NaOH. DNA was crosslinked to membrane using the autocross linking settings on UV Stratalinker 1800 (Stratagene). Probe generation, overnight hybridization at 42°C, and subsequent detection was performed using the DIG (digoxigenin) High prime DNA labeling and detection start kit II (Roche) following manufacturer's instructions. Films exposed to the Southern blots, at 2h and 24h timepoints, were developed in an EcomaxTM X-ray film processor (Protec).

2.4.6 Nucleic Acid Sequencing

Whole genome sequencing was performed by the Kansas State University Integrated Genome Facility (Manhattan, Kansas, USA). RNA sequencing was performed by University of Kansas Genome Sequencing Core (Lawrence, KS, USA).

2.4.7 Overlay Chrome Azurol-S Siderophore Assay

Overlay Chrome-Azurol-S (O-CAS) blue agar siderophore assay was adapted from Schwyn and Neilands (1987) and Pérez-Miranda et al., (2007). Solutions and protocol are listed for 500 mL, scaled as needed. The Chrome-Azurol-S -- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ Solution was slowly combined with the HDTMA solution while separately making the PIPES solution. The CAS- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ -HDTMA and PIPES solutions were autoclaved separately. After cooling to roughly 50°C post-autoclave, the CAS- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ -HDTMA solution was slowly added to the PIPES solution and mixed gently to make the final O-CAS solution. 0.9% agarose was added to the O-CAS solution, boiled to solubilize, and cooled to about 50°C before gently overlaying the O-CAS solution onto the agar plate containing a single point inoculated strain. The overlay solution was allowed to solidify on the bench at 25°C and photographs of plates were taken at 3h and 7h.

2.5 *Aspergillus* Genetic Manipulation Protocols

2.5.1 *Aspergillus nidulans* growth testing and genetic analysis

A. nidulans growth testing was performed as described in Pontecorvo et al. (1953). Fungal crosses and manipulations followed Todd et al., (2007a) and Todd et al., (2007b).

2.5.2 *Aspergillus nidulans* transformation

Transformation of *A. nidulans* strains were based on Andrianopoulos and Hynes (1988) with modification (Downes et al., 2013). The non-homologous end-

joining mutant *nkuAΔ* strain was used for targeted integration into the genome (Nayak et al., 2006).

2.5.3 5-FOA selection for loss of *AfpyrG* deletion cassettes

Uridine and uracil auxotrophs (*pyrG⁻*) after DNA-mediated transformation with wild type DNA fragments corresponding to deleted genes were selected using 1-2 mg/mL 5-fluoroorotic acid (5-FOA) (Zymo Research) added to protoplast media supplemented with 5 mM uracil and 10 mM uridine and appropriate supplementation for other auxotrophies. Transformants containing the *pyrG⁻* mutation, which leads to 5-FOA resistance, were picked to selective media after two days incubation at 37°C.

2.6 Gene Expression Characterization Protocols

2.6.1 β -galactosidase reporter gene assays

β -galactosidase assays were performed following Davis et al. (1988) and Pateman (1969) using soluble total protein extracts from strains harboring *gdhA-lacZ* reporter genes targeted at *amdS* (Downes et al., 2013). β -galactosidase specific activity is defined as $A_{420} \times 10^3 \text{ min}^{-1} \text{ mg}^{-1}$ of soluble protein. Protein concentrations were determined using Bio-Rad Assay reagent (Bio-Rad).

2.6.2 Reverse Transcriptase – quantitative PCR

Reverse Transcriptase – quantitative PCR (RT-qPCR) used total RNA extracts from mycelia to produce cDNA with qScript cDNA SuperMix (Quanta Biosciences). RT-qPCR data collection used a MyiQ thermocycler (Bio-Rad) with the iTAQ™ Universal SYBR® Green Supermix (Bio-Rad) and iQ5 v2.1 program (Bio-Rad). Fold change was calculated using the $\Delta\Delta C_T$ method with the β -tubulin coding gene, *benA*, as the reference housekeeping gene (May et al., 1987; Pfaffl, 2001; Schmittgen and Livak, 2008). Primers (IDT) were designed to specifically amplify cDNA by overlapping

a splice junction within the gene target. Primer sequences are provided within the specific chapters.

2.7 DNA and RNA Sequencing

2.7.1 Whole Genome Sequencing

Genomic DNA was isolated from mycelia grown in 100 mL of supplemented ANM with 10 mM ammonium tartrate in shaking flasks (160 RPM) for 16 h and ground under liquid nitrogen. The extraction protocol used is outlined in Lee and Taylor (1990), with an added RNase A (Sigma) step. The RNase was added to the supernatant of the initial phenol-chloroform-isoamyl alcohol purification step and incubated for 20 minutes. RNase was subsequently removed by additional phenol-chloroform-isoamyl alcohol purification. Additional chloroform purification was performed to remove any residual organic reagents in the solution. Purified DNA was sent to Kansas State University Integrated Genomics Facility (Manhattan, Kansas, USA) for library preparation and whole genome sequencing. Libraries were pooled and sequenced in a Midoutput paired end 150bp run on the NextSeq 500 Sequencer (Illumina).

2.7.2 RNA Sequencing

Total RNA from mycelia grown in 100 mL of supplemented ANM in shaking flasks for 16 h and isolated as described in Section 2.4.3 were sent to University of Kansas Genome Sequencing Core (Lawrence, KS, USA). Samples were prepared in biological triplicate. University of Kansas Genome Sequencing Core performed quality check of submitted RNA samples with a TapeStation 4150 (Aligent) before generating libraries with the NEBNext Stranded mRNA Library (NEB). Libraries were pooled and sequenced on the NextSeq550 High Output single read run (Illumina).

2.8 Bioinformatics and *in silico* analyses

2.8.1 Sequence identification and analysis

DNA and protein sequences were obtained through the Aspergillus Genome Database, AspGD (www.aspgd.org; (Arnaud et al., 2012)), the Saccharomyces Genome Database, SGD (www.yeastgenome.com; (Cherry et al., 2012)), the Fungal and Oomycete Informatics Resource, FungiDB (www.fungidb.org; (Amos et al., 2022)), the Broad Institute genomes database (www.broadinstitute.org), the NCBI protein database (www.ncbi.nlm.gov/protein), and the EMBL-EBI Pfam database (<http://pfam.xfam.org>; (Finn et al., 2014)). Protein sequence database searches used BLASTp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and percent identity and similarity were calculated with EMBOSS Needle (EMBL-EBI) with default parameters. DNA sequences were visualized, analyzed, and manipulated with Geneious version 5.3.5 and 2022.0 created by Biomatters (www.geneious.com). Alignments were generated with ClustalW2 or Clustal Omega (Larkin et al., 2007; Sievers et al., 2011) on the EMBL-EBI server (<http://www.ebi.ac.uk/Tools/msa>). Sequence alignments were shaded based on similarity using BoxShade 3.2 (K. Hofmann & M.D. Baron through (www.ch.embnet.org/software/BOX_form.html)) or the Sequence Manipulation Suite (<http://www.bioinformatics.org/SMS/index.html>; (Stothard, 2000)).

2.8.2 Phylogenetic Analysis

The Pfam database (Finn et al., 2014) was used to identify orthologs of the isocitrate/isopropylmalate dehydrogenase protein family (PF00180), the aminotransferase class IV protein family (PF01063), and Zn(II)C6 fungal-type DNA-binding domain (PF00172). Sequence alignment and phylogenetic trees were constructed using MAFFT (<https://mafft.cbrc.jp/alignment/software/>; (Katoh et al., 2019)). The neighbor-joining method with 100 bootstraps were used to generate consensus unrooted phylogenetic trees for each protein family. Tree visualization and label editing employed the Interactive Tree Of Life (iTOL) v4 program (<https://itol.embl.de>; (Letunic and Bork, 2019)).

2.8.3 Whole Genome Sequence Analysis

Whole genome sequence analysis was performed through the Galaxy Platform (www.usegalaxy.org; (Afgan et al., 2018)). FastQC tool was used to determine sequence read quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were mapped to the genome using the BWA-MEM tool for Illumina with default settings (Li and Durbin, 2009; Li and Durbin, 2010) to an updated *A. nidulans* FGSC A4 genome (Aspnd5rm) to which we have pre-release access (Adrian Tsang, Concordia University). Duplicate reads were marked using the MarkDuplicates tool (<http://broadinstitute.github.io/picard/>) and left aligned using BamLeftAlign (<http://arxiv.org/abs/1207.3907>). Single Nucleotide Polymorphisms (SNPs) were identified using the FreeBayes tool (<http://arxiv.org/abs/1207.3907>) with default setting except for the following parameters: minimum coverage (-l) to 4, polymorphism probability (-pvar) to 0.01, ploidy (-p) to 1, minimum mapping quality (-min-mapping-quality) to 1, and minimum alternate observations (-min-alternate-fraction) to 0.5. SNP characterization was determined with the 'SnpEff' SNP effect annotation tool (Cingolani et al., 2012) with Classic Effect names and amino acid variant annotation chosen.

2.8.4 RNA-Seq Analysis

RNA-Seq analysis was completed through the Galaxy Platform (www.usegalaxy.org; (Afgan et al., 2018)). FastQC tool was used to determine sequence read quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were aligned to the *A. nidulans* FGSC A4 genome (Aspnd5rm) (Adrian Tsang, Concordia University) using the HISAT2 tool (Kim et al., 2015) with default settings. Transcripts per gene were counted with the htseq-count tool with default parameters (Anders et al., 2015). Differential expression analysis was performed with the SeqMonk Mapped Sequence Data Analyser Program from the Babraham Institute (www.bioinformatics.babraham.ac.uk/projects/). Within SeqMonk, Replicate Sets were made for comparisons before checking quality with the RNA-Seq QC Plot.

DESeq2 analysis was performed using the internal RNA-Seq Analysis Pipeline. Differentially expressed gene list was then exported for subsequent analysis and interpretation.

2.8.5 Gene Ontology Analysis

Gene Ontology (GO) analysis was done through ShinyGO 0.76 (<http://bioinformatics.sdstate.edu/go/>) with the DESeq2 list of genes from the RNA-Seq Analysis (see above) (Ge et al., 2020). Parameters were as follows: 'Organisms' was *Aspergillus nidulans* STRINGdb, Pathway database was either GO Molecular Function or GO Biological Process, and # pathways to show was set to 10. All other parameters of the gene list were left as default. Visualization of GO analysis was done under the Chart heading, with pathways sorted by Category name, X-axis changed to Fold enrichment, and Color as $-\log_{10}(\text{FDR})$. Chart type was changed to barplot and aspect ratio to 2. Charts were then exported as a PNG file for subsequent figure formation.

Chapter 3 - The Branched Chain Amino Acid Biosynthesis Pathway

3.1 Abstract

The branched chain amino acids (BCAAs), isoleucine, leucine, and valine, are considered essential amino acids for mammals since they do not synthesize these amino acids themselves. Fungi, bacteria, and plants produce the BCAAs and the biosynthetic pathway has been best characterized in the yeast *Saccharomyces cerevisiae* and partially characterized in filamentous fungi. Isoleucine, leucine, and valine share the early steps of the BCAA biosynthetic pathway before diverging into pathway-specific steps. In addition, the final transamination step for all three BCAAs is catalyzed by the same enzyme. In the filamentous fungus *Aspergillus nidulans*, the genes for the first two dedicated steps of the leucine biosynthesis pathway have been characterized but the final two steps have not. Using sequence searches of the *A. nidulans* genome, we identified two genes that encode a β -isopropylmalate dehydrogenase enzyme and six genes that encode a BCAA aminotransferase enzyme, which constitute the penultimate step in leucine biosynthesis and the final step of the BCAA biosynthetic pathway, respectively. We determined the contributions of each gene to BCAA biosynthesis through combinations of gene knockouts and have determined that both β -isopropylmalate dehydrogenase genes contribute to leucine biosynthesis while only the two most highly expressed BCAA aminotransferase genes participate in BCAA biosynthesis.

3.2 Contribution Statement

This work was started by Dr. Damien Downes, Cameron Hunter and Pierre Migeon in the Todd lab at Kansas State University. Downes, Hunter, and Migeon constructed and confirmed the original single gene deletion strains, and the *leuD* Δ *leuE* Δ and *batA* Δ *batB* Δ double deletion strains and conducted preliminary growth characterization. Dr. Downes also did some characterization of the role of *leuD* Δ (Figure 3.5), the RNA-Seq analysis in Figures 3.5, 3.7 and 3.10A, and prepared the

BCAA biosynthetic pathway figure (Figure 3.1). Dr. Richard Todd performed the colinearity analyses. These experiments, analysis, and figures are included in this chapter to provide a well-rounded story of the BCAA biosynthetic pathway. The remaining work, including phylogenetic analyses (Figures 3.4 and 3.9), complementation analysis (Figures 3.6 and 3.13), RT-qPCR analysis (Figures 3.10B and 3.10C), growth tests (Figures 3.12 and 3.14), was conducted by Joel Steyer.

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3.3 Introduction

The branched chain amino acids (BCAAs) isoleucine, leucine, and valine can be produced de novo by bacteria, fungi, and plants but must be obtained through the diet of mammals. Leucine concentration plays many roles in different systems. In mammals, leucine levels indicate nutrient availability that controls the protein kinase mTORC1 (mammalian Target of Rapamycin Complex 1). mTORC1 regulates cellular processes such as cell growth, protein biosynthesis, and autophagy, and hyperactivation of mTORC1 is implicated in diseases such as cancer and diabetes (Saxton et al., 2016; Wolfson et al., 2016; Bar-Peled and Sabatini, 2014). Fungi, on the other hand, synthesize BCAAs for use in protein biosynthesis and secondary metabolite production, and BCAAs are implicated in virulence for opportunistic fungal pathogens (Yamamoto et al., 2017). BCAA auxotrophs of the fungal human pathogens *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus* show decreased pathogenicity (Do et al., 2015; Garcia et al., 2018; Kingsbury and McCusker, 2010; Oliver et al., 2012; Orasch et al., 2019; Richie et al., 2013), while the plant pathogens *Magnaporthe oryzae* and *Fusarium graminearum* require BCAA biosynthesis for full virulence (Du et al., 2013; Du et al., 2014; Liu et al., 2014; Liu et

al., 2015; Liu et al., 2016; Que et al., 2020). As such, these enzymes and their regulators can be considered potential drug and anti-fungal agent targets as the inactivation of these enzymes decrease disease severity, and in the case of mammals, target the pathogen and not the host.

The BCAA biosynthesis pathway is well characterized in the yeast *Saccharomyces cerevisiae* (Kohlhaw, 2003). Comparisons of the BCAA pathway with *A. fumigatus*, *Aspergillus niger*, and *Aspergillus nidulans* show similarities to and divergence from *S. cerevisiae* (MacDonald et al., 1974; Oliver et al., 2012; Polotnianska et al., 2004; Williams et al., 1996; Shimizu et al., 2010). Both *S. cerevisiae* and *A. nidulans* encode a single α -isopropylmalate (α -IPM) isomerase (LEU1 and LuA, respectively) (Hsu and Schimmel, 1984; Polotnianska et al., 2004; Skala et al., 1991). *S. cerevisiae* encodes a single dihydroxyacid dehydratase while *A. fumigatus* encodes two functional ones (Oliver et al., 2012; Velasco et al., 1993). Additionally, *S. cerevisiae* encodes two α -IPM synthetases, LEU4 and LEU9, but *A. nidulans* only encodes one, LeuC (Baichwal et al., 1983; Chang et al., 1984; Chang et al., 1985; Casalone et al., 2000; Downes et al., 2013). The penultimate step of the leucine biosynthesis pathway is catalyzed by the β -isopropylmalate (β -IPM) dehydrogenase LEU2 in *S. cerevisiae*. The bidirectional BCAA aminotransferase (BAT) proteins, BAT1 and BAT2, catalyze the final step in BCAA biosynthesis and the first step in their catabolism (Dickinson and Norte, 1993). While these final two steps have been characterized in *S. cerevisiae*, the *A. nidulans* orthologs were unknown.

In this chapter, we characterize the final two steps of the *A. nidulans* leucine biosynthetic pathway, the two β -IPM dehydrogenase genes and the six predicted BAT genes. We determine the roles for both β -IPM dehydrogenase genes in leucine biosynthesis and uncover roles for only two of the six BAT genes in BCAA production.

3.4 Material and Methods

3.4.1 Strain Construction

Aspergillus nidulans transformation was performed as described in Downes et al. (2013) using *nkuA* Δ non-homologous end joining mutants for targeted integrations (Nayak et al., 2006). Gene knockout constructs were generated by the *A. nidulans* whole genome deletion construct program (De Souza et al., 2013) and obtained through the Fungal Genetics Stock Center in Manhattan, KS (McCluskey et al., 2010). Gene knockout constructs were transformed into MH11068 (*pyrG89 pyrA4 nkuA* Δ ::*Bar*) and selected for uracil and uridine prototrophy to generate *leuD* Δ (AN0912 Δ ; RT411, Δ -7 to +1,431 bp), *leuE* Δ (AN2793 Δ ; RT413, Δ -6 to +1,233 bp), *batA* Δ (AN4323 Δ ; RT415, Δ +65 to +1,722 bp), *batB* Δ (AN5957 Δ ; RT440, Δ +25 to +1,395 bp), *batC* Δ (AN7878 Δ ; RT475, Δ -10 to +1,222 bp), *batD* Δ (AN7876 Δ ; RT419, Δ -7 to +1,297 bp), *batE* Δ (AN0385 Δ ; RT417, Δ +27 to +1,302 bp), and *batF* Δ (AN8511 Δ ; RT441, Δ -9 to +1,230 bp) strains. Selection media for putative transformants was supplemented with either 2mM leucine for *leuD* Δ and *leuE* Δ transformants or 2mM ILV (2mM each) for *bat* Δ transformants. The selectable marker, *Aspergillus fumigatus pyrG* (*AfpyrG*), incompletely complemented the pyrimidine auxotrophy in the *batC* Δ mutant due to position effect. Full complementation of the *pyrG89* mutation by *AfpyrG* was observed in all other deletion mutants generated. Knockout construct integration by single homologous double-crossover at the correct gene locus was confirmed by Southern blot with the 982bp *KpnI*-*SspI* *AfpyrG* fragment or diagnostic PCR. Meiotic crossing was used to generate the double, triple, and quadruple mutants used in this study and was confirmed by Southern blot or diagnostic PCR. Repair of the *leuD* Δ mutant was achieved by introduction the *leuD* PCR product (-960 to +3216) amplified from MH1 and selected for resistance to 1 mg ml⁻¹ 5-fluoroorotic acid to select for loss of *AfpyrG* in the absence of exogenous leucine. The *leuD* Δ *leuE* Δ mutant was complemented with the plasmid pJS249, which contains wild type *leuE* PCR product (-913 to +2877) amplified from MH1 and cloned into the pGEMTeasy vector, by transformation and selected for leucine prototrophy. The

batA Δ *batB* Δ mutant was complemented with wild type *batA* (-717 to +2558) or *batB* (-725 to +2187) gene using plasmids (pJS244 and pJS255, respectively) generated as described above. Transformants were selected for ILV prototrophy.

3.4.2 Strains used in this chapter

A. nidulans strains used in this chapter are described in Table 3.1

Table 3.1 *A. nidulans* strains used

Strain	Origin	Genotype ^a
MH1	M.J. Hynes	<i>biA1</i>
MH10865	R.B. Todd	<i>yA1 pabaA1 pyrG89 argB::fmdS-lacZ areAΔ(5')::riboB</i>
MH11068	M.J. Hynes	<i>pyrG89 pyroA4 nkuAΔ::Bar</i>
RT411	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuDΔ::AfpYrG</i>
RT412	RT411 X MH10865	<i>yA1 pabaA1 pyrG89 leuDΔ::AfpYrG</i>
RT413	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuEΔ::AfpYrG</i>
RT414	RT413 X MH10865	<i>yA1 pabaA1 pyrG89 leuEΔ::AfpYrG</i>
RT415	Transformant of MH11068	<i>pyrG89 batAΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT416	RT415 X MH10865	<i>yA1 pyrG89 pabaA1 batAΔ::AfpYrG</i>
RT417	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar batEΔ::AfpYrG</i>
RT418	RT417 X MH10865	<i>yA1 pabaA1 pyrG89 batEΔ::AfpYrG</i>
RT419	Transformant of MH11068	<i>pyrG89 batDΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT440	Transformant of MH11068	<i>pyrG89 batBΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT441	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar batFΔ::AfpYrG</i>
RT444	RT411 X RT414	<i>pyrG89 pyroA4 leuEΔ::AfpYrG leuDΔ::AfpYrG</i>
RT454	RT418 X RT419	<i>yA1 pabaA1 pyrG89 batDΔ::AfpYrG batEΔ::AfpYrG</i>
RT457	RT416 X RT440	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i>
RT458	RT412 X RT453	<i>yA1 pabaA1 pyrG89 amdS::AfpYrA-gdhA(-753 bp)-lacZ leuDΔ::AfpYrG</i>
RT466	RT441 X RT454	<i>pabaA1 pyrG89 batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT475	Transformant of MH11068	<i>pyrG89 batCΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT520	RT457 X RT466	<i>batBΔ::AfpYrG batAΔ::AfpYrG batEΔ::AfpYrG</i>
RT523	RT457 X RT466	<i>pabaA1 batBΔ::AfpYrG batAΔ::AfpYrG batFΔ::AfpYrG</i>
RT524	RT457 X RT466	<i>pabaA1 batBΔ::AfpYrG batAΔ::AfpYrG batEΔ::AfpYrG</i>
RT525	RT457 X RT466	<i>batBΔ::AfpYrG pyroA4 batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT526	RT457 X RT466	<i>batBΔ::pyrG+ batFΔ::AfpYrG+ nkuAΔ::Bar</i>
RT531	RT457 X RT466	<i>batBΔ::pyrG+ batEΔ::AfpYrG+ batFΔ::pyrG+ nkuAΔ::Bar</i>
RT636	RT525 X RT524	<i>batBΔ::AfpYrG batEΔ::AfpYrG</i>
RT642	RT525 X RT523	<i>batBΔ::AfpYrG batAΔ::AfpYrG pyroA4 batFΔ::AfpYrG batEΔ::AfpYrG</i>

Strain	Origin	Genotype ^a
RT645	RT415 X RT466	<i>pabaA1 batAΔ::AfpyrG pyroA4 batFΔ::AfpyrG</i>
RT647	RT415 X RT466	<i>pabaA1 batAΔ::AfpyrG batFΔ::AfpyrG batEΔ::AfpyrG</i>
RT648	RT415 X RT466	<i>batAΔ::AfpyrG pyroA4 batEΔ::AfpyrG</i>

^a all strains carry *veA1*

3.4.3 Genomic and RT-qPCR primers

Primers used in this chapter are described in Tables 3.2 and Table 3.3.

Table 3.2 Genomic PCR primer sets for leucine biosynthesis genes

Target	Primer Name ^A	Sequence (5' → 3')
<i>batA</i>	An4323FGSC5'F	CTCAGACGAGCGAGATTAC
	An4323FGSC3'R	CTACGGATCCCTACCTAGAA
	<u>ANID_04323.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGCTCAGACGAGCGA GATTAC
	<u>ANID_04323.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCCTACGGATCCCTAC CTAGAA
<i>batB</i>	An5957FGSC5'F	CTAGTGCAGCAAGAGATCC
	An5957FGSC3'R	AAGGACGACTTGAGGACTAC
	<u>ANID_05957.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGCTAGTGCAGCAAGA GATCC
	<u>ANID_05957.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCAAGGACGACTTGAG GACTAC
<i>batC</i>	AN7878FGSC5'F	GATGAGTTCCCATAGTGAGC
	AN7878FGSC3'R	GGGACTCATTAGTGAGAAG
	<u>ANID_07878.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGGATGAGTTCCCAT GTGAGC
	<u>ANID_07878.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCGGGGACTCATTAGT GAGAAG
<i>batD</i>	<u>ANID_07876.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGGCTAGATCTGACTC TTTGGC
	<u>ANID_07876.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCGTGCATATCTACAT GGGTGG
<i>batE</i>	AN0385_5'F	GATTCCTGAGTAGGGATACG
	AN0385_3'R	GAGTCTGAATCTGCCTCTG
	<u>ANID_00385.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGGATTCTGAGTAGG G ATACG
	<u>ANID_00385.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCGAGTCTGAATCTGC CTCTG
<i>batF</i>	An8511FGSC5'F	GCTCATGATACTAGCCCTCT
	An8511FGSC3'R	GGCTAGTACTGCTTTGATCC
	<u>ANID_08511.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGGCTCATGATACTAG CCCTCT
	<u>ANID_08511.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCGGCTAGTACTGCTT TGATCC
<i>leuD</i>	AN0921FGSC5'F	ATCATCCTCTCTACTCCAGC
	AN0921FGSC3'R	GTATGTGGGTAGCATACTCG
	<u>ANID_00921.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGTGTACGAGACTTCT ACTGCC
	<u>ANID_00921.1 3'R</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGTGTACGAGACTTCT ACTGCC
<i>leuE</i>	AN2793FGSC5'F	TAGTTGGTACGTAGGTGGC
	AN2793FGSC3'R	GTGAAAGGAGAGATGAGGAG
	<u>ANID_02793.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGTAGTTGGTACGTAG GTGGC
	<u>ANID_02793.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCGTGAAAGGAGAGAT GAGGAG

^A Underlined primers were used to amplify the deletion constructs.

Table 3.3 RT-qPCR primer sets for leucine biosynthesis genes

Target	Primer Name	Sequence (5' → 3')
<i>benA</i>	benA_RT_F	CCTGCTCCGCTSTCTTCC
	benA_RT_R	GACTGTTCTTGCTCTGGAT
<i>leuC</i>	leuC_RT_F	TCAAAGATCCCTCTAAGAAATACA
	leuC_RT_R	TTGTTCGCCATCCATAGG
<i>luA</i>	luA_RT_F	TCTTGATCTATATCGACAGACA
	luA_RT_R	TGACGAGGTAGGGATGTT
<i>leuD</i>	leuD_RT_F	ATTGGTGGCCCAGAATGG
	leuD_RT_R	AATTGCATGGTCGCAAGTTG
<i>leuE</i>	leuE_RT_F	AAATCTGAGACCGTGTTT
	leuE_RT_R	ATTCGTCCATTGCATAATC
<i>batA</i>	batA_RT_F	GCTTCAGTGCTACTCGTG
	batA_RT_R	TGAACATGTGATCGGTAAAG
<i>batB</i>	batB_RT_F	CGAAGTTGTTGAGAAGAC
	batB_RT_R	TGATGAAGAATGCAGTCC
<i>batC</i>	batC_RT_F	TCTTTCAGTACCACATC
	batC_RT_R	TTATCGTAGGGAGTGATTTG
<i>batD</i>	batD_RT_F	GGGATGAAAGCCTATCGT
	batD_RT_R	TAGAAACAGCATCACAAGAAC
<i>batE</i>	batE_RT_F	CTCAATCATTCCCTCCTCCTC
	batE_RT_R	CATGGCCGTAACTTCGC
<i>batF</i>	batF_RT_F	AAGTTCGGTAAATGGTCTG
	batF_RT_R	CTCCTGTCCGTAGTTGAA

3.5 Results

3.5.1 Identification of the β -isopropylmalate dehydrogenase genes

The leucine biosynthesis pathway contains four enzymatic steps, the first two of which have been previously characterized in *A. nidulans* (Figure 3.1). The penultimate step in the leucine biosynthesis pathway is catalyzed by β -IPM dehydrogenase and is encoded by one gene, *LEU2*, in *S. cerevisiae* (Andreadis et al., 1984; Toh-e et al., 1980). However, *A. niger* contains two orthologs to *LEU2*, *leu2A* and *leu2B* (Williams et al., 1996). By using the *S. cerevisiae* Leu2p gene sequence as the query in BLASTp searches, we determined that *A. nidulans* also contained two β -IPM dehydrogenase genes, encoded by AN0912 and AN2793. We designated AN0912 as *leuD* and AN2793 as *leuE*. Pairwise comparisons showed that LeuD and LeuE had 50.5% identity and 67.3% similarity to each other (Table 3.4). When expanding the comparison to include LEU2, Leu2A, and Leu2B, we determined that there was strong sequence conservation throughout the protein, including the substrate binding loop and the NAD binding motif (Figure 3.2). We then investigated if *leuD* or *leuE* had conserved colinearity within the *Aspergillus* species. Out of the 27 *Aspergillus* genomes available on FungiDB, *leuD* is found in a highly conserved region in all 27 species. However, *leuE* lacks colinearity with the 24 identified orthologs in the 27 *Aspergillus* species (Figure 3.3A and 3.3B).

We used phylogenetic analysis to determine the relationship between LeuD and LeuE (Figure 3.4). LeuD and LeuE formed separate clades with their orthologs. The LeuD clade position is consistent with the known *Aspergillus* evolutionary tree (Galagan et al., 2005).

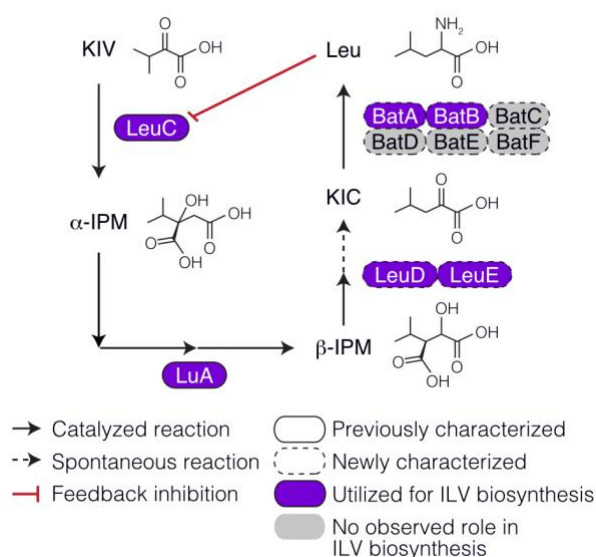


Figure 3.1 Leucine biosynthesis in *Aspergillus nidulans*.

Pathway of committed leucine (Leu) biosynthesis enzymes (rounded rectangles). The stages involving generation of α -isopropylmalate (α -IPM) from α -ketoisovalerate (KIV) by α -IPM synthetase (LeuC) and subsequent conversion to β -isopropylmalate (β -IPM) by α -IPM isomerase (LuA) have previously been characterized. The two β -IPM dehydrogenase enzymes (LeuD and LeuE), which generate α -ketoisocaproate (KIC), and two BCAA aminotransferases (BatA and BatB), which also function in isoleucine and valine biosynthesis and isoleucine, leucine, and valine (ILV) catabolism, were characterized in this work from eight candidate coding genes.

```

A. nig Leu2B      1 MSGTRAYNIIVLPDGI GPEVMAEAIKVLRTFNS..SSMOHLQEBLIGGISIDTHGHSV
A. nid AN2793    1 ~MSEKSYNIIVLPDGI GPEVMAEATKLSLFNT..STVRRRTQTELLIGGCSIDTHGKSV
S. cer Leu2p     1 ~MSAPKKIIVLPDGVGGETAEAIKVLKASDVRSNVKKDFENHLIGGAIDATGVPL
A. nig Leu2A     1 ~MPAYNIIVVFAGDHWGPEVTAEAIKVLRVHEKSRDDITLNLDHLLGGASIDATANPL
A. nid AN0912    1 ~MPSYNIIVVFAGDHC GPEVTAEAVKILRVHEKSRDDITNLQDHLGGCSIDATGSPL

Substrate binding loop
A. nig Leu2B      59 TDPVKDAIVADAVLFAVGGSRVDHIRRGLDGPFCGLLIQVRKAMDIYANLRPCSVDVPS
A. nid AN2793    58 TDAVTPAAVSSDAVLFAVGGPKVDHIRRGLDGPFCGLLIQVRKAMDIYANLRPCSVDSPE
S. cer Leu2p     59 TDEALAAKRNADAVLLGAVGGPKNGT...GAVRPEQGLLKIRKELQYANLRPCNFASDS
A. nig Leu2A     58 TDEALAAKRNADAVLLGAVGGPKNGT...GAVRPEQGLLNVRKEMGTFCNLRPCNFAPS
A. nid AN0912    58 TDALEAAKRNADAVLLGAVGGPKNGT...GAVRPEQGLLKIRKEMGTFCNLRPCNFAPS

E[QKR]XLLXXR
A. nig Leu2B      119 REIARDSPFRQEVIEGVDFVVVRENCGGAYFGKKVSEE..NYAMDEWGYSTTEIQRIR
A. nid AN2793    118 REIARDSPFRQDVIEGVDFVVVRENCGGAYFGKKVSEED..DYAMDEWGYSSSEIQRITR
S. cer Leu2p     116 L...LDSPKKPQFAKGTDFVVVRELGGIYFGKRKEDDGDGVWDSQYTVPEVORITR
A. nig Leu2A     115 L...VERSPKKASVCEGVDFNIIRELGGIYFGDRKKMTA.AATMDTEPYSRAETERTP
A. nid AN0912    115 L...VESPPLRPEICRGVDFNIIRELGGIYFGERKEDDGSGFALDEPYSRAETERTR

A. nig Leu2B      177 LAARLALRHPPFPVHSLDKANVLASSRLWRRVVENTLSVFPQVKLVHOLADSAAMIMA
A. nid AN2793    176 LAARLALRHPPFPVHSLDKANVLASSRLWRRVVEKTSVFPQVKLVHOLADSAAMIMA
S. cer Leu2p     173 MAAPMALQHPPPLPVHSLDKANVLASSRLWRKTVEETIKNFFPLKVOBOLIDSAAMILV
A. nig Leu2A     171 LRAHMLAQHPPPLPVHSLDKANVLASSRLWRKTVEETIMAKFFPOLKIBHOLIDSAAMIMV
A. nid AN0912    172 LCAHMLALQHPPPLPVHSLDKANVLASSRLWRKTVEETIMAKFFPOLKIBHOLIDSAAMIMV

A. nig Leu2B      237 TDPRLNGLVILADNTFGDMLSDQAGSLIGLGLVPSASLDGLPHPGKQKRVGLYEPTHG
A. nid AN2793    236 TNPRLNGLVILADNTFGDMVSDQAGSLVGLGLVPSASLDGLPKGEQKRVHGLYEPTHG
S. cer Leu2p     233 KNPRLNGLVILHSTNMFGDIISDEASVIPGSLGLLPSASLASLPD..KN.TAPGLYEPTHG
A. nig Leu2A     230 KNPRLNGLVILHSTNLFGDIISDEASVIPGSLGLLPSASLSQIDP..GKGNVGLYEPTHG
A. nid AN0912    232 KDPRLNGLVILHSTNLFGDIISDEASVIPGSLGLLPSASLSIPD..GKGNVGLYEPTHG

NAD binding
A. nig Leu2B      297 SAPDTAGKNIAFPAMILCVSLMFRYSFNMEAEARQIEDAVRAVLDRGURTPDLGGNSST
A. nid AN2793    296 SAPDTAGKNIAFPAMILCVSLMFRYSFNMEAEARQIEAAVRVTLDKGIRTSDLGGSTGT
S. cer Leu2p     290 SAPDLPK.NKVNPIATILSAAMMLKLSLNLPEEGRAIEDAVKKVLDAGIRTDGLGGNSST
A. nig Leu2A     288 SAPDTAGRGIVNPVAAILSVAMMMQYSFGRFDEARAIEAARNVLESQVTRTGDHGGKATT
A. nid AN0912    290 SAPDISGKRGIVNPVAAILSVGLMMQYSFALFEARAIVATAVSNVIEAGVTRTGDHGGKAST

HGSAPDI
A. nig Leu2B      357 QTFGDAVVVAALQGGKY--
A. nid AN2793    356 RTFGDAVVVAALKGEH--
S. cer Leu2p     349 TGVGDAAVAEEVKKIILA*
A. nig Leu2A     348 SEVGDAAVAEELEKLLK-
A. nid AN0912    350 KEVGDAAVAEELEKLLKK

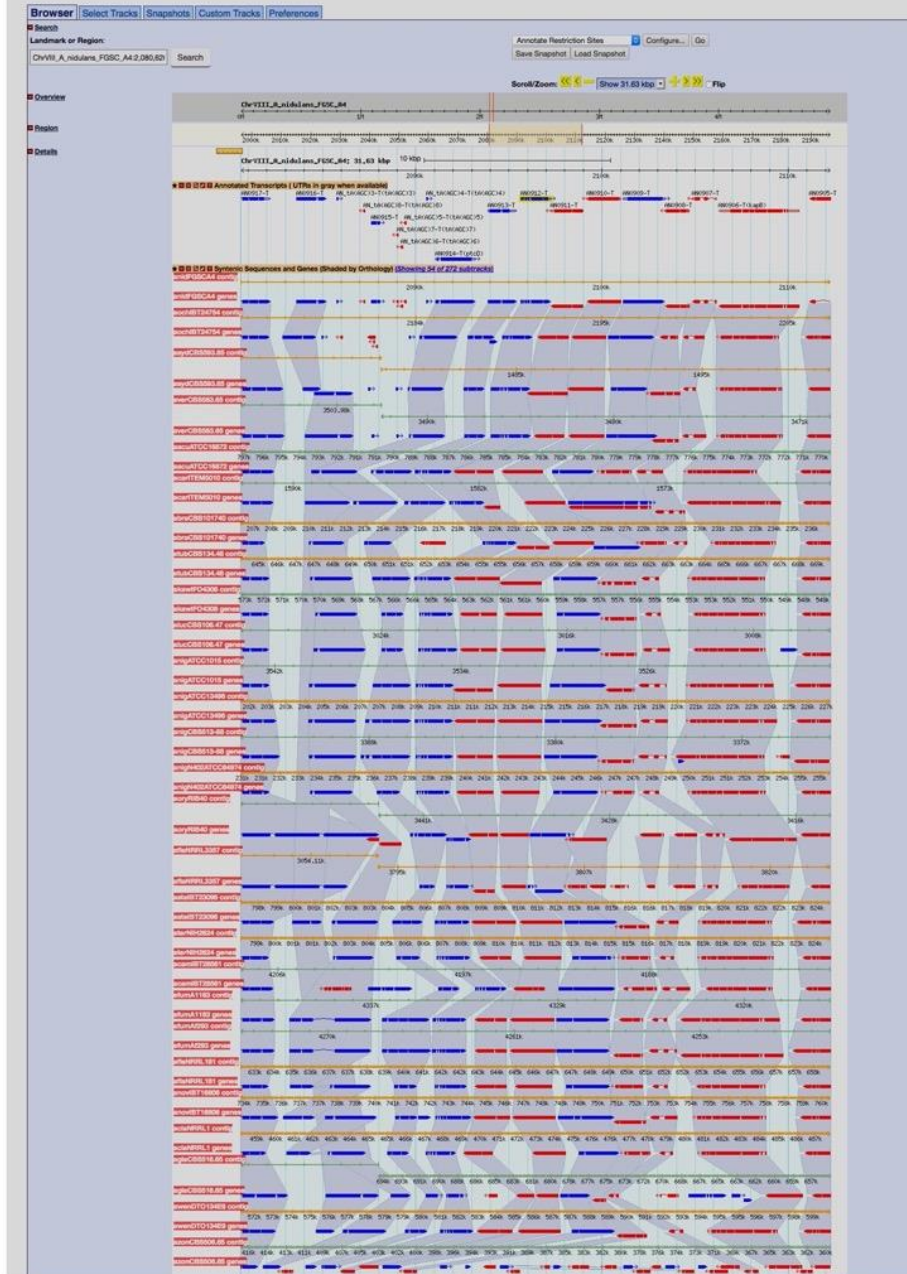
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Figure 3.2 Protein sequence alignment of β -IPM dehydrogenases.

Clustal Omega alignment of Leu2p from *S. cerevisiae* (*S. cer*) with AN0912 (LeuD) and AN2793 (LeuE) from *A. nidulans* (*A. nid*) and Leu2A and Leu2B from *A. niger* (*A. nig*). The substrate-binding loop (green) and the NAD-binding motif (blue). Shading was performed with Boxshade with a minimum of 0.6 identity (black) or similarity (gray).

AN0912 (*leuD*)

FungiDB GBrowse v2.48: 31.63 kbp from ChrVIII_A_nidulans_FGSC_A4:2,080,628..2,112,257



AN2793 (*leuE*)

FungiDB GBrowse v2.48: 31.39 kbp from ChrVI_A_nidulans_FGSC_A4:2,782,077..2,813,467



Figure 3.3 Colinearity of β -isopropylmalate dehydrogenase genes in *Aspergilli*.

The colinearity of syntenic regions for (A) AN0912 (*leuD*) and (B) AN2793 (*leuE*) was illustrated using the GBrowse genome browser of FungiDB with genomes displayed in the order: *Aspergillus nidulans* FGSC A4, *Aspergillus ochraceoroseus* IBT 24754, *Aspergillus sydowii* CBS 593.65, *Aspergillus versicolor* CBS 583.65, *Aspergillus aculeatus* ATCC 16872, *Aspergillus carbonarius* ITEM 5010, *Aspergillus brasiliensis* CBS 101740, *Aspergillus tubingensis* CBS 134.48, *Aspergillus kawachii* IFO 4308, *Aspergillus luchuensis* CBS 106.47, *Aspergillus niger* ATCC 1015, *Aspergillus niger* ATCC 13496, *Aspergillus niger* CBS 513.88, *Aspergillus niger* strain N402 (ATCC64974), *Aspergillus oryzae* RIB40, *Aspergillus flavus* NRRL3357, *Aspergillus steynii* IBT 23096, *Aspergillus terreus* NIH2624, *Aspergillus campestris* IBT 28561, *Aspergillus fumigatus* A1163, *Aspergillus fumigatus* Af293, *Aspergillus fischeri* NRRL 181, *Aspergillus novofumigatus* IBT 16806, *Aspergillus clavatus* NRRL 1, *Aspergillus glaucus* CBS 516.65, *Aspergillus wentii* DTO 134E9, *Aspergillus zonatus* CBS 506.65.

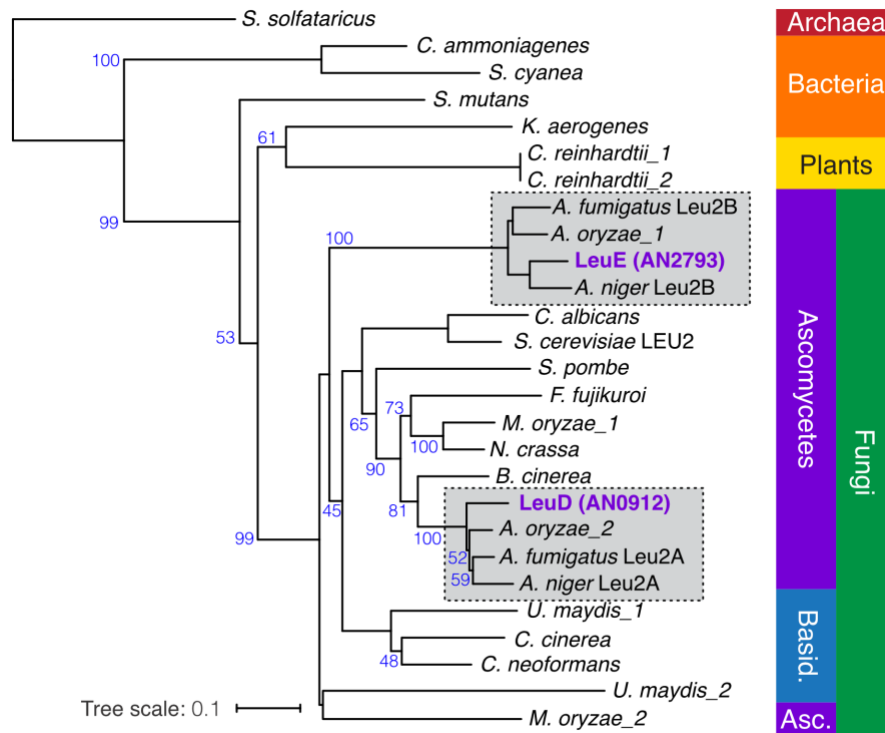


Figure 3.4 Phylogenetic analysis of β -IPM dehydrogenase genes.

Unrooted phylogeny of β -IPM dehydrogenases. Bootstrap support (100 replicates) greater than 40% is shown. Protein sequences for *A. nidulans* were downloaded from AspGD, sequences for *S. cerevisiae* were downloaded from SGD, and all other sequences came from Pfam or NCBI. Archaea, *Sulfolobus solfataricus* (Q9UXB2.1); Bacteria, *Corynebacterium ammoniagenes* (D5NZR1.1), *Klebsiella aerogenes* (WP_077203698.1), *Streptococcus mutans* (Q8DTG3.1), *Saccharomonospora cyanea* (H5XNC6.1); Basidiomycota (Basid.), *Coprinopsis cinerea* (A8NYJ8.1), *Cryptococcus neoformans* (Q5KP37.1), *Ustilago maydis* (1, XP_011387179.1; 2, XP_011391948.1); Ascomycota (Asc.), *A. fumigatus* Leu2A (Q4WRM6.1), Leu2B (Q4WLG7.1), *A. nidulans* LeuD (AN0912), LeuE (AN2793), *A. niger* Leu2A (P87256.1), Leu2B (P87257.1), *A. oryzae* (1, Q2TYA5.1; 2, Q877A9.1), *Botrytis cinerea* (XP_001546815.1) *Candida albicans* (C4YTB1.1), *Fusarium fujikuroi* (C1L3C2.1), *M. oryzae* (1, G4N5B0.1; 2, G4NIK0.1), *Neurospora crassa* (P34738.2), *Saccharomyces cerevisiae* Leu2p (YCL018W), *Schizosaccharomyces pombe* (P18869.1); Planta, *Chlamydomonas reinhardtii* (1, A8I7N4.1; 2, A8I7N8.1). The scale

bar corresponds to the branch length for an expected number of 0.1 substitutions per site. The two distinct *Aspergillus* clades are boxed.

Table 3.4 Pairwise protein sequence comparisons of β -IPM dehydrogenase genes.

Protein ^A	Systematic Name	Leu2p		Leu2A		Leu2B	
		% Identity	% Similarity	% Identity	% Similarity	% Identity	% Similarity
Leu2p	YCL018W	100	100	ND ^B	ND	ND	ND
LeuD	AN0912	62.8	79.6	87.7	94.5	50.7	66.3
LeuE	AN2793	50.1	64.8	53.3	67.7	84.9	82.2

^A *A. nidulans* LeuD and LeuE β -IPM dehydrogenase full protein sequences aligned pairwise and compared with *S. cerevisiae* Leu2p and *A. niger* Leu2A and Leu2B

^B ND: not determined

3.5.2 Characterization of the roles of LeuD and LeuE in leucine biosynthesis

As leucine biosynthesis pathways in other fungi only contain one β -IPM dehydrogenase, we investigated if either or both *leuD* and *leuE* participated in leucine biosynthesis in *A. nidulans*. Deletion mutants of *leuD* and *leuE* were generated by gene replacement (Figure 3.5A; See Materials and Methods). Leucine auxotrophy occurs when genes required for leucine biosynthesis are deleted (Downes et al., 2013; Polotnianka et al., 2004). In the case of *leuD* Δ and *leuE* Δ single deletion mutants, strict leucine auxotrophy was not observed (Figure 3.5B). The *leuE* Δ strain was indistinguishable from the wild type strain but the *leuD* Δ strain showed leaky leucine auxotrophy, meaning that there was a reduction of colony growth without exogenous leucine. Growth was restored to the wild type phenotype when supplemented with exogenous leucine. Transformation of the *leuD* gene into the *leuD* Δ strain also restored leucine prototrophy (Figure 3.6A). To determine if the leaky leucine phenotype of the *leuD* Δ mutant was due to LeuE function, we constructed a *leuD* Δ *leuE* Δ double mutant by meiotic crossing. We found that the *leuD* Δ *leuE* Δ double mutant was a strict leucine auxotroph, only growing when exogenous leucine was added to the media.

To complement the tight leucine auxotrophy of the *leuD* Δ *leuE* Δ double mutant, we transformed a plasmid containing wild type *leuE* and directly selected for leucine prototrophy (Figure 3.6B to D). Leaky leucine auxotrophy consistent with a *leuD* Δ single mutant phenotype was observed in single-copy integration transformants. However, multi-copy integration transformants showed stronger growth than the *leuD* Δ strains. This indicated that additional copies of *leuE* were able to partially suppress the *leuD* Δ phenotype. We then determined if expression differences between *leuD* Δ and *leuE* Δ accounted for the differences in leucine prototrophy. Using reverse transcription-quantitative PCR (RT-qPCR), we found that *leuD* had approximately 64-fold higher expression than *leuE* after 16h growth in 10mM ammonium-minimal media. RNA-Seq data showed that *leuD* had higher expression than *leuE* on three nitrogen sources: ammonium (35-fold higher), alanine (12-fold higher) and glutamine (13-fold higher) (Figure 3.5C). The leucine biosynthesis

pathway is regulated by feedback inhibition of leucine. We determined the effects of the *leuD* Δ deletion mutation on the preceding steps of the pathway, *luA* and *leuC*, and *leuE* by RT-qPCR. We also investigated the effects of *leuD* Δ mutation on *gdhA*, which encodes the main nitrogen assimilation enzyme NADP-glutamate dehydrogenase and is coregulated with leucine biosynthesis, using the enzymatic activity of LacZ expressed from the *gdhA-lacZ* translational fusion reporter gene (Downes et al., 2013; Polotnianka et al., 2004). All three leucine biosynthesis genes and *gdhA-lacZ* showed increased expression in the *leuD* Δ mutant as compared to wild type levels (Figure 3.5D and E). Therefore, we conclude that while the *leuD* Δ mutation results in reduced leucine production, other leucine biosynthesis genes and *gdhA* are upregulated to compensate for the loss.

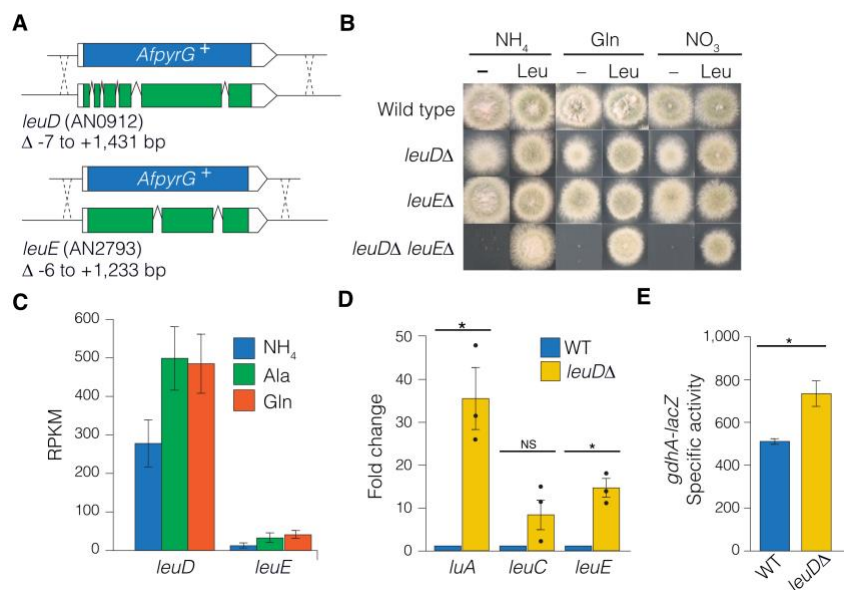


Figure 3.5 *leuD* encodes the major β -IPM dehydrogenase.

A) Strategy for the deletion of the two putative β -IPM dehydrogenase genes, *leuD* and *leuE* using cassettes that gene replaced the coding region with the *A. fumigatus pyrG* selectable marker (*Afp_{pyrG}⁺*). **B)** Wild-type (MH1), *leuD*Δ (RT411), *leuE*Δ (RT413), and *leuD*Δ *leuE*Δ (RT444) strains were grown at 37°C for 2 days on solid supplemented ANM with or without 2 mM leucine (Leu) and with 10 mM ammonium (NH₄), glutamine (Gln), and nitrate (NO₃) as the nitrogen source. **C)** Mean reads per kilobase per million mapped reads (RPKM) from RNA-seq of MH1 grown at 37°C for 16 h in supplemented liquid ANM with 10 mM ammonium (NH₄), glutamine (Gln), and alanine (Ala). **D)** RT-qPCR quantification of mean fold change in transcript expression in *leuD*Δ (RT411) strain compared to the wild type (MH1) grown at 37°C for 16 h in supplemented liquid ANM–10 mM ammonium and 2 mM leucine. Bars indicate the mean fold change from three independent biological replicates (circles). *, $P \leq 0.05$. NS, not significant using two-tailed Student's t test with equal distribution. **E)** LacZ specific activity for wild-type (MH12101) and *leuD*Δ (RT458) strains, which contain the –753 bp *gdhA-lacZ* reporter construct. Strains were grown at 37°C for 16 h in supplemented liquid ANM with 10 mM ammonium and 2 mM leucine ($n = 3$). *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$; NS, not significant; using one-way ANOVA. For panels C to E, error bars depict standard error of the mean ($n=3$).

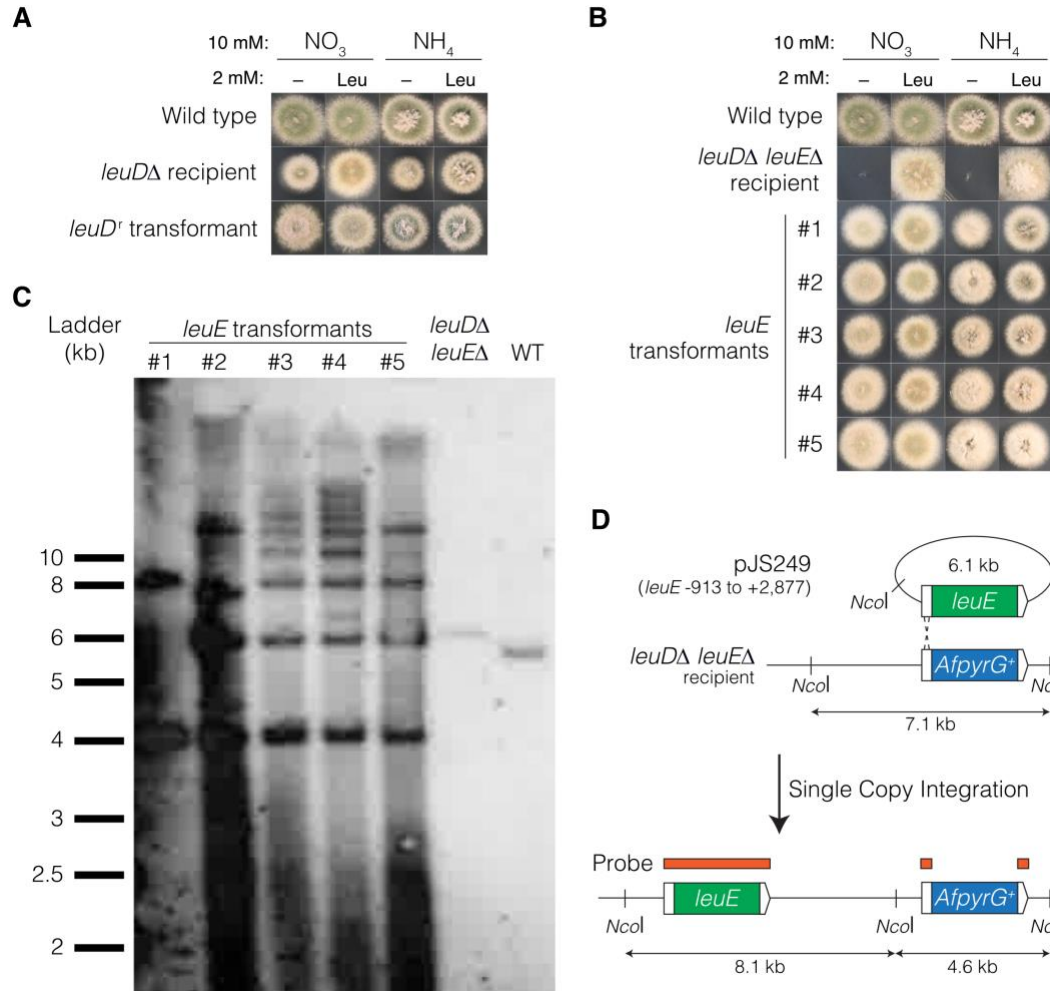


Figure 3.6 Complementation analysis of deleted β -IPM dehydrogenase genes.

A) Transformants of the *leuDΔ* mutant (RT411) with PCR-amplified wild type *leuD* DNA were grown at 37°C for 2 d on solid supplemented ANM with (Leu) or without (–) 2 mM leucine, and with 10 mM ammonium (NH₄) or nitrate (NO₃) as the nitrogen source. *leuD^r* indicates the reconstructed wild type *leuD* by gene replacement of *leuDΔ::Afp_{pyrG}*. **B)** Wild type (MH1), *leuDΔ leuEΔ* (RT444), and transformants of RT444 with a plasmid carrying wild type *leuE* were grown at 37°C for 2 d on solid supplemented ANM with (Leu) or without (–) 2 mM leucine, and with 10 mM ammonium (NH₄) or nitrate (NO₃) as the nitrogen source. Transformant #1 is a single copy *leuE* integrant by homology via the *leuEΔ* flanking sequence, while transformants #2–#5 are multicopy transformants. **C)** Southern analysis of *NcoI*-digested genomic DNA from *leuE* transformants, the *leuDΔ leuEΔ* recipient, and wild type, hybridized with the *leuE* plasmid insert as probe. **D)** Single copy homologous integration of the

leuE plasmid into the *leuD* Δ *leuE* Δ recipient strain. Only integration via crossover at the left flank of *leuE* is shown. *Nco*I restriction sites and restriction fragment lengths are indicated. Sequences bound by the *leuE* probe are represented by the orange boxes.

3.5.3 Identification of the six branched chain amino acid aminotransferase genes

The final step of the BCAA biosynthesis pathway is the reversible BCAA aminotransferase (BAT) step (Figure 3.1). While *S. cerevisiae* encodes two BAT proteins, Bat1p and Bat2p, there are six predicted BAT genes in the *A. nidulans* genomes (Eden et al., 1996; Kispal et al., 1996; Shimizu et al., 2010). We used BLASTp searches to confirm that these were all the BAT genes in the genome and designated them BatA (AN4323), BatB (AN5957), BatC (AN7878), BatD (AN7876), BatE (AN0385), and BatF (AN8511). Pairwise sequence comparisons showed that all *A. nidulans* BAT genes had >21% identity and >31% similarity to the Bat1p and Bat2p proteins and had strong conservation of the NAD cofactor binding residues and complete conservation of the catalytic lysine residue (Table 3.5 and Figure 3.7). In *S. cerevisiae*, the two BAT proteins are localized and function in different cellular compartments. Bat1p is localized to the mitochondria while Bat2p is mostly cytoplasmic (Kispal et al., 1996). Subcellular localizations of the *A. nidulans* BATs are currently unknown so we used DeepLoc-1.0, TargetP v1.1, and Predotar targeting signal predictions to calculate the likeliest location for each BAT (Almagro Armenteros et al., 2017; Emanuelsson et al., 2000; Nielsen et al., 1997; Small et al., 2004). All three algorithms predicted BatA and BatC, similar to Bat1p, to be mitochondrial (Tables 3.6-3.8). Deeploc-1.0 predicted the other BATs to localize to the cytoplasm (Table 3.8). Protein sequence alignment revealed that these three putative mitochondrial BATs have extended N termini with a predicted mitochondrial targeting signal (Figure 3.7).

We investigated the colinearity of the six *A. nidulans* BAT genes using the same method as we did for the β -IPM dehydrogenase genes (Figure 3.8). The *batA* and *batB* orthologs are highly colinear throughout all 27 species of Aspergilli while *batE* displayed moderate colinearity with 13 of its orthologs. The other three BAT genes, *batC*, *batD*, and *batF* lacked orthologs in many *Aspergillus* species, with *batD* orthologs in *A. niger* and *A. oryzae*. *batC* and *batD* are located in the same region of the *A. nidulans* genome, separated by ~2 kbp within the aspercryptins secondary metabolite biosynthetic gene cluster (Andersen et al., 2013; Chiang et al., 2016;

Henke et al., 2016; Inglis et al., 2013). While the physical linkage between these two would suggest gene duplication from unequal crossover, the genes have low sequence homology with 28.9% protein sequence identity.

We next determined the relationship between the six BATs using phylogenetic analysis (Figure 3.9). Two separate groups were formed within the fungi. Group I is the larger of the two groups, consisting of 37 out of the 52 fungal BATs. It contains BatA, BatB, BatC, both *S. cerevisiae* Bat1p and Bat2p, and one BAT protein from each of the other fungi investigated. Group II only had 15 of the 52 proteins and was almost exclusively Pezizomycotina (*Aspergillus*, *Penicillium*, *Fusarium*, *Neurospora*, *Magnaporthe*). Additionally, we noticed that BatC and BatD are in separate groups, consistent with each gene being separately recruited to the aspercryptins biosynthetic gene cluster.

Table 3.5 Pairwise protein sequence comparisons of BAT genes.

Protein ^A	Systematic Name	Bat1p		Bat2p	
		% Identity	% Similarity	% Identity	% Similarity
Bat1p	YHR208W	100	100	73.5	81.2
Bat2p	YJR148W	73.5	81.2	100	100
BatA	AN4323	49.3	59.7	49.0	59.2
BatB	AN5957	40.5	54.4	43.4	58.6
BatC	AN7878	44.9	62.9	45.8	59.9
BatD	AN7876	24.3	41.3	23.8	41.4
BatE	AN0385	24.2	36.6	24.9	39.0
BatF	AN8511	21.7	31.8	25.1	35.8

^A *A. nidulans* BatA, BatB, BatC, BatD, BatE, and BatF branched-chain amino acid aminotransferase full-length protein sequences were aligned pairwise and compared with *S. cerevisiae* Bat1p and Bat2p.

```

Sc_Bat1p      1 ~~~~~~MLO
Sc_Bat2p      1 ~~~~~~
AN4323        1 MRFGCFLRLSLNCKLRLVTQEAPFNLHYDLRYPSSAILLATGATADMKSLHQLARPRALPSFAAPRAQASAR
AN5957        1 ~~~~~~
AN7878        1 ~~~~~~MAR
AN7876        1 ~~~~~~
AN0385        1 ~~~~~~
AN8511        1 ~~~~~~

Sc_Bat1p      4 RHSLKL.GKFSIRTTLATGPTASKLKITRNPNPS..KPRPNEELV.....EGQTF...FDHMLT
Sc_Bat2p      1 ~~~~~~MTLAPLDASKVKITTTQHAS..KPKPNSELV.....FGKSF...FDHMLT
AN4323        71 SNRLWQRCFSATRAASEGQOLDPSKLTITKTSTPK..ELLPAKDLV.....FGKNF...FDHMLT
AN5957        1 ~~~~~~MGSIGNTLAELDASVVKITRSTELRHVPLPGSLDEL.....SHSYC...FDHMLT
AN7878        4 LLNRWHLPALVRRH...YSSRFQSLREKTSQPK..PLPDSTELQ.....EGRSF...FDHMLT
AN7876        1 ~~~~~~MSTFAEPRSDIEWE.....KVGVSFLDVNGHVES
AN0385        1 ~~~~~~MASQSFPFPPVDTIDWS.....NIGPKVREVNGHVES
AN8511        1 ~~~~~~MTEWKVTIIHADLTSAALTPANGDVQC

Sc_Bat1p      58 IPWSAKEGVTGTHIKPYGNLSLDPSACVPHAFEL.EGLKAYRTQON.TITMFRPDKNMA.MNKSAARIC
Sc_Bat2p      41 AEWTAEGKGTPEIKPYGNLSLDPSAVVPHAFEL.EGMKAYRTVDN.KITMFRPDMNMK.MNKSAORIC
AN4323        126 VEWTAAGGWLAPQIVPYQKLDPSACVPHAFEC.EGMKAYKDSKG.QIRLFRPDKNMQ.LNKSASARIA
AN5957        48 ARWTAAGGWETPEVKPFQNLSTPPASCLHAFEC.EGMKVYRGFDG.KLRIFRPDLNGE.LNSAVRAS
AN7878        55 LEWTTTQGWSDAQHTPYDNLRLDPSACVLAFTC.EGMKAYKDPHG.NARLFRPEENLA.LNRSAAALA
AN7876        31 DPNYQGTGTHSEPVFVQDHYLVKHGLAPGLN.GQQV.EGMKAYRDPNG.QIOTFRPTDHAL.MRSCDAVS
AN0385        33 HYTPAKKSHSPKLVKSPYPIHGMAPGLN.GQQA.EGLKAFRHNNNSKITFRPDRNAL.MRCSASFIS
AN8511        28 QFSRKFKSHAPLFIEDPFRVHGLAPVFN.GQEV.EGLKGIPTESQWPP.HHPLH.....

Sc_Bat1p      127 LPTFEESEELIKLTGKLTIEQDK..HLVPQGNGLSLYIRPTMIGTSKGLGVTSEALLYVITSVPVGYVYKT
Sc_Bat2p      110 LPTFDPEELITLIGKLIQODK..CLVPEGKGLSLYIRPTLIGTTAGLGSTDDRALLYVICCPVGYVYKT
AN4323        195 LPTVDGEALTTLIGELVKLDS..RFIPDARGHSLYIRPTMIGTQSTLG.GPPGSAALLYVIAVPVGYVYPT
AN5957        117 LPSFRFQELKTKTAKLMQIDGLRWLKDQPGRFLYRPTLIGSGTQLGQAAPAEALLTIAVWPDPDA.T
AN7878        124 LPTFEESEGLVLEFLANVVDLEK..RFIPHLPGHSLYIRPTLLGTDTSIS.SRPRSALLYVIASEMGDVYFAN
AN7876        100 IPSIPESLPHASVNLAVAKN.SEFVPPHASEAAMYIRPLAFSGGGMPPAAGPYKFFVYALFFCAYHGT
AN0385        103 IIPVPEDLPLEAVELAVGAN.AGFVPPHETGAAMYIRPLIFGSSAQGLGSPPEEYTFVYVFMPTGVYHGV
AN8511        85 .SPGSRTPFPRSVNLAVAN.AEFVPSHESQGMLYIRPTIFGSSACIQTPDCKYTFCYVTVAVYNGI

Sc_Bat1p      195 GKFA.....VRLBATDYATRAWPGGVGDR*LGANAPCIVPOLQAARKGYQONLWLFQ...EKNI
Sc_Bat2p      178 GKFA.....VRLBATDYATRAWPGGCGDR*LGANAPCVLPOLQAASRGYQONLWLFQ...NNNI
AN4323        263 GKFA.....ISLBATDYAVRAWPGGVGDR*LGANAPCIVPOLQAASRGYQONLWLFQ...EEYV
AN5957        186 RPKATPGELGLKLTAPDPTIRAWPGGEGYALGAN.GPSLAAGKAQAQGFDOVLWLFQ...DRQV
AN7878        192 GKMA.....VTLCATRSVPRAWPGGVGEF*VGCNAPSIVPEEAAEAGSQONLWLLADRESGEEFV
AN7876        .LP.....VDAAVL...EELDRAPLGVGNV*VGCNAPVLKWSDKARKEGFGITLHDSKT...RGEI
AN0385        172 .HA.....VDALIL...EDFDRAAPEGTGS*VGCNAPVLRHSAKAHAEGFGITLHDSRT...RSEI
AN8511        153 .NP.....LDALIL...EGFDRAAPRGTCG*VGCNAPVMKWSQARREGVATLHDSAT...RSEI

Sc_Bat1p      253 TVGTMNVPFFVFLNKVVGKKELVTAPLDG.TILEGTRDSVLTLARDKLDP....QENDINERYYTITE
Sc_Bat2p      236 TVGTMNAPFFVKDKSTGKKELVTAPLDG.TILEGTRDSVLTLARDKLDP....SEMTISERYFTIGE
AN4323        321 TVGTMNLFIALKNKETGKKELVTAPLDG.TILEGTRDSVLTLARDKLDP....KQNOVSERKIRMAE
AN5957        252 TVGASNFPIVWENAOCTGKKELVTAPLENQLILPGTRRSVLELARSRLNQAVGDLEAVEVVEKTFITWD
AN7878        254 TACTNMLPVVWSSSTGKKELVTAPLDG.TILPGTRMSVLELARELEG...DRSGIEVVERRTIMRE
AN7876        226 DFFSGFVGIKYTESSGGEKGYTLVVPNSQCHIKS.TSSTSVVEVAR.S.....LGVREVRPIPYDE
AN0385        229 DFFSGMIAVKKNKESGK.VTLVQPDSPNVIDS.TAASVCEIGKLW.....FGYDVEKRRIPYEE
AN8511        210 EFSAGFVGVKREKADG..DGVAVVVPDGGNVVDS*TVRCILAVAA.R.....MGWAVERRVVKYEE

Sc_Bat1p      317 VSTRAGQELLEAFSGGAAVVSPIKEIGWNNEDIHVPLLPGEQ...CGALTKQVAQWIADIOYGRV..N
Sc_Bat2p      300 VTERSKNGELLEAFSGGAAIVSPIKEIGWKGEQINIPLLPGEQ...TGPLAKEVAQWINGIOYGET..E
AN4323        385 VEEAADDEGRLLLEVFGAGAAIVSPVRTISYRGRLVNCGLKETEE...AGEIASOMKNWIEGIOYGEDE..E
AN5957        322 VEAANREGRVVVEAFVCGAFFITPVKLIRNGAVD..IQLLKPGQ...TAGYAAQIKSWIEAVMYGKDGA
AN7878        320 LAAASREGRLLEVFGAGAVVVSPPVRSIRWGDQCISCGLRDGE...AGPMSLQMKTWIEEVOYGLV..E
AN7876        287 LEF.....FDEVLAAGAAAMITSHRSITHRSKDQVFRYKTSDE...PGACEKLSRHKGIQKDEKDT
AN0385        289 LNE.....FDEVMAAGAAALVPIRSITRRSSGNRFYECCGGEAGGCEVCVKLLRTKGIQKQKIEET
AN8511        269 LNS.....FSEELACGAVTVIPIRSITCKSRGNRFTYDEASV.SKPPYPAKLAATGDIQRKVKET~

Sc_Bat1p      382 YGNWSKTVDLNL~~~~~
Sc_Bat2p      365 HGNWSRVVTDLNL~~~~~
AN4323        450 H.PWSYVL~~~~~
AN5957        387 NHEWSYIENESEK~~~~~
AN7878        385 H.PWSYRV~~~~~
AN7876        348 F.GWLKRVEEVTV~~~~~
AN0385        353 L.GWNRVVKAPPAEWVGAADKEEAGIEVPQACRMICTASNNY~~~~~
AN8511        331 ~~~~~~

```

Figure 3.7 Protein sequence alignment of BAT enzymes.

Clustal Omega alignment of Bat1p and Bat2p from *S. cerevisiae* with BatA (AN4323), BatB (AN5957), BatC (AN7878), BatD (AN7876), BatE (AN0385), and BatF (AN8511) from *A. nidulans*. The co-factor binding residues (green), and mitochondrial-targeting signals (blue) are highlighted. The catalytic lysine is marked with a star above and below. Shading was performed with Boxshade with a minimum of 0.5 identity (black) or similarity (gray).

Table 3.6 TargetP prediction of BAT subcellular localization.

Protein	TargetP v1.1					
	mTP	SP	Other	Loc.	RC	TPlen
Bat1p	0.723	0.035	0.265	M	3	17
Bat2p	0.118	0.067	0.84	–	2	-
BatA	0.515	0.182	0.252	M	4	79
BatB	0.131	0.088	0.819	–	2	-
BatC (AtnH)	0.956	0.019	0.074	M	1	18
BatD (AtnJ)	0.085	0.091	0.908	–	1	-
BatE	0.103	0.06	0.903	–	1	-
BatF	0.053	0.206	0.719	–	3	-

TargetPv1.1 predicts the probability of targeting to specific subcellular compartments. mTP: mitochondrial Targeting Peptide. SP: Signal Peptide. Loc: Predicated localization, where M is mitochondrial and – is any other location. RC: Reliability Class, a measure of the size of the difference between the highest and second highest output scores, on a scale from 1 to 5 and 1 indicating the strongest prediction. TPlen: Predicted presequence length.

Table 3.7 Predotar subcellular localization predictions for BATs.

Protein	Predotar V1.03			
	Mito.	ER	Other	Loc.
Bat1p	0.88	0.01	0.12	M
Bat2p	0.03	0.3	0.94	–
BatA	0.56	0.01	0.44	M
BatB	0	0.01	0.99	–
BatC (AtnH)	0.86	0.01	0.14	M
BatD (AtnJ)	0	0.01	0.99	–
BatE	0	0.01	0.99	–
BatF	0	0.01	0.99	–

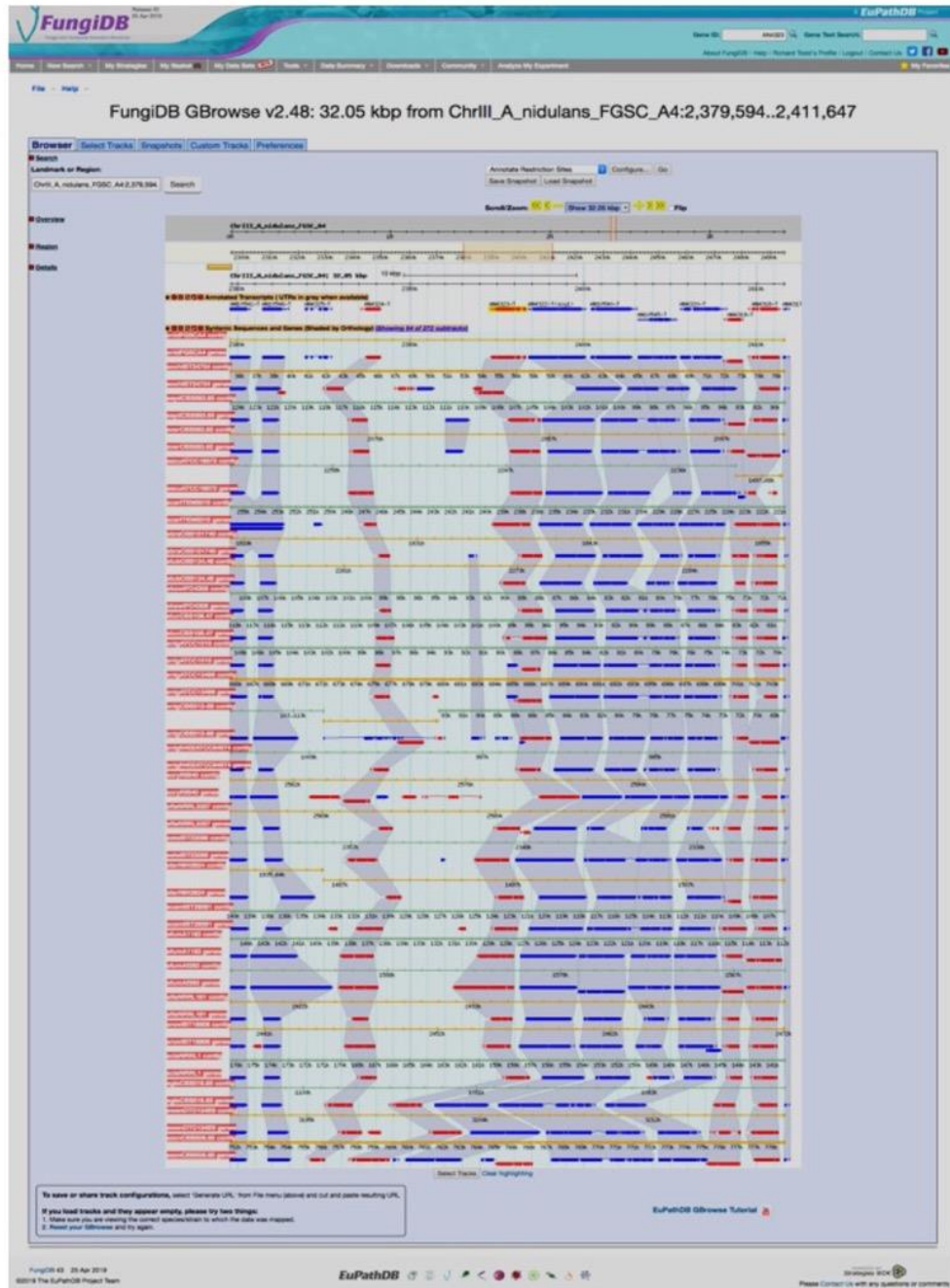
Predotar v1.03 predicts the probability of targeting to a specified subcellular compartment. Mito: Mitochondrial. ET: Endoplasmic reticulum. Loc: Predicted location where M is mitochondrial and – is undetermined.

Table 3.8 DeepLoc-1.0 predictions of subcellular localization of BATs.

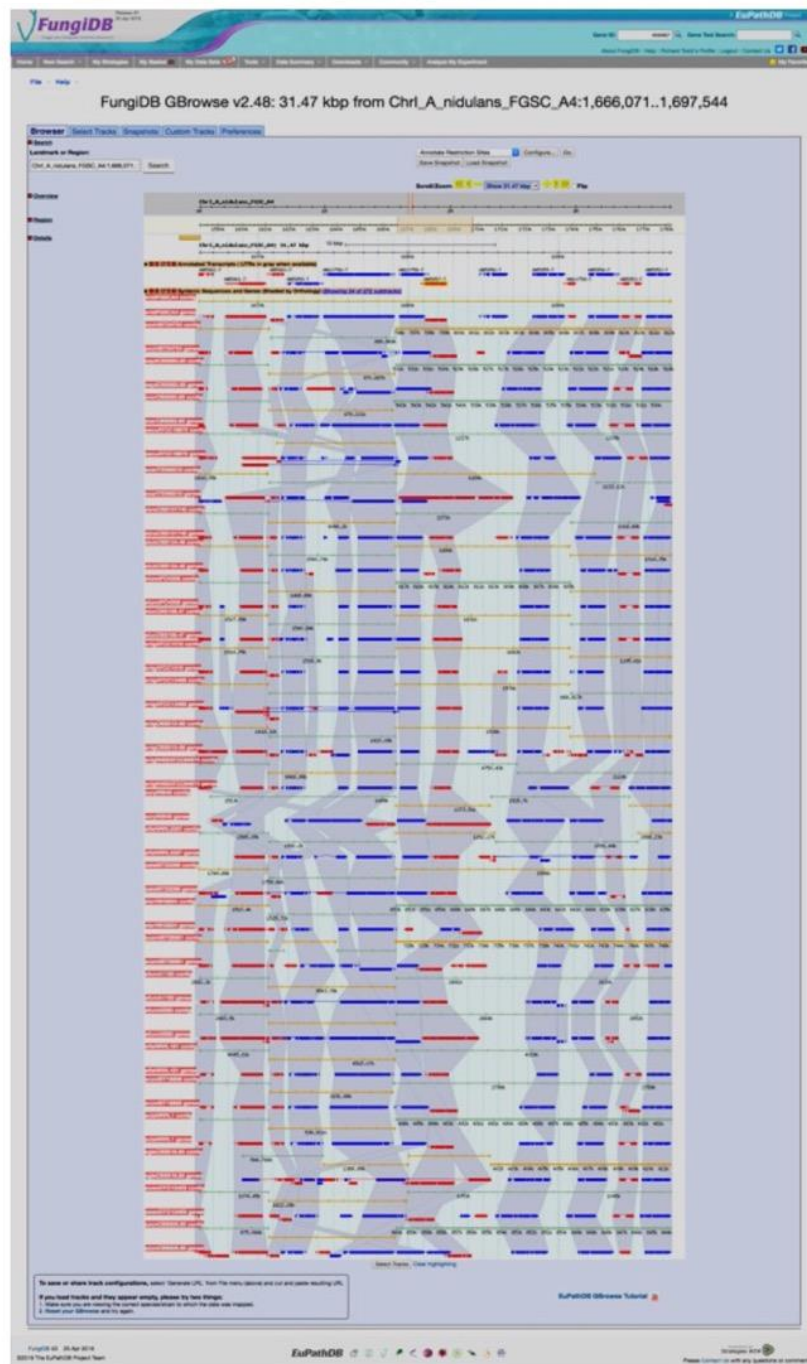
Protein	DeepLoc-1.0											
	Loc.	Type	Nuc.	Cyto.	EC	Mito.	CM	ER	Plastid	Golgi	Lyso. / Vac.	Perox.
Bat1p	M	Sol.	0	0.0009	0.0002	0.9981	0	0	0.0002	0	0	0.0006
Bat2p	C	Sol.	0.0019	0.9107	0.0027	0.0217	0.001	0.001	0.0067	0.0001	0.0028	0.0516
BatA	M	Sol.	0.0001	0.0049	0.0001	0.6932	0	0	0.2912	0	0	0.0105
BatB	C	Sol.	0.0008	0.5518	0.0032	0.0657	0.0018	0.0019	0.0058	0.0001	0.0028	0.366
BatC	M	Sol.	0	0.0006	0.0001	0.9984	0	0	0.0004	0	0	0.0005
BatD	C	Sol.	0.0005	0.9125	0.0006	0.0034	0.0002	0.0011	0.0017	0	0.0005	0.0795
BatE	C	Sol.	0.0009	0.8171	0.0016	0.0202	0.0006	0.0015	0.0234	0	0.0016	0.133
BatF	C	Sol.	0.0056	0.8688	0.0077	0.0113	0.0017	0.0071	0.0366	0.0006	0.0128	0.0478

Loc: Predicted location where M is mitochondria and C is cytoplasm. Type indicates either soluble (Sol.) or membrane (Mem.). Nuc: Nucleus. Cyto: Cytoplasm. EC: Extracellular. Mito: Mitochondria. CM: Cell Membrane. ER: Endoplasmic reticulum. Golgi: Golgi apparatus. Lyso./Vac.: Lysosome/Vacuole. Perox.: Peroxisome.

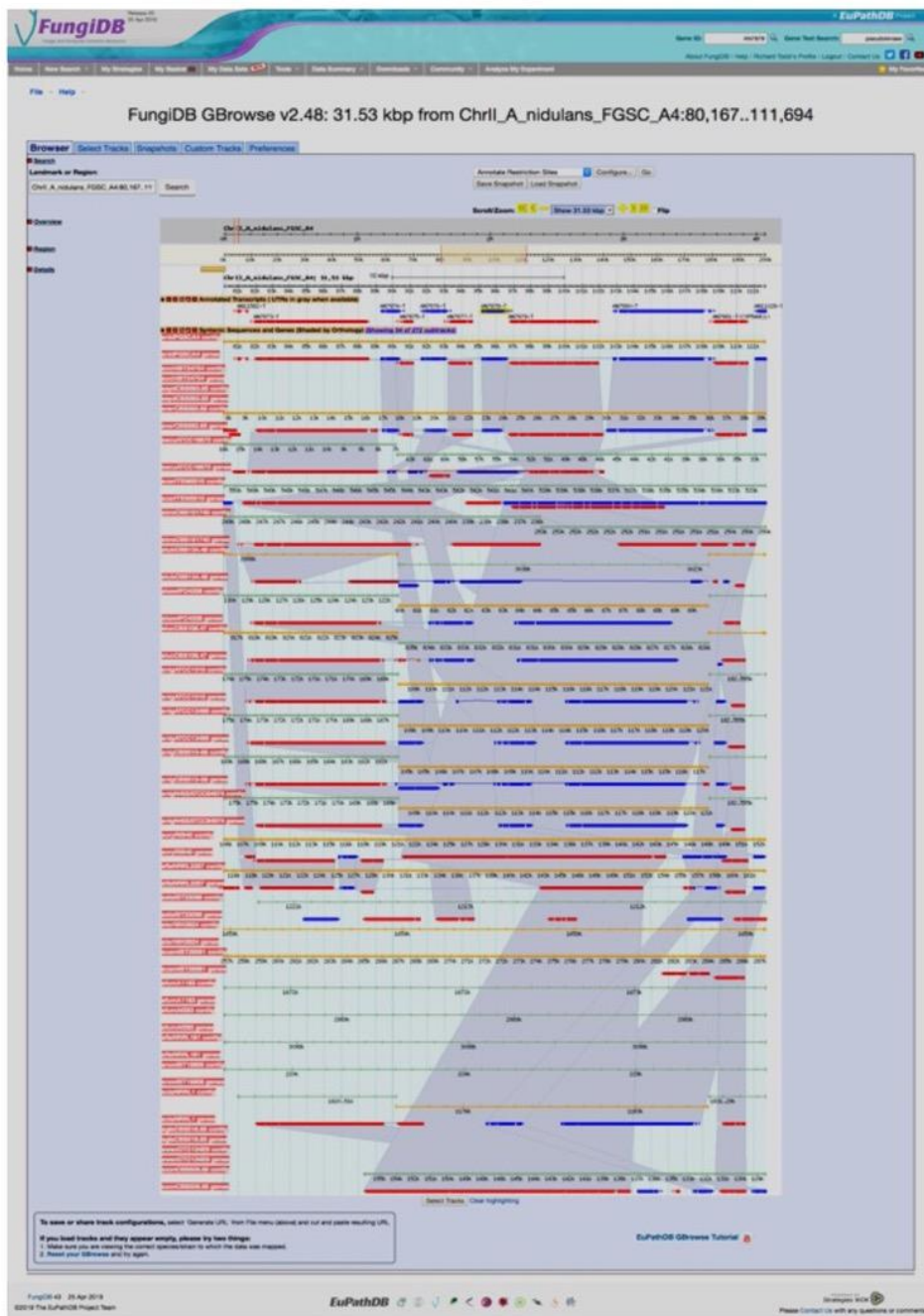
AN4323 (*batA*)



AN5957 (*batB*)



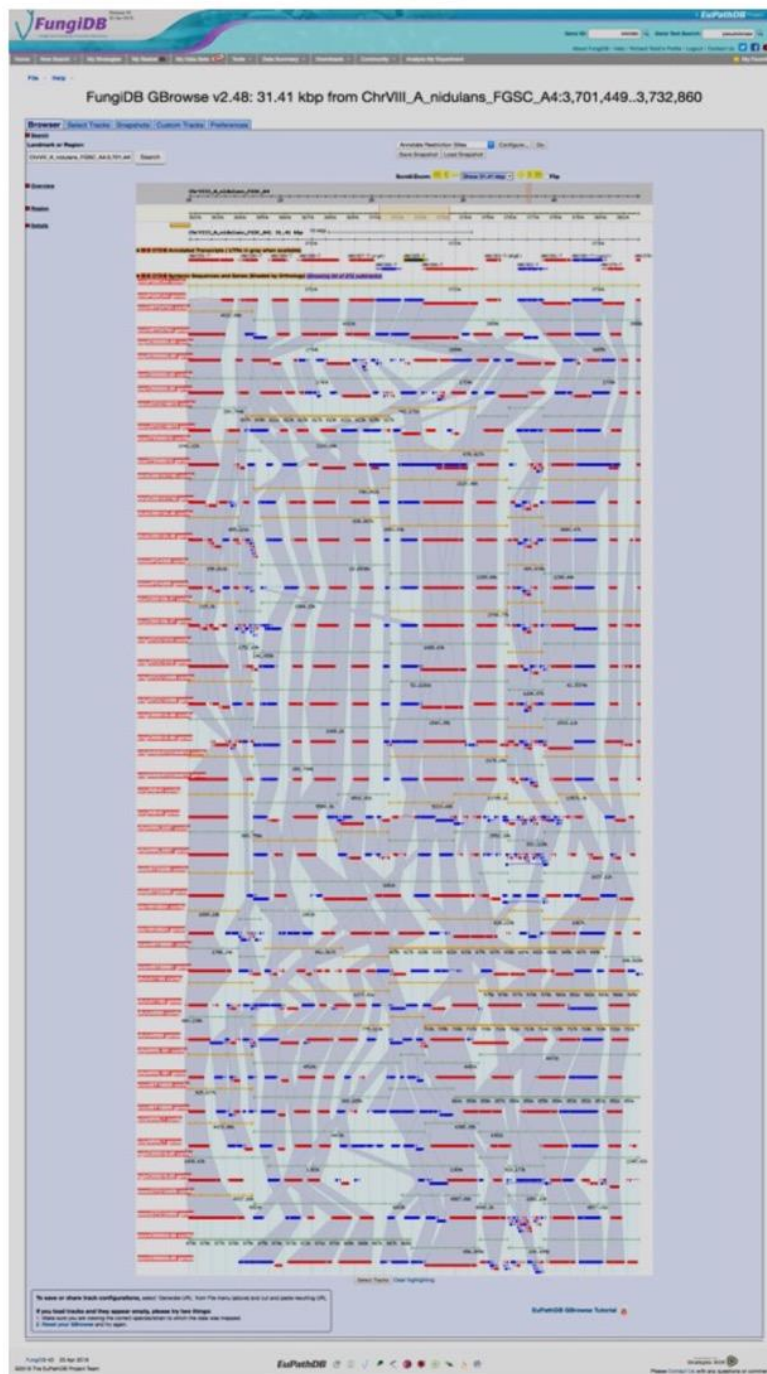
AN7878 (*batC*)



AN7876(batD)



AN0385 (*batE*)



AN8511 (*batF*)

Figure 3.8 Colinearity of BAT genes in Aspergilli.

The colinearity of syntenic regions for **(A)** AN4323 (*batA*), **(B)** AN5957 (*batB*), **(C)** AN7878 (*batC*), **(D)** AN7876 (*batD*), **(E)** AN0385 (*batE*), and **(F)** AN8511 (*batF*) was illustrated using the GBrowse genome browser of FungiDB with *Aspergillus* genomes displayed in the same order as in Figure 3.3.

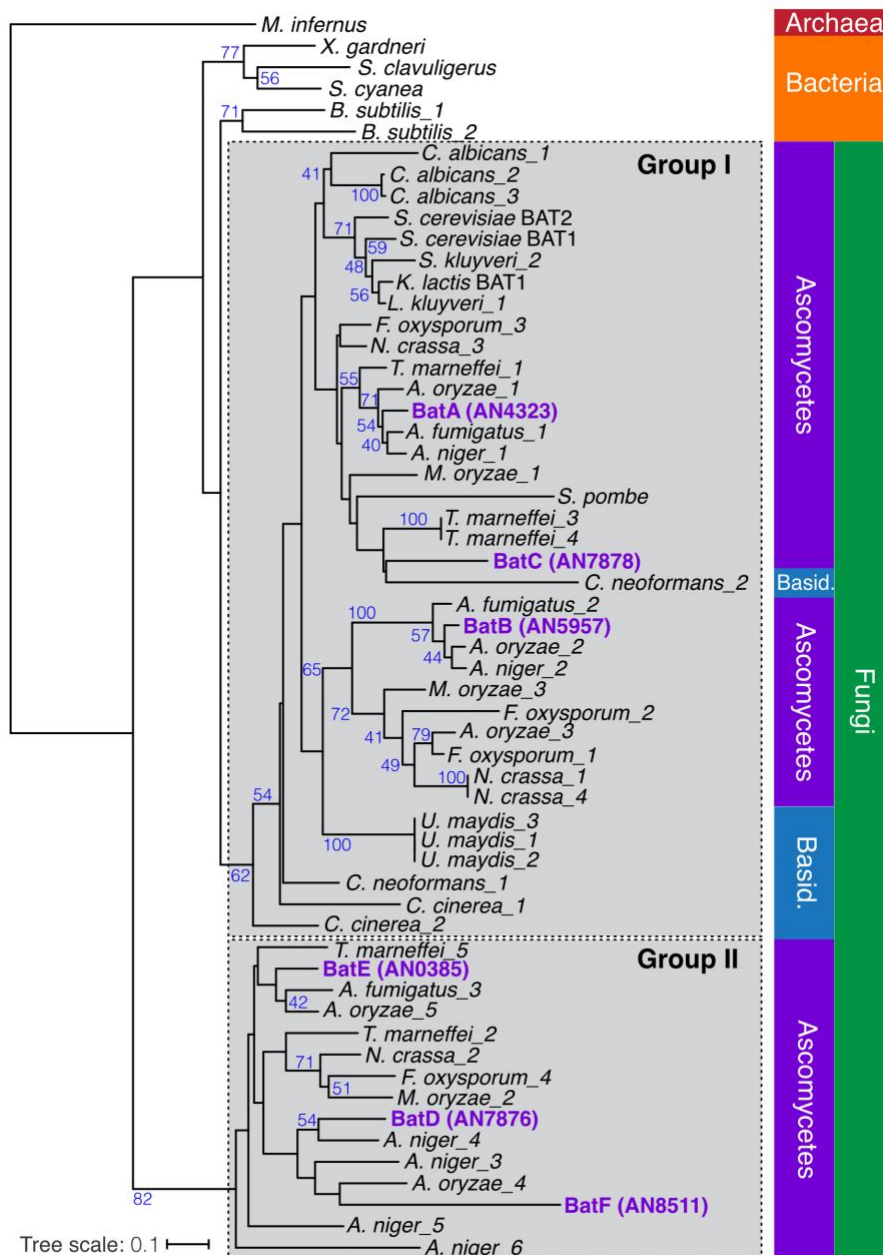


Figure 3.9 Phylogenetic Tree of BCAA aminotransferases.

Unrooted phylogeny of BCAA aminotransferases. Bootstrap support (100 replicates) greater than 40% is shown. Protein sequences for Aspergilli were downloaded from AspGD, sequences for *S. cerevisiae* were downloaded from SGD, and all other sequences came from Pfam or NCBI. Archaea: *Methanocaldococcus infernus* (D5VSZ6.1); Bacteria: *Bacillus subtilis* (1, O31461.1; 2, P39576.5), *Streptomyces clavuligerus* (B5H0M8.1), *S. cyanea* (H5XQS6.1), *Xanthomonas gardneri* (F0C966.1); Basidiomycota (Basid.): *C. cinerea* (1, A8N0B4.2; 2, A8N0V2.2), *C. neoformans* (1,

Q5K761.1; 2, Q5KD20.1), *U. maydis* (1, XP_011386074.1; 2, NC_026478.1:289079-290305; 3, CM003140.1:289079-290305); Ascomycota: *A. fumigatus* (1, Afu4g06160; 2, Afu2g10420; 3, Afu1g01680), *A. nidulans* (BatA, AN4323; BatB, AN5957; BatC, AN7878; BatD, AN7876; BatE, AN0385; BatF, AN8511), *A. niger* (1, An04g00430; 2, An02g06150; 3, An09g01990; 4, An10g00620; 5, An01g06530; 6, An05g01100), *A. oryzae* (1, AO090023000977; 2, AO090011000598; 3, AO090023000123; 4, AO090011000044; 5, AO090005000936), *C. albicans* (1, Q59YS9.1; 2, Q5AHJ9.1; 3, Q5AHX4.1), *Fusarium oxysporum* (1, F9FH16.1; 2, F9FH71.1; 3, F9FL84.1; 4, F9FPH4.1), *M. oryzae* (1, G4MK83.1; 2, G4MNR9.1; 3, G4NDD5.1), *N. crassa* (1, Q9HEB7.2; 2, Q7SFT9.2; 3, Q7S699.1; 4, Q1K779.1), *S. cerevisiae* (Bat1p, YHR208W; Bat2p, YJR148), *S. pombe* (O14370.2), *Kluyveromyces lactis* (XP_451451.1), *Saccharomyces kluyveri* (1, CM000688.1:1070225-1071418; 2, CM000688.1:c1071418-1070225), and *Talaromyces marneffe* (1, XP_002144420.1; 2, XP_002147519.1; 3, XP_002148544.1; 4, XP_002148548.1; 5, XP_002152979.1). The scale bar corresponds to the branch length for an expected number of 0.1 substitutions per site. The two distinct fungal BAT clades are boxed.

3.5.4 Determining gene expression profiles for the major BAT-encoding genes

We characterized expression of the six BAT-encoding genes using RNA-Seq from wild type mycelia grown on ammonium, alanine, and glutamine. RNA-Seq analysis showed that *batA* was the most highly expressed on all three nitrogen sources (Figure 3.10A). *batB* had the next highest levels of expression, with increased expression on alanine and glutamine, while *batC*, *batD*, and *batE* had intermediate levels of expression. *batF* was not expressed under any of the three conditions tested. Since *batC* (*atnH*) and *batD* (*atnJ*) were shown to be involved in secondary metabolism (see below) (Chiang et al., 2016; Henke et al., 2016), we focused on the four remaining BAT genes. We used RT-qPCR of RNA prepared from samples grown on ammonium, alanine, or nitrate and measured expression of *batA*, *batB*, *batE*, and *batF*. Consistent with the RNA-Seq analysis, *batF* was not detectable. The expression levels of the three other *bat* genes did not vary substantially between conditions (Figure 3.10B).

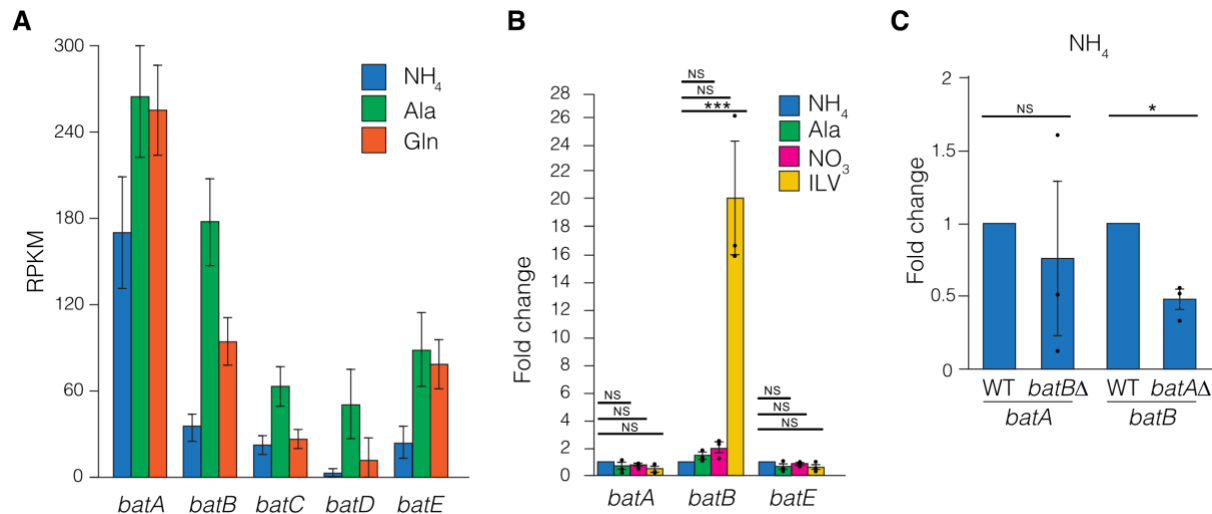


Figure 3.10 Transcriptome analysis of BAT-encoding genes.

A) Mean reads per kilobase per million mapped reads (RPKM) from RNA-seq of MH1 grown at 37°C for 16 h in supplemented liquid ANM with 10mM ammonium (NH₄), glutamine (Gln), and alanine (Ala). Error bars depict SEM (n=3). **B**) RT-qPCR to measure expression levels of *batA*, *batB*, and *batE* under anabolic conditions compared with catabolic conditions. The wild type (MH1) was grown for 16 h in supplemented liquid ANM with 10 mM ammonium (NH₄), nitrate (NO₃), or alanine (Ala) (anabolic conditions) or 3.3 mM (each) ILV (catabolic conditions). Mean fold change (bars) in expression is shown relative to the wild type on 10 mM ammonium for three independent replicates (circles). ***, $P \leq 0.0001$; NS, not significant, using a two-tailed Student's t test with equal variance. *batF* was not detected by either RNA-seq or RT-qPCR. **C**) RT-qPCR of *batA* and *batB* in the wild-type (MH1), *batA*Δ (RT415), or *batB*Δ (RT440) strains grown for 16 h in supplemented liquid ANM with 10mM ammonium. Mean fold change in expression (bars) relative to the wild type for three independent replicates (circles) is shown. *, $P \leq 0.05$; NS, not significant, using a two-tailed Student's t test with equal variance.

3.5.5 Genetic analysis of the six BAT-encoding genes

The number of BAT-encoding genes in *A. nidulans* suggests neofunctionalization or specialization for isoleucine, leucine, or valine production or breakdown. To determine which BAT-encoding genes were required for BCAA biosynthesis, we constructed single deletion mutants of each of the six genes (Figure 3.11). Growth tests of the single *bat* deletion mutants showed that all were BCAA prototrophs, indicating that each of the six bats were dispensable for BCAA production (Figure 3.12A). While investigating the role of the six *bat* genes in BCAA biosynthesis, *batC* and *batD* were published by others to be involved in aspercryptins biosynthesis. Named *atnH* and *atnJ* (formerly *batC* and *batD* respectively), these two genes are thought to produce three different types of BCAAs component of aspercryptins, specifically 2-aminocaprylic acid, 2-aminododecanoic acid, and 2-aminodecanoic acid (Chiang et al., 2016; Henke et al., 2016). Since these two genes were shown to be involved in other metabolic processes, we focused the rest of our study on the four other BAT genes.

We constructed double, triple, and quadruple mutants of *batA* Δ , *batB* Δ , *batE* Δ , and *batF* Δ by meiotic crossing and confirmed by either Southern blot or diagnostic PCR. The *batA* Δ *batB* Δ double mutant, which is a deletion of the two most related and highly expressed genes, was a strict BCAA auxotroph (Figure 3.12B). The *batA* Δ *batB* Δ *batE* Δ and *batA* Δ *batB* Δ *batF* Δ triple mutants and the *batA* Δ *batB* Δ *batE* Δ *batF* Δ quadruple mutants showed the same BCAA auxotrophy as the *batA* Δ *batB* Δ double mutant. However, any of the double or triple *bat* deletion mutants that contained either *batA* or *batB* were BCAA prototrophs. Therefore, BatA and BatB have overlapping roles as the major BAT enzymes in BCAA biosynthesis. Introduction of either wild type *batA* or *batB* into the *batA* Δ *batB* Δ double mutant restored BCAA prototrophy (Figure 3.13A). As *leuD* deletion caused a compensatory upregulation of its paralog *leuE*, we investigated if the loss of either *batA* or *batB* caused upregulation of the remaining *bat* gene. RT-qPCR showed that there was no upregulation of either *batA* or *batB* in a *batB* Δ or *batA* Δ mutant (Figure 3.10C). This indicates that wild type expression levels of either *batA* or *batB* is sufficient for BCAA prototrophy.

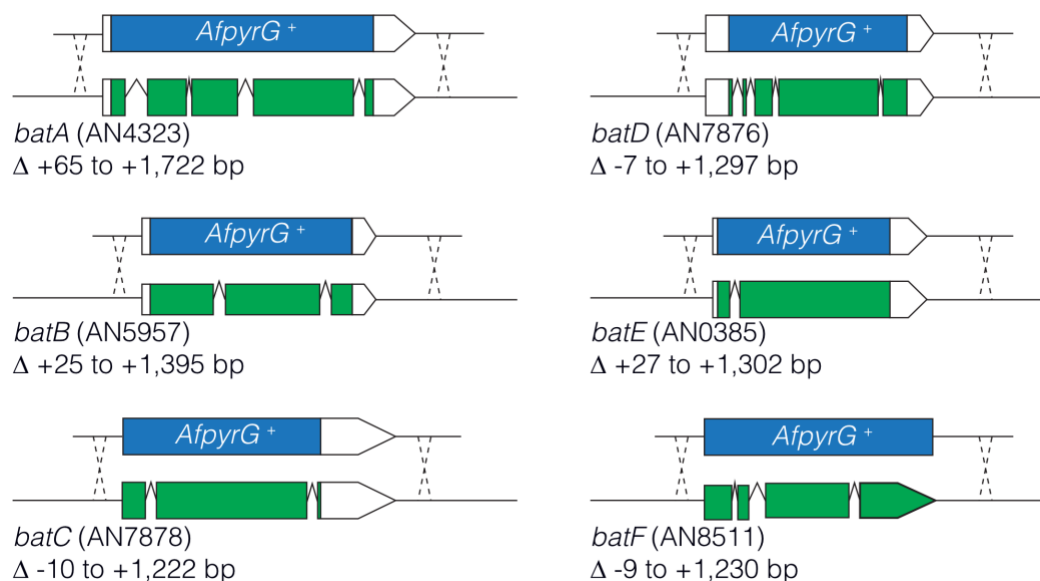


Figure 3.11 Gene Knockout Cassettes for BATs.

Strategy for the deletion of the six candidate BAT-encoding genes *batA*, *batB*, *batC*, *batD*, *batE*, and *batF* using cassettes that gene replaced the coding region with the *A. fumigatus pyrG* selectable marker (*AfpYrG+*).

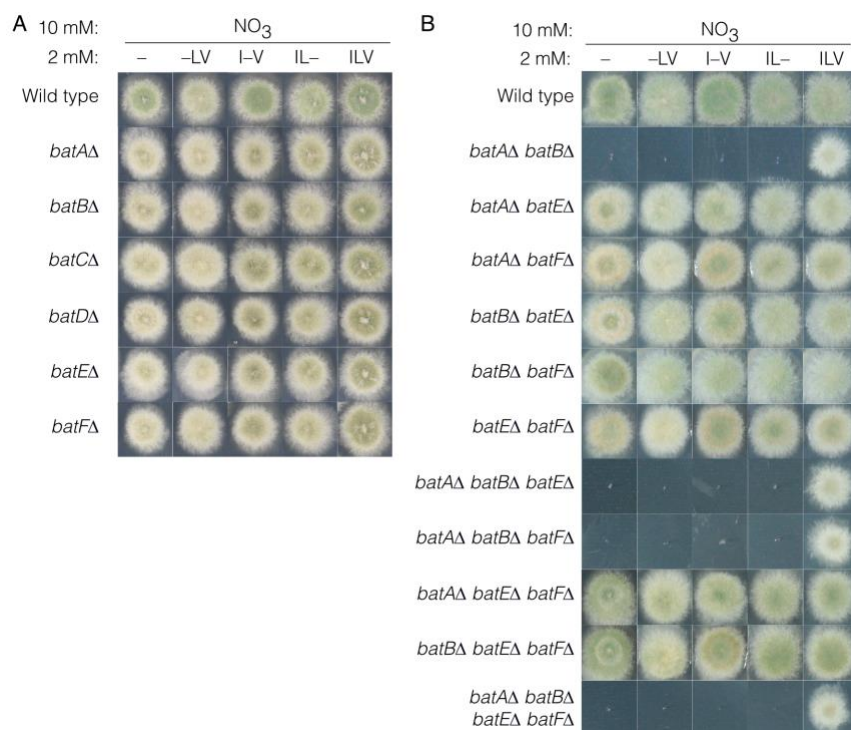


Figure 3.12 Growth testing of the single and combinatorial BAT gene deletions.

A) Wild-type (MH1), *batA*Δ (RT415), *batB*Δ (RT440), *batC*Δ (RT475), *batD*Δ (RT419), *batE*Δ (RT417), and *batF*Δ (RT441) strains were grown on supplemented ANM solid media for 2 days with 10 mM nitrate as the predominant nitrogen source and combinations of 2 mM (each) isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. –, an omitted amino acid. **B)** Wild-type (MH1), *batA*Δ *batB*Δ (RT457), *batA*Δ *batE*Δ (RT648), *batA*Δ *batF*Δ (RT645), *batB*Δ *batE*Δ (RT636), *batB*Δ *batF*Δ (RT526), *batE*Δ *batF*Δ (RT466), *batA*Δ *batB*Δ *batE*Δ (RT520), *batA*Δ *batB*Δ *batF*Δ (RT523), *batA*Δ *batE*Δ *batF*Δ (RT647), *batB*Δ *batE*Δ *batF*Δ (RT531), and *batA*Δ *batB*Δ *batE*Δ *batF*Δ (RT642) strains grown on supplemented ANM solid media for 2 days with 10 mM nitrate (NO_3) as the predominant nitrogen source and combinations of 2 mM each isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. –, an omitted amino acid.

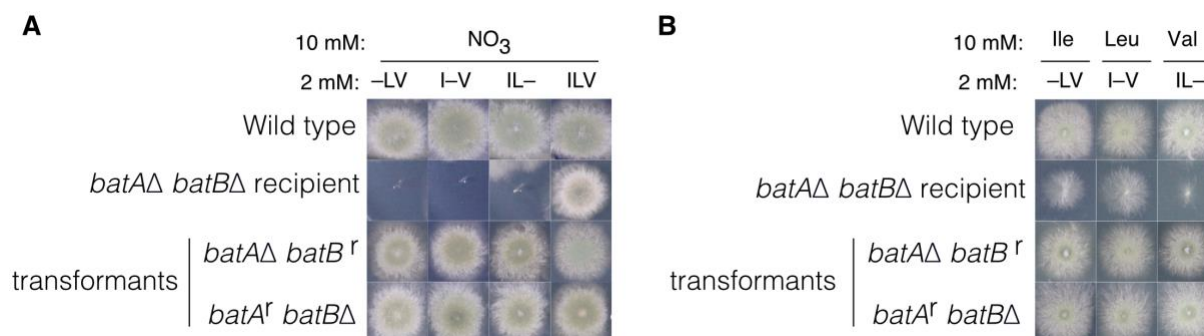


Figure 3.13 Repair analysis of deleted BAT-encoding genes.

A) Repair analysis of the *batAΔ batBΔ* biosynthetic growth phenotype with wild type DNA of either *batA* or *batB*. Wild type (MH1), *batAΔ batBΔ* (RT457) and transformants of RT457 with PCR-amplified wild type *batA* (*batA^r batBΔ*) or *batB* (*batAΔ batB^r*) DNA, grown under ILV anabolic conditions at 37°C for 2 days on solid supplemented ANM with 10 mM nitrate (NO₃) as the nitrogen source and combinations of 2 mM each of isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. *batA^r* and *batB^r* indicate the reconstructed wild type allele. **B)** Repair analysis of the *batAΔ batBΔ* catabolic growth phenotype with wild type DNA of either *batA* or *batB*. Strains from panel A grown under ILV catabolic conditions at 37°C for 2 days on solid supplemented ANM with 10 mM of either Isoleucine (Ile), Leucine (Leu), or Valine (Val) as the predominant nitrogen source and combinations of 2 mM each of isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. In **A** and **B**, – represents an omitted amino acid.

3.5.6 Characterization of BAT-encoding genes in BCAA catabolism

The BATs constitute the last step in BCAA biosynthesis but also are the first step in BCAA catabolism (Dickinson and Norte, 1993). We investigated the roles of each of the BATs in catabolism using each of the BCAAs as the primary nitrogen source, supplemented with lower amounts of the other two BCAAs to ensure supplementation of the BCAA auxotrophy (Figure 3.14A). Single *bat* deletion mutants were able to utilize the three BCAAs as a nitrogen source. In the single, double, triple, and quadruple mutants, strains lacking *batB* had a reduced colony morphology in comparison to strains with wild type *batB*. Additionally, the *batA* Δ *batB* Δ double, triple, and quadruple mutants had severe reduction in colony growth. This reduction was greater on isoleucine and valine than on leucine. We further investigated the *batB* Δ phenotype by examining wild type, *batA* Δ , *batB* Δ , and *batA* Δ *batB* Δ on increasing amounts of equimolar ILV (Figure 3.14B). We found that the *batB* Δ mutant has reduced colony morphology in comparison to either *batA* Δ mutant or wild type at each concentration but grew better than the *batA* Δ *batB* Δ double mutant. Transformation of either wild type *batA* or *batB* into the *batA* Δ *batB* Δ double mutant restored BCAA utilization (Figure 3.13B). We next examined if there was a difference in relative expression levels between *batA*, *batB*, *batE*, and *batF* under catabolic conditions (Figure 3.10B). While both *batA* and *batE* expression levels were similar under either biosynthetic or catabolic conditions, *batB* was substantially elevated during ILV catabolism. This, in combination with the growth tests, indicates that BatB is the primary catabolic enzyme. *batF* was not detectable under this condition. There was no compensatory upregulation of *batA* or *batB* under catabolic conditions in the *batB* Δ or *batA* Δ mutants, respectively (Figure 3.14C). Therefore, the *A. nidulans* BatA and BatB are the primary enzymes for both BCAA biosynthesis and utilization. We did not observe any phenotypes for the *batE* Δ and *batF* Δ mutants in BCAA biosynthesis or utilization.

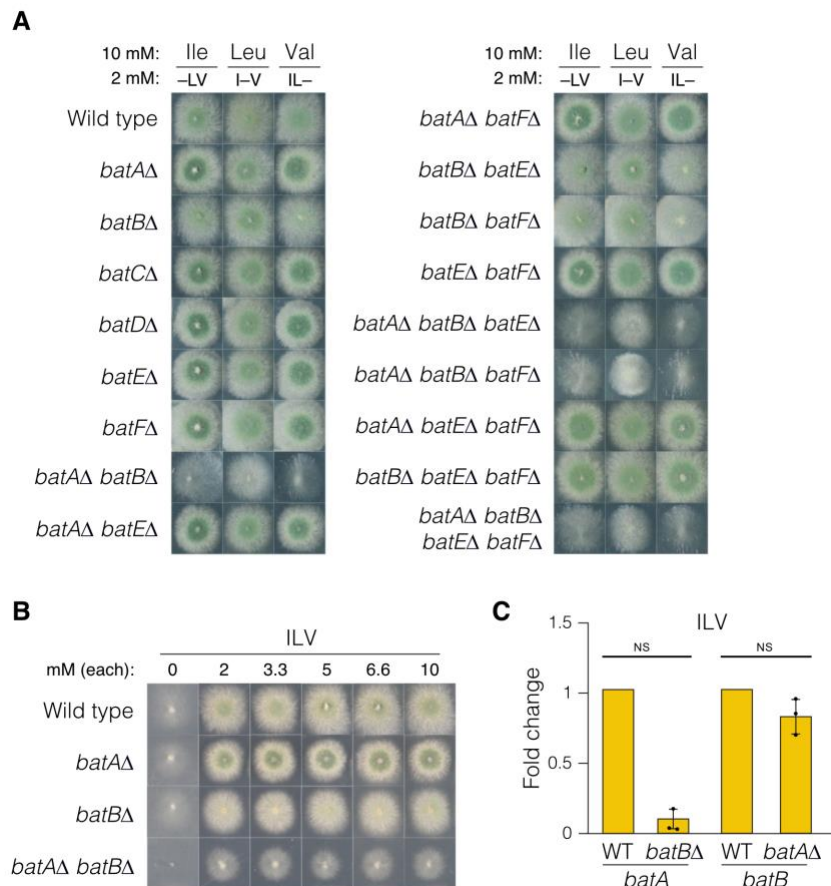


Figure 3.14 Single and combinatorial analysis of BAT-encoding genes during BCAA catabolism.

A) Wild-type (MH1), *batAΔ* (RT415), *batBΔ* (RT440), *batCΔ* (RT475), *batDΔ* (RT419), *batEΔ* (RT417), *batFΔ* (RT441), *batAΔ batBΔ* (RT457), *batAΔ batEΔ* (RT648), *batAΔ batFΔ* (RT645), *batBΔ batEΔ* (RT636), *batBΔ batFΔ* (RT526), *batEΔ batFΔ* (RT466), *batAΔ batBΔ batEΔ* (RT520), *batAΔ batBΔ batFΔ* (RT523), *batAΔ batEΔ batFΔ* (RT647), *batBΔ batEΔ batFΔ* (RT531), and *batAΔ batBΔ batEΔ batFΔ* (RT642) strains were grown on supplemented ANM solid media for 2 days with 10 mM isoleucine (Ile), leucine (Leu), or valine (Val) as the predominant nitrogen source and combinations of 2 mM each isoleucine (I), leucine (L), and valine (V) to supplement auxotrophies. –, an omitted amino acid. **B)** Wild-type (MH1), *batAΔ* (RT415), *batBΔ* (RT440), and *batAΔ batBΔ* (RT457) strains were grown on supplemented ANM solid media for 2 days at 37°C with increasing equimolar concentrations of isoleucine (I), leucine (L), and valine (V). **C)** RT-qPCR of *batA* and *batB* in the wild-type (MH1), *batAΔ* (RT415), or *batBΔ* (RT440) strains grown for 16 h in supplemented liquid ANM with 3.3 mM

(each) isoleucine (I), leucine (L), and valine (V), i.e., catabolic conditions. Mean fold change in expression (bars) relative to the wild type for three independent replicates (circles) is shown. NS, not significant using two-tailed Student's t test with equal variance.

3.6 Discussion

Our work has characterized the genes involved in the final two steps of the *A. nidulans* leucine biosynthesis pathway and thus we have characterized which genes are involved in the final step of BCAA biosynthesis pathway for isoleucine, leucine, and valine. We have determined that there is divergence between yeast and aspergilli in the number of genes encoding enzymes for each step. *S. cerevisiae* has two genes that encode α -IPM synthetases, Leu4p and Leu9p. Homodimers and heterodimers of the two proteins show varying levels of sensitivity to leucine levels in *S. cerevisiae* (Baichwal et al., 1983; Casalone et al., 2000; Chang et al., 1984; Chang et al., 1985; López et al., 2015). In *A. nidulans*, only one gene encodes an α -IPM synthetase, LeuC (Downes et al., 2013). The next step converts α -IPM to β -IPM by an isopropylmalate isomerase, encoded by a single gene, *LEU1* and *luA*, in both *S. cerevisiae* and *A. nidulans*, respectively. Divergence again occurs at the conversion of β -IPM into ketoisocaproate as *S. cerevisiae* encodes a single β -IPM dehydrogenase, Leu2p, but *A. nidulans* has two, LeuD and LeuE (Andreadis et al., 1984; Toh-e et al., 1980). The last step of the BCAA biosynthesis pathway is catalyzed by the branched chain amino acid aminotransferase (BAT) enzyme. *S. cerevisiae* has two BAT-encoding genes (Eden et al., 1996; Kispal et al., 1996). In *A. nidulans*, there are six genes that encode BAT enzymes; however our data indicates that BatA and BatB are the primary enzymes in BCAA biosynthesis. The steps that have dimeric enzymes, namely α -IPM synthetase, β -IPM dehydrogenase, and BAT, differ in gene number but the monomeric α -IPM isomerase gene is nonduplicated (López et al., 2015; Colón et al., 2011; Hsu and Schimmel, 1984; Parsons and Burns, 1969; Bigelis and Umbarger, 1976). The α -IPM synthetase and BAT gene duplication in *S. cerevisiae* was a result of the ancestral whole-genome duplication and the paralogous enzymes now exhibit functional diversification after the acquisition of fermentative metabolism (Escalera-Fanjul et al., 2019).

The *Aspergillus* lineage did not have a whole-genome duplication event but other mechanisms or events can cause gene duplication (Galagan et al., 2005). Generally, gene duplication can lead to robustness by neofunctionalization but in

some cases confers genome fragility (Diss G et al., 2017; Escalera-Fanjul et al., 2019; Wapinski et al., 2007). We determined that both *leuD* and *leuE* function in leucine biosynthesis, although lower expression and prototrophy of the *leuE* Δ mutant indicates that *leuE* plays a secondary role to *leuD*. In this case, gene duplication provided redundancy as loss of *LeuD* resulted in upregulation of *leuE* to compensate. Our analysis also showed that each of the six *A. nidulans* BATs are dispensable. Yet, when we combined deletions of BAT encoding genes, we discovered that BatA and BatB are the primary enzymes for BCAA biosynthesis and utilization. The *A. nidulans* BatA counterpart in *S. cerevisiae* is Bat1p and both are mitochondrial isoforms and predominantly biosynthetic. BatB and Bat2p correspond to each other and they are cytoplasmic isoforms and are predominately catabolic (Colón et al., 2011; Montalvo-Arredondo et al., 2015; Takpho et al., 2018b). Gene duplication provided genome robustness as BatA and BatB can be biosynthetic or catabolic. This distribution of biosynthetic or catabolic roles is observed in *Lachancea kluyveri* (Montalvo-Arredondo et al., 2015). In contrast, *Kluyveromyces lactis* is thought to predate the whole genome duplication event that is observed in *S. cerevisiae* and thus has one BAT gene capable of both BCAA biosynthesis and degradation (Colón et al., 2011).

New functions in different metabolic pathways may account for the dispensability of the remaining four BATs in *A. nidulans*. Although we did not observe a role for *batE* in BCAA biosynthesis and catabolism, it has been shown that *batE* is upregulated during hypoxia. This upregulation occurs in the absence of glucose-to-ethanol fermentation and increased BCAA biosynthesis to generate NAD⁺ to survive under anaerobic stress (Shimizu et al., 2016; Shimizu et al., 2010). Under our normoxic laboratory conditions, we did not find a role for *batE* in BCAA metabolism. *batC* and *batD*, now called *atnH* and *atnJ* respectively, are characterized as part of the aspercryptins biosynthetic gene cluster. *atnH* and *atnJ* are thought to be the transaminases of unusual BCAAs: 2-aminocaprylic acid, 2-aminododecanoic acid, and 2-aminodecanoic acid (Chiang et al., 2016; Henke et al., 2016). Aspercryptins contain three BCAAs in their structure: isoleucine or valine, 2-aminocaprylic acid, and 2-aminododecanoic acid or 2-aminodecanoic acid. We did not find a role of *batF* in BCAA metabolism as it was undetectable under our growth conditions and in both our

and publically available RNA-Seq data under multiple conditions (Sibthorp et al., 2013). *batF* is located near the terriquinone A (*tdi*) biosynthetic gene cluster, but unlikely to contribute to terriquinone A as it is not part of the established *tdi* biosynthetic gene cluster (Inglis et al., 2013; Baichwal et al., 1983; Bouhired et al., 2007; Kelly et al., 2009; Balibar et al., 2007).

The lack of a leucine biosynthesis pathway in mammals and the established reduction in virulence of leucine metabolic mutants in fungi makes the fungal leucine biosynthesis enzymes strong candidates for antifungal treatments (Do et al., 2015; Kingsbury and McCusker, 2010; Oliver et al., 2012; Orasch et al., 2019; Long et al., 2018; Kingsbury et al., 2004). Duplication and redundancy of BCAA biosynthesis pathway enzymes of aspergilli must be considered for effective antifungal treatments, as targeting one enzyme may not be sufficient for leucine auxotrophy and thus reduced virulence. Overall, this study has annotated the *A. nidulans* leucine biosynthesis pathway. We have determined the relative roles of *leuD* and *leuE* in leucine biosynthesis and for *batA* and *batB* in BCAA metabolism. While the roles of *batC* (*atnH*) and *batD* (*atnJ*) have been reported in aspercryptins production, the roles of *batE* and *batF* remain undetermined.

3.7 Future Directions

Our research has characterized the full leucine biosynthesis pathway in *A. nidulans*, defining the roles for the β -IPM dehydrogenase enzymes, LeuD and LeuE, and the reversible BCAA aminotransferase enzymes, BatA and BatB, which act in biosynthesis and catabolism of all three proteinogenic BCAAs. The roles of BatE and BatF remain undetermined but may still participate in BCAA biosynthesis or catabolism under other conditions, such as hypoxia for the *batE* orthologs. Identifying *batE* regulators or artificially upregulated *batE* expression by inducible promoter in the *batA* Δ *batB* Δ double mutant may provide insights into the function of BatE as a BCAA aminotransferase. As *batF* is located near the *tdi* secondary metabolite gene cluster, it may be involved in secondary metabolite production in some capacity. One possibility would be that *batF* is involved in making alternatives of terriquinone A and subject to repression under normal lab conditions or under hypoxia.

Chapter 4 - Suppressor Of Branched Chain Amino Acid Auxotrophy

4.1 Abstract

The branched chain amino acids (BCAAs), isoleucine, leucine, and valine are synthesized de novo in filamentous fungi. BCAAs are necessary for fungal growth, protein synthesis, and secondary metabolite production and are implicated in pathogenicity of numerous fungal pathogens. The final step of the BCAA biosynthesis pathway is catalyzed by the branched chain amino acid aminotransferase (BAT) genes. In *Aspergillus nidulans*, two BAT genes, *batA* and *batB*, are necessary for BCAA production, two others are involved in biosynthesis of the secondary metabolite aspercryptins, and the function of the remaining two remains unknown. Loss of both *batA* and *batB* genes causes tight BCAA auxotrophy but having either *batA* or *batB* restores BCAA prototrophy. Using plate testing of the *batA*Δ *batB*Δ double mutant on various combinations of BCAAs, we discovered spontaneous suppressor mutants arise after four days when supplemented with isoleucine and valine but lacking leucine. We isolated ten independent suppressors of BCAA auxotrophy and used Whole Genome Sequencing and Single Nucleotide Polymorphism (SNP) analysis as a strategy to work towards identifying the causative suppressor mutation. Each suppressor mutant carried between 158-205 SNPs that are not found in the *batA*Δ *batB*Δ double deletion parent, with a total of 267 SNPs unique to the mutants. Six genes carried the same non-synonymous mutations in all ten of the suppressor mutants. Based on literature review, one of the affected genes, AN5995, is the strongest candidate causative gene as it is predicted to encode a protein with an SPT2 domain and is an ortholog of *S. cerevisiae* SPT2, which is a repressor of transcription that interacts with the histone complex and is part of the hypoxia regulation response. The loss of repression by AN5995 during normoxic conditions could de-repress the expression of *batC*, *batD*, and *batE*, the remaining branched chain amino acid aminotransferase genes.

4.2 Introduction

Genome integrity and stability is necessary for successful sexual reproduction and propagation. The *Aspergillus nidulans* genome is estimated to be about 31 million base pairs with 8 chromosomes (Galagan et al., 2005). The genome contains about 10,700 protein coding genes (Galagan et al., 2005). DNA is replicated constantly throughout the lifetime of the organism. Faithful replication of the DNA sequence is important as deviation from the DNA template can alter the encoded protein functionality. Changes in the DNA sequence can occur through several different events, such as a single base pair change through faulty replication known as a single nucleotide polymorphism (SNP) or larger changes like deletion or insertion of multiple base pairs due to unequal crossover between chromosomes (Loewe, 2008). These events can range from innocuous to catastrophic, depending on where they occur.

SNPs are characterized by a single base pair change in the DNA sequence. They can occur across any region of the genome, including protein coding regions. These mutations are classified based on the effect they have on the sequence. Synonymous substitutions are sequence changes in the coding region that do not alter the encoded amino acid, while nonsynonymous substitutions are base pair changes that cause either a different amino acid to be encoded or the protein to be prematurely terminated through an early stop codon. SNPs arise naturally at low levels through unfaithful replication of the DNA sequence by DNA polymerase (Falaschi and Kornberg, 1966). SNPs can also be induced at high frequency by mutagenesis with toxic compounds or conditions (Boeke et al., 1987; Downes et al., 2014a; Scott and Maley, 1981; Álvarez-Escribano et al., 2019).

Most SNPs have no phenotypic effect due to the location of the base pair change in the genome while a small subset of SNPs have phenotypic effects. One type of effect is called a suppressor mutant in which the new SNP or set of SNPs suppress a mutant phenotype, typically to restore colony growth or morphology on a specific set of conditions (Prelich, 1999). Suppressors have been shown in *A. nidulans* to occur for nitrogen and carbon metabolism, temperature sensitivity, and light conditions related to sexual reproduction and conidiation (Seo et al., 2003; Arst and Cove, 1973; Mooney et al., 1990; Jarvik and Botstein, 1975; Roberts et al., 1979).

Suppressor phenotypes can inform alternative mechanisms for regulating and catalyzing primary metabolism in the face of a genetic obstacle, such as a loss of function mutation affecting an enzyme. They can also contribute to genetic analysis studies by identifying additional genes in a metabolic or regulatory pathway that would not normally be observed in laboratory conditions.

In this chapter, spontaneous sectors that suppress the tight BCAA auxotrophy of the *batA* Δ *batB* Δ mutant (described in Chapter 3 and Steyer et al., (2021)) were selected in the absence of leucine supplementation. Preliminary characterization of these sectors revealed vegetative stability of the revertant phenotype on media lacking BCAA supplementation. Whole genome sequencing and SNP analysis of ten spontaneous sectors was used to identify candidate suppressor genes.

4.3 Materials & Methods

4.3.1 Isolation of mutant sectors

Spontaneous suppressor mutants were isolated from RT457 conidia selected by stab-inoculation to supplemented *Aspergillus* minimal media (ANM) with 10mM nitrate as the primary nitrogen source and 2mM isoleucine and 2mM valine after 4 days of growth at 37°C. Sectors were picked and isolated to supplemented ANM with 10mM nitrate lacking supplementation with BCAAs for 2 days at 37°C.

4.3.2 Strains used

A. nidulans used in this chapter are described in Table 4.1

Table 4.1 *A. nidulans* strains used.

Strain	Origin ^a	Genotype ^b
RT456	RT416 X RT440	<i>yA1 pyrG89 pabaA1 batAΔ::AfpYrG batBΔ::AfpYrG</i>
RT457	RT416 X RT440	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i>
RT651	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT652	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT653	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT654	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT655	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT656	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT657	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT658	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT659	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"
RT660	RT457 sector	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i> "sector"

^a sectors were selected on supplemented ANM containing 10 mM nitrate and 2mM isoleucine and 2 mM valine.

^b all strains carry *veA1*

4.3.3 Whole Genome Sequencing Library Preparation

Genomic DNA was isolated from mycelia grown in 100 mL of supplemented ANM with 10 mM ammonium tartrate in shaking flasks (160 RPM) for 16 h and ground under liquid nitrogen. The extraction protocol used is outlined in Lee and Taylor (1990), with an added RNase A (Sigma) step. The RNase was added to the supernatant of the initial phenol-chloroform-isoamyl alcohol purification step and incubated for 20 minutes. RNase was subsequently removed by additional phenol-chloroform-isoamyl alcohol purification. Additional chloroform purification was performed to remove any residual organic reagents in the solution. Purified DNA was sent to Kansas State University Integrated Genomics Facility (Manhattan, Kansas, USA) for library preparation and whole genome sequencing. Libraries were pooled

and sequenced in a Mid-output paired end 150bp run on the NextSeq 500 Sequencer (Illumina).

4.3.4 Whole Genome Sequencing Analysis

Whole genome sequence analysis was performed through the Galaxy Platform (www.usegalaxy.org) (Afgan et al., 2018). FastQC tool was used to determine sequence read quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were mapped to the genome using the BWA-MEM tool for Illumina with default settings (Li and Durbin, 2009; Li and Durbin, 2010) to an updated *A. nidulans* FGSC A4 genome (Aspnd5rm) to which we have pre-release access (Adrian Tsang, Concordia University). Duplicate reads were marked using the MarkDuplicates tool (<http://broadinstitute.github.io/picard/>) and left aligned using BamLeftAlign (<http://arxiv.org/abs/1207.3907>). Single Nucleotide Polymorphisms (SNPs) were identified using the FreeBayes tool (<http://arxiv.org/abs/1207.3907>) with default setting except for the following parameters: minimum coverage (-l) to 4, polymorphism probability (-pvar) to 0.01, ploidy (-p) to 1, minimum mapping quality (-min-mapping-quality) to 1, and minimum alternate observations (-min-alternate-fraction) to 0.5. SNP characterization was determined with the SnpEff tool (Cingolani et al., 2012) with Classic Effect names and amino acid variant annotation chosen.

4.4 Results

4.4.1 Discovery of spontaneous suppressor mutant sectors

The *batA*Δ *batB*Δ double mutant is a tight BCAA auxotroph and requires exogenous isoleucine, leucine, and valine for growth at two days at 37°C (Steyer et al., 2021). Downes (2015) reported growth of the *batA*Δ *batB*Δ double mutant when supplemented with isoleucine and valine upon extended 4 days incubation at 37°C. We investigated if there was a delayed growth phenotype in the *batA*Δ *batB*Δ double mutant (RT457) on supplemented ANM with nitrate as the primary nitrogen source and with exogenous isoleucine and valine. After four days, some of the *batA*Δ *batB*Δ double mutant colonies had some growth. These colonies had very poor,

asymmetrical growth and colony size varied between sectors, some appearing slightly larger than others at the four day time point. This growth pattern is consistent with the formation of spontaneous mutant sectors. These spontaneous sectors occurred on plates supplemented with only isoleucine and valine. We did not observe any growth of the *batA* Δ *batB* Δ double mutant on any other combinations of isoleucine, leucine, and valine at the end of four days except when supplemented with all three BCAAs (Figure 4.1). We isolated these colonies and propagated them to supplemented ANM plates with nitrate that lacked BCAA supplementation and observed consistent and symmetrical growth between independent sectors in two days. When the isolation of BCAA auxotrophy suppressors from the *batA* Δ *batB* Δ double mutant was repeated, we observed similar poor, asymmetrical growth on supplemented ANM with nitrate and isoleucine and valine after four days, and then subsequent strong growth of the isolated suppressors without any BCAA supplementation. This indicated that the *batA* Δ *batB* Δ double mutant was somehow able to overcome the BCAA auxotrophy frequently, given enough time and the right conditions. Subsequent plate testing showed that the suppression of BCAA auxotrophy phenotype was stably inherited through asexual conidia as conidia from the spontaneous sectors showed suppression of the BCAA auxotrophy and restored growth in two days when propagated. While it is possible that an epigenetic mechanism could underlie the basis of the spontaneous suppressor phenotype sectors, suppressors in *A. nidulans* often prove to be spontaneous mutants (Shwab et al., 2007; Bok et al., 2009; Pfannenstiel and Keller, 2019; Williams et al., 2008).

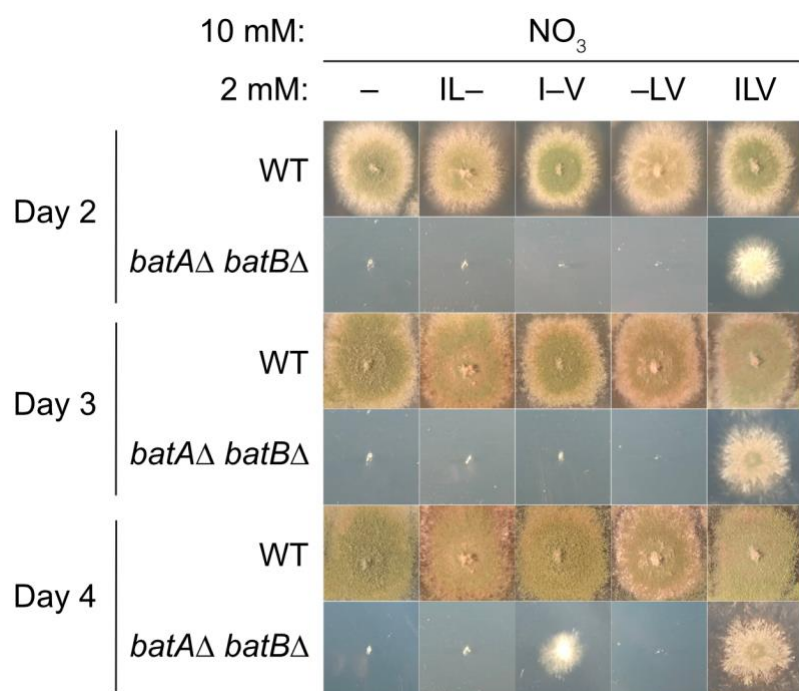


Figure 4.1 Growth of the *batAΔ batBΔ* double mutant with incomplete supplementation.

Wildtype (MH1) and *batAΔ batBΔ* (RT457) were grown at 37°C for a period of 4d with photos taken on day 2, 3, and 4. Grown on solid ANM supplemented with biotin and pyridoxine and containing 10 mM nitrate (NO₃) as the nitrogen source and combinations of 2 mM each of isoleucine (I), leucine (L), and valine (V). – represents an omitted amino acid.

4.4.2 Outcrossing Spontaneous Suppressor Mutants

We isolated ten of the BCAA auxotrophy suppressor sectors, named RT651 – RT660, and attempted to cross each of them with another *batA*Δ *batB*Δ double mutant (RT456). RT456 has different nutrient markers and a conidial color marking to aid crossing and genetic analysis. Our hypothesis was that if the suppressor phenotype was due to mutation and suppression of BCAA auxotrophy was caused by mutations in one gene, we would observe a one-to-one ratio for segregation of suppression of BCAA auxotrophy in the haploid progeny. Between ten crosses, we were only able to obtain between 10 and 50 spores per cross. This was an extremely low number to obtain in a cross. *A. nidulans* crosses normally generate 10,000-100,000 progenies per cleistothecium (fruiting body) (Pontecorvo et al., 1953). This indicated that the suppressor mutation also affected the ability to sexually reproduce successfully.

4.4.3 Determining candidate suppressor genes through Whole Genome Sequencing

To determine the causative single nucleotide polymorphism (SNP) in the spontaneous suppressor mutants, we performed whole genome sequencing on the ten suppressor mutants and the *batA*Δ *batB*Δ double mutant parent from which they were derived, RT457. Sequence comparison of the new mutants to the *batA*Δ *batB*Δ double mutant would reveal SNPs that occurred during selection of the suppressor mutants. By sequencing multiple mutants, we could determine if any of the SNPs were shared by all of the mutants, and if those shared mutations could that indicate a possible mechanism of suppression of the BCAA auxotrophy.

We hypothesized that the suppressor mutation might be within or in the cis-regulatory region of one of the remaining branched chain amino acid aminotransferase genes with no obvious role in BCAA biosynthesis, or one of the BCAA pathway regulators, specifically *leuB*, *leuR*, AN7596, *tamA*, *areA*, or *cpcA*. Whole Genome Sequencing and SNP analysis revealed 158-204 SNPs per mutant compared with the *batA*Δ *batB*Δ double mutant parent (Table 4.2). Notably, multiple identical SNPs arose independently and were common to multiple mutants. There was a total of 267 unique

SNPs in the ten mutants compared with the *batA*Δ *batB*Δ double mutant parent (Table 4.2). The 267 SNPs were found in or adjacent to roughly 117 genes and of those 267 SNPs, 45 of them were in protein coding regions (Table 4.3). None of the SNPs occurred in or adjacent to *batE*, *batF*, *batC* (*atnH*), *batD* (*atnJ*), *leuB*, *leuR*, *AN7596*, *tamA*, *areA* or *cpcA* suggesting that the suppressor phenotype did not occur from a mutation in genes with presumed or confirmed roles in BCAA biosynthesis and regulation.

Table 4.2 Number of SNPs per sector mutant.

Strain Number	RT651	RT652	RT653	RT654	RT655	RT656	RT657	RT658	RT659	RT660
Number of SNPs	163	187	169	176	170	164	205	158	196	178

Total number of new SNPs per individual sector mutants absent from the RT457 parent.

Table 4.3 SNPs found only in the suppressor mutants.

Chrom.	Start	AN Number	Effected Strains	Function	Gene or Intergenic	SNP Type
Chr_I	287,189	AN6407	RT652, RT657	Ortholog(s) have oxidoreductase activity and role in steroid metabolic process	Upstream	
Chr_I	475,283	AN6341	RT657	Protein with similarity to <i>Saccharomyces cerevisiae</i> Crn1p	Upstream	
Chr_I	475,293	AN6341	RT652, RT660	Protein with similarity to <i>Saccharomyces cerevisiae</i> Crn1p	Upstream	
Chr_I	967,140	AN6195	RT653	Transcriptional regulator that mediates carbon catabolite repression	Upstream	
Chr_I	967,151	AN6195	RT651	Transcriptional regulator that mediates carbon catabolite repression	Upstream	
Chr_I	1,113,516	AN6141	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Ortholog(s) have glutaminase activity, role in negative regulation of apoptotic process, pyridoxine biosynthetic process and cytoplasm localization	Upstream	
Chr_I	1,161,880	AN12442	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Has domain(s) with predicted DNA binding, sequence-specific DNA binding RNA polymerase II transcription factor activity, zinc ion binding activity and role in regulation of transcription, DNA-templated, transcription, DNA-templated	Upstream	
Chr_I	1,561,074	AN5995	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Ortholog of <i>A. fumigatus</i> Af293: Afu2g10110, <i>A. niger</i> CBS 513.88: An16g04920, <i>A. oryzae</i> RIB40: AO090011000625, <i>Aspergillus wentii</i> : Aspwe1_0045120 and <i>Aspergillus sydowii</i> : Aspsy1_0031289	Protein Coding	Non-Syn; Missense
Chr_I	1,755,691	AN5934	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Ortholog(s) have role in budding cell bud growth, microtubule cytoskeleton organization, tRNA wobble uridine modification and cytosol, nucleus localization	Upstream	

Chr_I	1,784,683	AN5927	RT651, RT654	Ortholog of <i>A. fumigatus</i> Af293: Afu2g10790, <i>A. oryzae</i> R/B40: AO090011000964, <i>Aspergillus wentii</i> : Aspwe1_0117218, <i>Aspergillus sydowii</i> : Aspsy1_0132184 and <i>Aspergillus terreus</i> NIH2624: ATET_01498	Upstream	
Chr_I	2,038,245	AN5849	RT653, RT658	Has domain(s) with predicted sequence-specific DNA binding RNA polymerase II transcription factor activity, zinc ion binding activity, role in regulation of transcription, DNA-templated and nucleus localization	Upstream	
Chr_I	3,158,668	AN11527	RT651, RT654	Protein of unknown function	Upstream	
Chr_I	3,158,678	AN11527	RT654, RT655, RT657, RT658	Protein of unknown function	Upstream	
Chr_I	3,367,029	AN6850	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660		Upstream	
Chr_I	3,588,850	AN10868	RT657	Ortholog of <i>A. niger</i> CBS 513.88: An14g01330, An02g11890, An14g01620 and <i>A. oryzae</i> R/B40: AO090012000045, AO090011000315, AO090701000905	Protein Coding	Non-Syn; Missense
Chr_I	3,728,237	AN6966	RT653, RT660	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_I	3,728,440	AN6966	RT651, RT654, RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Syn; Silent
Chr_I	3,728,505	AN6966	RT651, RT654, RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense

Chr_I	3,728,570	AN6966	RT654, RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_I	3,728,579	AN6966	RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_I	3,729,798	AN6965	RT659	Has domain(s) with predicted role in cell wall macromolecule catabolic process	Upstream	
Chr_I	3,748,012	AN6972	RT651, RT652, RT653, RT654, RT658, RT659, RT660	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_I	3,748,719	AN6972	RT659	Protein of unknown function	Upstream	
Chr_I	3,748,746	AN6972	RT655, RT658, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,771	AN6972	RT652, RT655, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,784	AN6972	RT652, RT655, RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,828	AN6972	RT652, RT655, RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,860	AN6972	RT652, RT655, RT656, RT657, RT658, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,879	AN6972	RT652, RT655, RT656, RT657	Protein of unknown function	Upstream	
Chr_I	3,748,881	AN6972	RT656, RT657	Protein of unknown function	Upstream	
Chr_I	3,748,882	AN6972	RT652, RT655, RT656, RT657	Protein of unknown function	Upstream	
Chr_I	3,748,891	AN6972	RT652, RT654, RT655, RT656, RT657, RT659	Protein of unknown function	Upstream	

Chr_I	3,748,919	AN6972	RT652, RT654, RT655, RT656, RT657, RT658, RT659	Protein of unknown function	Upstream	
Chr_I	3,748,925	AN6972	RT652, RT654, RT655, RT656, RT657, RT658	Protein of unknown function	Upstream	
Chr_I	3,749,142	AN6972	RT653, RT654, RT655, RT657	Protein of unknown function	Upstream	
Chr_I	3,749,177	AN6972	RT655, RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,749,601	AN6972	RT652, RT657, RT658, RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,632	AN6972	RT652, RT658, RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,825	AN6972	RT652, RT654, RT657, RT658, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,855	AN6972	RT652, RT654, RT657, RT658, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,878	AN6972	RT651, RT652, RT654, RT656, RT657, RT658, RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,904	AN6972	RT652, RT654, RT656, RT657, RT658, RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,749,988	AN6972	RT651, RT652, RT654, RT656, RT657, RT659, RT660	Protein of unknown function	Upstream	

Chr_I	3,750,010	AN6972	RT654, RT656, RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,750,018	AN6972	RT651, RT652, RT654, RT656, RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,750,027	AN6972	RT659	Protein of unknown function	Upstream	
Chr_I	3,750,028	AN6972	RT657, RT659	Protein of unknown function	Upstream	
Chr_I	3,750,582	AN6972	RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,750,597	AN6972	RT659	Protein of unknown function	Upstream	
Chr_I	3,750,743	AN6972	RT652, RT654, RT655	Protein of unknown function	Upstream	
Chr_I	3,750,764	AN6972	RT652, RT654, RT655, RT660	Protein of unknown function	Upstream	
Chr_I	3,750,777	AN6972	RT652, RT654, RT655	Protein of unknown function	Upstream	
Chr_I	3,750,778	AN6972	RT655	Protein of unknown function	Upstream	
Chr_I	3,750,792	AN6972	RT654, RT655, RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,750,831	AN6972	RT659, RT660	Protein of unknown function	Upstream	
Chr_I	3,750,873	AN6972	RT652, RT659	Protein of unknown function	Upstream	
Chr_I	3,750,880	AN6972	RT659	Protein of unknown function	Upstream	
Chr_II	51,827	AN7863	RT653	Protein of unknown function	Upstream	
Chr_II	279,196	AN7937	RT654	Protein responsive to Concanamycin A	Upstream	
Chr_II	489,219	AN8005	RT652, RT656	Putative GNAT-type acetyltransferase	Upstream	
Chr_II	572,945	AN8037	RT660	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_II	2,003,819	AN10506	RT660	Ortholog of <i>A. fumigatus</i> Af293: Afu5g14780, <i>A. niger</i> CBS 513.88: An18g01640, <i>A. oryzae</i> R1B40: AO090103000417, <i>Aspergillus versicolor</i> : Aspve1_0173600 and <i>Aspergillus niger</i> ATCC 1015: 42671-mRNA	Upstream	
Chr_II	2,283,126	AN4033	RT654	Ortholog(s) have tubulin binding activity, role in tubulin complex assembly and prefoldin complex localization	Upstream	

Chr_II	3,084,713	AN3776	RT651, RT652	Has domain(s) with predicted role in transmembrane transport and integral component of membrane localization	Protein Coding	Non-Syn; Missense
Chr_II	3,382,582	AN3684	RT654	Has domain(s) with predicted DNA binding, sequence-specific DNA binding RNA polymerase II transcription factor activity, zinc ion binding activity and role in regulation of transcription, DNA-templated, transcription, DNA-templated	Upstream	
Chr_II	3,782,513	AN3551	RT654	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_II	3,783,773	AN3551	RT657	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_II	3,785,330	AN3551	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_II	3,845,234	AN3538	RT651, RT652, RT653, RT654, RT655, RT658, RT659, RT660	Protein of unknown function	Upstream	
Chr_II	4,065,885	AN3493	RT657	Predicted threonine-type endopeptidase	Upstream	
Chr_III	4,048		RT654, RT656, RT657, RT660		Intergenic	
Chr_III	5,141		RT659		Intergenic	
Chr_III	5,161		RT659		Intergenic	
Chr_III	5,167		RT659		Intergenic	
Chr_III	5,215		RT659		Intergenic	
Chr_III	34,354	AN5090	RT659	Predicted ADP ribosylation factor (Arf) GTPase	Protein Coding	Non-Syn; Missense
Chr_III	114,685	AN5069	RT651, RT657, RT658	Ortholog of <i>A. nidulans</i> FGSC A4: AN2683, <i>A. fumigatus</i> Af293: Afu5g02860, Afu5g14320, <i>A. niger</i> CBS 513.88: An09g04830, An14g05730 and <i>A. oryzae</i> R1B40: AO090102000482, AO090001000197	Upstream	

Chr_III	425,792	AN4975	RT660	Has domain(s) with predicted oxidoreductase activity and role in oxidation-reduction process	Upstream	
Chr_III	451,972	AN4965	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Ortholog(s) have cytosol, nucleus localization	Protein Coding	Non-Syn; Missense
Chr_III	576,924	AN4920	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Putative calcium-transporting mitochondrial ATPase involved in calcium homeostasis	Protein Coding	Non-Syn; Missense
Chr_III	1,201,839	AN4722	RT652	Predicted glycosylphosphatidylinositol (GPI)-anchored protein	Protein Coding	Non-Syn; Missense
Chr_III	1,202,242	AN4722	RT651	Predicted glycosylphosphatidylinositol (GPI)-anchored protein	Protein Coding	Syn; Silent
Chr_III	1,386,300	AN11973	RT659	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_III	1,679,208	AN4562	RT657	Basic leucine zipper (bZIP) type transcription factor involved in regulation of secondary metabolism	Protein Coding	Non-Syn; Missense
Chr_III	2,020,154	AN4455	RT657	Ortholog of <i>A. fumigatus</i> Af293: Afu4g07490, <i>A. niger</i> CBS 513.88: An04g01850, <i>A. oryzae</i> RIB40: AO090023000827, <i>Aspergillus wentii</i> : Aspwe1_0167139 and <i>Aspergillus sydowii</i> : Aspsy1_0095020	Protein Coding	Non-Syn; Missense
Chr_III	2,295,551	AN4364	RT656	Ortholog of <i>A. fumigatus</i> Af293: Afu4g06520, <i>A. niger</i> CBS 513.88: An04g00860, <i>A. oryzae</i> RIB40: AO090023000934, <i>Aspergillus wentii</i> : Aspwe1_0252118 and <i>Aspergillus sydowii</i> : Aspsy1_0062282	Protein Coding	Frame Shift
Chr_III	2,355,787	AN4359	RT659	Has domain(s) with predicted zinc ion binding activity	Protein Coding	
Chr_III	2,458,233	AN12153	RT652	Protein of unknown function	Upstream	
Chr_III	2,458,237	AN4310	RT652	Ortholog(s) have cyclin-dependent protein serine/threonine kinase inhibitor activity	Upstream	
Chr_III	2,458,240	AN4310	RT652	Ortholog(s) have cyclin-dependent protein serine/threonine kinase inhibitor activity	Upstream	

Chr_III	2,458,257	AN4310	RT657	Ortholog(s) have cyclin-dependent protein serine/threonine kinase inhibitor activity	Upstream	
Chr_III	2,458,261	AN4310	RT652	Ortholog(s) have cyclin-dependent protein serine/threonine kinase inhibitor activity	Upstream	
Chr_III	2,842,802	AN8777	RT651, RT653, RT659	Acetamidase, produces ammonium and acetate from acetamide, allowing utilization of acetamide as sole carbon or nitrogen source	Upstream	
Chr_III	3,149,987	AN8675	RT659	ORF that was absent from the original release of version 4 of the <i>A. nidulans</i> annotation, but present in a previous version	Protein Coding	Non-Syn; Missense
Chr_III	3,214,688	AN8664	RT655, RT656	Ortholog(s) have cytosol, nucleus localization	Upstream	
Chr_IV	72,306	AN7400	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Ortholog of <i>Aspergillus versicolor</i> : Aspve1_0037710 and <i>Aspergillus sydowii</i> : Aspsy1_0038876	Protein Coding	Codon Deletion
Chr_IV	493,888	AN12374	RT659	Protein of unknown function	Protein Coding	Stop Gained
Chr_IV	917,897	AN8648	RT653	Has domain(s) with predicted RNA binding, RNA-DNA hybrid ribonuclease activity, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_IV	1,143,450	AN7068	RT657	Putative vanillyl alcohol oxidase	Upstream	
Chr_IV	1,306,377	AN7019	RT657	Protein with similarity to histone deacetylases	Upstream	
Chr_IV	1,352,590	AN7003	RT657	Ortholog(s) have cell surface, cytosol localization	Protein Coding	Non-Syn; Missense
Chr_IV	1,352,597	AN7003	RT652	Ortholog(s) have cell surface, cytosol localization	Protein Coding	Non-Syn; Missense
Chr_IV	1,997,902	AN7576	RT653	Predicted Rho GTPase activating protein	Upstream	
Chr_IV	2,159,686	AN10987	RT657	Ortholog(s) have cytosol, nucleus localization	Protein Coding	Syn-Silent
Chr_IV	2,216,630	AN7640	RT654	Ortholog of <i>A. nidulans</i> FGSC A4: AN5292, <i>A. fumigatus</i> Af293: Afu5g12490, Afu5g13780, <i>A. niger</i> CBS 513.88: An14g06730 and <i>A. oryzae</i> R1B40: AO090120000374, AO090020000661	Protein Coding	

Chr_IV	2,216,632	AN7640	RT653, RT654	Ortholog of <i>A. nidulans</i> FGSC A4: AN5292, <i>A. fumigatus</i> Af293: Afu5g12490, Afu5g13780, <i>A. niger</i> CBS 513.88: An14g06730 and <i>A. oryzae</i> R/B40: AO090120000374, AO090020000661	Protein Coding	Non-Syn; Missense
Chr_IV	2,216,633	AN7640	RT654	Ortholog of <i>A. nidulans</i> FGSC A4: AN5292, <i>A. fumigatus</i> Af293: Afu5g12490, Afu5g13780, <i>A. niger</i> CBS 513.88: An14g06730 and <i>A. oryzae</i> R/B40: AO090120000374, AO090020000661	Protein Coding	Non-Syn
Chr_IV	2,647,973	AN7775	RT653, RT657	Has domain(s) with predicted role in response to stress and integral component of membrane localization	Protein Coding	Non-Syn; Missense
Chr_IV	2,647,974	AN7775	RT660	Has domain(s) with predicted role in response to stress and integral component of membrane localization	Protein Coding	Non-Syn; Missense
Chr_IV	2,647,979	AN7775	RT654, RT660	Has domain(s) with predicted role in response to stress and integral component of membrane localization	Protein Coding	Non-Syn; Missense
Chr_IV	2,682,457	AN7440	RT652, RT654, RT659	Ortholog(s) have role in CENP-A containing nucleosome assembly, chromatin silencing at centromere, mitotic sister chromatid segregation and nucleoplasm localization	Protein Coding	Non-Syn; Missense
Chr_IV	2,708,375	AN7796	RT653	Has domain(s) with predicted role in transmembrane transport and integral component of membrane localization	Upstream	
Chr_IV	2,735,698	AN12090	RT659	Has domain(s) with predicted O-methyltransferase activity	Protein Coding	Syn; Silent
Chr_IV	2,852,971	AN7830	RT652	Has domain(s) with predicted nucleic acid binding, zinc ion binding activity	Downstream	
Chr_IV	2,852,992	AN7830	RT652	Has domain(s) with predicted nucleic acid binding, zinc ion binding activity	Downstream	
Chr_IV	2,881,994		RT651		Intergenic	
Chr_IV	2,882,035		RT651		Intergenic	
Chr_IV	2,882,053		RT657		Intergenic	
Chr_V	2,127		RT651, RT652, RT654, RT655, RT660		Intergenic	

Chr_V	2,138		RT651, RT652, RT654, RT655, RT660		Intergenic	
Chr_V	8,762		RT658, RT659		Intergenic	
Chr_V	9,357		RT653, RT654, RT656, RT658, RT660		Intergenic	
Chr_V	9,695		RT651, RT656, RT658, RT660		Intergenic	
Chr_V	9,750		RT651, RT653, RT658, RT660		Intergenic	
Chr_V	10,397		RT653, RT654, RT655, RT656, RT658		Intergenic	
Chr_V	10,398		RT653, RT654, RT655, RT656		Intergenic	
Chr_V	10,399		RT653, RT654, RT655, RT656, RT658		Intergenic	
Chr_V	10,746		RT652, RT653, RT654, RT657		Intergenic	
Chr_V	11,734		RT654, RT655, RT660		Intergenic	
Chr_V	11,916		RT654, RT656, RT658, RT659, RT660		Intergenic	
Chr_V	12,002		RT654, RT655, RT656, RT658, RT660		Intergenic	
Chr_V	12,425		RT652, RT653, RT658, RT660		Intergenic	
Chr_V	12,494		RT652, RT653, RT654, RT657, RT658		Intergenic	

Chr_V	12,966		RT651, RT657, RT658		Intergenic	
Chr_V	13,402		RT651, RT652, RT653, RT655, RT658, RT660		Intergenic	
Chr_V	13,512		RT654, RT655, RT660		Intergenic	
Chr_V	13,614		RT651, RT654, RT656, RT660		Intergenic	
Chr_V	14,323		RT651, RT652, RT654, RT658		Intergenic	
Chr_V	14,510		RT651, RT652, RT655, RT656, RT658, RT659		Intergenic	
Chr_V	15,144		RT651, RT652, RT653, RT658		Intergenic	
Chr_V	68,470		RT656, RT659		Upstream	
Chr_V	68,492	AN0004	RT654		Upstream	
Chr_V	98,766	AN8313	RT655	Protein of unknown function	Protein Coding	Syn; Silent
Chr_V	278,357		RT657		Upstream	
Chr_V	278,362		RT657		Upstream	
Chr_V	279,760		RT659		Upstream	
Chr_V	544,318	AN8453	RT652, RT653	Protein with pectate lyase activity, involved in degradation of pectin	Downstream	
Chr_V	652,738	AN8484	RT655	Predicted glycosylphosphatidylinositol (GPI)-anchored protein	Upstream	
Chr_V	682,831	AN8494	RT657	Has domain(s) with predicted 2-dehydropantoate 2-reductase activity, NADP binding, coenzyme binding, oxidoreductase activity, oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor activity	Upstream	
Chr_V	932,375	AN8577	RT657	Ortholog of <i>Aspergillus terreus</i> NIH2624: ATET_10228	Protein Coding	Non-Syn; Missense
Chr_V	971,953		RT654		Protein Coding	Syn; Silent

Chr_V	1,301,970	AN10636	RT655, RT658	Ortholog(s) have Golgi apparatus, endoplasmic reticulum localization	Protein Coding	Syn; Silent
Chr_V	1,301,975	AN10636	RT651, RT653, RT655, RT657	Ortholog(s) have Golgi apparatus, endoplasmic reticulum localization	Protein Coding	Non-Syn; Missense
Chr_V	1,301,980	AN10636	RT658	Ortholog(s) have Golgi apparatus, endoplasmic reticulum localization	Protein Coding	Non-Syn; Missense
Chr_V	1,305,399	AN5141	RT652	Ortholog of <i>A. fumigatus</i> Af293: Afu1g07280, <i>A. niger</i> CBS 513.88: An07g09830, <i>A. oryzae</i> R/B40: AO090012000982, <i>Aspergillus wentii</i> : Aspwe1_0172497 and <i>Aspergillus sydowii</i> : Aspsy1_0033440	Protein Coding	Syn; Silent
Chr_V	1,354,004	AN5126	RT660	Ortholog of <i>A. fumigatus</i> Af293: Afu1g07490, Afu2g04420, <i>A. niger</i> CBS 513.88: An11g03310, An07g10050, <i>A. oryzae</i> R/B40: AO090012000999 and <i>Aspergillus wentii</i> : Aspwe1_0046371	Upstream	
Chr_V	1,443,846	AN0376	RT655	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Syn; Silent
Chr_V	1,443,867	AN0376	RT655	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Syn; Silent
Chr_V	1,443,885	AN0376	RT655, RT657	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Syn; Silent
Chr_V	1,443,974	AN0376	RT652, RT657, RT659, RT660	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn;
Chr_V	1,443,994	AN0376	RT652, RT657	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn;
Chr_V	1,444,007	AN0376	RT652, RT657, RT659, RT660	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense

Chr_V	1,444,033	AN0376	RT652, RT657	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn
Chr_V	1,444,078	AN0376	RT652, RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_V	1,529,517	AN5782	RT660	Has domain(s) with predicted catalytic activity and role in metabolic process	Upstream	
Chr_V	2,050,091		RT654, RT656, RT657, RT658		Protein Coding	Codon Change, Insertion
Chr_V	2,176,802	AN5573	RT659	Ortholog of <i>A. fumigatus</i> Af293: Afu4g11630, <i>A. niger</i> CBS 513.88: An04g04730, <i>A. oryzae</i> R1B40: AO090003001056, <i>Aspergillus wentii</i> : Aspwe1_0166629 and <i>Aspergillus sydowii</i> : Aspsy1_0088453	Protein Coding	Non-Syn; Missense
Chr_V	2,220,734	AN10693	RT651, RT654	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_V	2,651,994	AN5430	RT655	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_V	2,654,264	AN5429	RT656	Ortholog of <i>A. nidulans</i> FGSC A4: AN8498, AN8310, AN11121, <i>A. fumigatus</i> Af293: Afu3g01350 and <i>A. niger</i> CBS 513.88: An12g08560, An09g02370, An13g02790, An13g02620	Protein Coding	Non-Syn; Missense
Chr_V	3,177,721	AN5254	RT654	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity and role in RNA-dependent DNA replication	Upstream	
Chr_V	3,177,726	AN5254	RT654	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity and role in RNA-dependent DNA replication	Upstream	
Chr_V	3,177,809	AN5254	RT651	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity and role in RNA-dependent DNA replication	Upstream	

Chr_V	3,184,707	AN8648	RT654	Has domain(s) with predicted RNA binding, RNA-DNA hybrid ribonuclease activity, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in RNA-dependent DNA replication	Upstream	
Chr_VI	53,256	AN9221	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Putative transcription factor	Protein Coding	Non-Syn; Missense
Chr_VI	382,271	AN9120	RT659	Ortholog(s) have DNA binding, DNA-directed RNA polymerase activity, RNA binding activity and role in chromatin silencing by small RNA, transcription from RNA polymerase II promoter	Upstream	
Chr_VI	1,269,655	AN10388	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Has domain(s) with predicted UDP-N-acetylmuramate dehydrogenase activity, flavin adenine dinucleotide binding, oxidoreductase activity, oxidoreductase activity, acting on CH-OH group of donors activity	Upstream	
Chr_VI	1,697,739	AN3136	RT657	Has domain(s) with predicted acid-amino acid ligase activity	Protein Coding	Non-Syn; Missense
Chr_VI	1,744,717	AN3121	RT654	Ortholog of <i>A. fumigatus</i> Af293: Afu3g12790, <i>A. niger</i> CBS 513.88: An02g08810, <i>Neosartorya fischeri</i> NRRL 181: NFIA_064510 and <i>Aspergillus wentii</i> : Aspwe1_0025248	Upstream	
Chr_VI	1,744,733	AN3121	RT651	Ortholog of <i>A. fumigatus</i> Af293: Afu3g12790, <i>A. niger</i> CBS 513.88: An02g08810, <i>Neosartorya fischeri</i> NRRL 181: NFIA_064510 and <i>Aspergillus wentii</i> : Aspwe1_0025248	Upstream	
Chr_VI	1,910,632	AN3075	RT655	Zinc finger protein of unknown function	Downstream	
Chr_VI	1,910,638	AN3075	RT655	Zinc finger protein of unknown function	Downstream	
Chr_VI	2,307,901	AN2941	RT651, RT657, RT660	Ortholog of <i>A. nidulans</i> FGSC A4: AN5437, <i>A. fumigatus</i> Af293: Afu3g08250, Afu6g13630, <i>A. niger</i> CBS 513.88: An02g11860 and <i>A. oryzae</i> R1B40: AO090005001453, AO090103000325	Protein Coding	Syn; Silent

Chr_VI	2,496,627	AN5672	RT652, RT654, RT656, RT660	Has domain(s) with predicted catalytic activity and role in metabolic process	Upstream	
Chr_VI	2,560,565	AN2861	RT657, RT659	Putative F-box protein	Protein Coding	Syn; Silent
Chr_VI	3,381,968	AN5093	RT659	Protein of unknown function	Protein Coding	Codon Insertion
Chr_VI	3,384,921	AN5093	RT654	Protein of unknown function	Upstream	
Chr_VI	3,385,529	AN5093	RT659	Protein of unknown function	Upstream	
Chr_VII	83,737		RT653		Upstream	
Chr_VII	83,774		RT653		Upstream	
Chr_VII	800,161	AN1443	RT654	Ortholog of <i>A. fumigatus</i> Af293: Afu8g04310, <i>A. niger</i> CBS 513.88: An16g08760, <i>A. oryzae</i> R1B40: AO090103000048, <i>Aspergillus wentii</i> : Aspwe1_0118145 and <i>Aspergillus sydowii</i> : Aspsy1_0028793	Protein Coding	Non-Syn; Missense
Chr_VII	855,196	AN1463	RT657	Predicted glycosylphosphatidylinositol (GPI)-anchored protein	Upstream	
Chr_VII	855,203	AN1463	RT657	Predicted glycosylphosphatidylinositol (GPI)-anchored protein	Upstream	
Chr_VII	1,230,076	AN1577	RT659	Has domain(s) with predicted substrate-specific transmembrane transporter activity, transmembrane transporter activity, role in transmembrane transport and integral component of membrane, membrane localization	Upstream	
Chr_VII	1,527,434	AN1659	RT652, RT657, RT660	Putative amino acid transporter	Protein Coding	Non-Syn; Missense
Chr_VII	1,527,437	AN1659	RT659	Putative amino acid transporter	Protein Coding	Non-Syn; Missense
Chr_VII	1,704,296	AN1717	RT655	Protein of unknown function	Upstream	
Chr_VII	2,842,557	AN2098	RT657	Ortholog of <i>Aspergillus wentii</i> : Aspwe1_0046221, <i>Aspergillus versicolor</i> : Aspve1_0038126, <i>Aspergillus zonatus</i> : Aspzo1_0128775 and <i>Aspergillus sydowii</i> : Aspsy1_0026522	Upstream	

Chr_VII	3,514,948	AN10285	RT652	Ortholog of <i>A. fumigatus</i> Af293: Afu5g10660, <i>A. niger</i> CBS 513.88: An14g04040, <i>A. oryzae</i> R1B40: AO090010000472, <i>Aspergillus wentii</i> : Aspwe1_0050403 and <i>Aspergillus terreus</i> NIH2624: ATET_07619	Upstream	
Chr_VII	3,722,337	AN2374	RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660	Has domain(s) with predicted oxidoreductase activity and role in metabolic process	Upstream	
Chr_VII	3,912,706	AN2426	RT654	Histone H4.2, core histone protein	Upstream	
Chr_VII	4,032,515	AN2466	RT652	Has domain(s) with predicted substrate-specific transmembrane transporter activity, transmembrane transporter activity, role in transmembrane transport and integral component of membrane, membrane localization	Protein Coding	Syn; Silent
Chr_VII	4,153,052	AN2503	RT653	Ortholog(s) have role in response to endoplasmic reticulum stress	Protein Coding	Non-Syn; Missense
Chr_VII	4,368,654	AN7830	RT659	Has domain(s) with predicted nucleic acid binding, zinc ion binding activity	Upstream	
Chr_VII	4,368,658	AN2564	RT652	Has domain(s) with predicted catalytic activity, pyridoxal phosphate binding activity and role in biosynthetic process	Upstream	
Chr_VII	4,368,688	AN2564	RT659	Has domain(s) with predicted catalytic activity, pyridoxal phosphate binding activity and role in biosynthetic process	Upstream	
Chr_VII	4,368,723	AN2564	RT653	Has domain(s) with predicted catalytic activity, pyridoxal phosphate binding activity and role in biosynthetic process	Upstream	
Chr_VII	4,470,250	AN2595	RT653, RT657, RT659	Protein of unknown function	Upstream	
Chr_VII	4,520,614	AN2610	RT652, RT653, RT655	Putative cytochrome P450	Upstream	
Chr_VII	4,550,384	AN5093	RT659	Protein of unknown function	Upstream	

Chr_VII	4,553,278		RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660		Intergenic	
Chr_VIII	6,947	AN10649	RT651, RT652, RT653, RT655, RT656	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_VIII	7,245	AN10649	RT651, RT652, RT653, RT656, RT657	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Stop Gained
Chr_VIII	7,290	AN10649	RT651, RT652	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_VIII	8,484	AN10649	RT653	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_VIII	8,493	AN10649	RT653	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_VIII	8,522	AN10649	RT653, RT657	Has domain(s) with predicted RNA binding, RNA-directed DNA polymerase activity, nucleic acid binding activity and role in DNA integration, RNA-dependent DNA replication	Protein Coding	Non-Syn; Missense
Chr_VIII	12,175	AN5248	RT651, RT653, RT656, RT657, RT658	Protein of unknown function	Protein Coding	Non-Syn; Missense
Chr_VIII	14,187	AN5248	RT651, RT652, RT653, RT657	Protein of unknown function	Protein Coding	Syn

Chr_VIII	18,280	AN5248	RT655, RT657	Protein of unknown function	Upstream	
Chr_VIII	18,307	AN5248	RT657	Protein of unknown function	Upstream	
Chr_VIII	18,385	AN5248	RT652, RT657	Protein of unknown function	Upstream	
Chr_VIII	18,406	AN5248	RT652, RT657	Protein of unknown function	Upstream	
Chr_VIII	18,418	AN5248	RT652, RT657	Protein of unknown function	Upstream	
Chr_VIII	18,486	AN5248	RT653, RT657, RT658, RT660	Protein of unknown function	Upstream	
Chr_VIII	18,736	AN5248	RT654, RT660	Protein of unknown function	Upstream	
Chr_VIII	18,748	AN5248	RT653, RT654, RT660	Protein of unknown function	Upstream	
Chr_VIII	18,775	AN5248	RT654, RT660	Protein of unknown function	Upstream	
Chr_VIII	18,776	AN5248	RT654	Protein of unknown function	Upstream	
Chr_VIII	18,777	AN5248	RT653, RT654	Protein of unknown function	Upstream	
Chr_VIII	19,242	AN5248	RT651, RT653, RT654, RT657	Protein of unknown function	Upstream	
Chr_VIII	19,259	AN5248	RT651, RT652, RT653, RT657	Protein of unknown function	Upstream	
Chr_VIII	19,266	AN5248	RT651, RT653, RT657	Protein of unknown function	Upstream	
Chr_VIII	19,297	AN5248	RT651, RT653, RT657, RT658	Protein of unknown function	Upstream	
Chr_VIII	19,315	AN5248	RT651, RT653, RT657	Protein of unknown function	Upstream	
Chr_VIII	19,320	AN5248	RT651, RT653	Protein of unknown function	Upstream	
Chr_VIII	19,337	AN5248	RT651, RT653	Protein of unknown function	Upstream	
Chr_VIII	19,346	AN5248	RT651, RT653	Protein of unknown function	Upstream	
Chr_VIII	19,365	AN5248	RT651	Protein of unknown function	Upstream	
Chr_VIII	19,627	AN5248	RT653, RT654, RT658, RT659	Protein of unknown function	Upstream	
Chr_VIII	19,639	AN5248	RT653, RT659	Protein of unknown function	Upstream	
Chr_VIII	19,695	AN5248	RT653, RT654, RT655, RT659	Protein of unknown function	Upstream	
Chr_VIII	19,704	AN5248	RT653, RT654, RT659	Protein of unknown function	Upstream	

Chr_VIII	19,766	AN5248	RT653, RT654, RT659	Protein of unknown function	Upstream	
Chr_VIII	33,030		RT656		Upstream	
Chr_VIII	451,229	AN9371	RT653	Ortholog of <i>Aspergillus tubingensis</i> : Asptu1_0038687, <i>Aspergillus brasiliensis</i> : Aspbr1_0072586, <i>Aspergillus kawachii</i> : Aspka1_0182707 and <i>Aspergillus acidus</i> : Aspfo1_0149057	Upstream	
Chr_VIII	953,715	AN10170	RT659	Ortholog(s) have ATPase activity, role in negative regulation of G2/M transition of mitotic cell cycle and cytosol, nucleus localization	Upstream	
Chr_VIII	1,014,140	AN10169	RT656	Short-chain dehydrogenase	Protein Coding	Non-Syn; Missense
Chr_VIII	1,341,344	AN1163	RT659	Putative chaperone	Upstream	
Chr_VIII	1,843,104	AN0999	RT656	Adenylate cyclase-associated protein	Protein Coding	Syn; Silent
Chr_VIII	3,312,247	AN10104	RT660	Has domain(s) with predicted role in cell wall macromolecule catabolic process	Upstream	
Chr_VIII	3,740,358	AN10070	RT659	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	3,740,374	AN10070	RT652	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	3,740,377	AN10070	RT660	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	3,740,380	AN10070	RT653	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	

Chr_VIII	3,741,464	AN10070	RT657, RT658	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	3,741,487	AN10070	RT658	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	3,741,506	AN10070	RT658	Ortholog of <i>A. fumigatus</i> Af293: Afu1g01790, <i>A. niger</i> CBS 513.88: An01g06420, <i>A. oryzae</i> R/B40: AO090005000908, <i>Aspergillus wentii</i> : Aspwe1_0049751 and <i>Aspergillus sydowii</i> : Aspsy1_0144393	Upstream	
Chr_VIII	4,077,063	AN0276	RT654	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,837,044	AN0022	RT657, RT660	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,837,052	AN0022	RT660	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,837,060	AN0022	RT660	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,837,081	AN0022	RT660	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,837,094	AN0022	RT660	Putative alpha-1,4-galactosidase	Upstream	
Chr_VIII	4,911,707	AN0006	RT655	Ortholog of <i>Neosartorya fischeri</i> NRRL 181: NFIA_005440, <i>Aspergillus versicolor</i> : Aspve1_0175571, <i>Aspergillus clavatus</i> NRRL 1: ACLA_062940, ACLA_090730 and <i>Aspergillus sydowii</i> : Aspsy1_0047904	Upstream	
Chr_VIII	4,920,761		RT651, RT652, RT653, RT654, RT655, RT656, RT657, RT658, RT659, RT660		Intergenic	
Chr_VIII	4,920,773		RT659		Intergenic	

List of all SNPs found in the ten suppressor mutants (RT651-RT660). Chrom.: Chromosome. Non-Syn: Non-Synonymous. Syn: Synonymous.

There were six protein coding region SNPs that occurred in all ten of the sectors, five of them being missense mutants and one being a codon deletion (Table 4.4). Mutations in AN4920, AN7400, and AN9921 appear unlikely to suppress loss of BAT activity. AN4920 (*pmcB*) plays a role in calcium ion homeostasis (Findon et al., 2010). AN7400 is predicted to encode an extracellular membrane protein. AN9921 is mis-annotated as a fusion of two genes encoding a transcription factor (AlnR) and membrane protein (AlnG) involved in asperlin biosynthesis (Grau et al., 2018). AN4965 encodes an uncharacterized protein in *A. nidulans*, but orthologs in other fungi have domains involved in the CCR-NOT complex and woronin body formation (Tang et al., 2020). AN5995 encodes a protein of unknown function in *A. nidulans* but orthologs of AN5995 have SPT2 domains.

Table 4.4 Shared protein coding SNPs in the suppressor mutants.

	AN Number	Function	SNP Type
1	AN5995	Orthologs contain an SPT2 domain	Missense
2	AN3551	Protein of unknown function	Missense
3	AN4965	CCR4-NOT Complex, Woronin body (Tang et al., 2020)	Missense
4	AN4920	Putative calcium-transporting mitochondrial ATPase PmcB, involved in calcium homeostasis (Findon et al., 2010)	Missense
5	AN7400	Extracellular membrane protein	Codon Deletion
6	AN9921	Mis-annotated fusion between AlnR, the asperlin gene cluster transcription factor, and AlnG, a transmembrane protein (Grau et al., 2018)	Missense

List of the six shared protein coding region SNPs of the spontaneous suppressor mutants sequenced.

We investigated the common mutated genes, focusing on AN5995 as the potential causative suppressor mutant gene. The *SPT2* gene found in *S. cerevisiae* encodes a protein involved in chromatin remodeling, transcription elongation, and genome stability (Kruger and Herskowitz, 1991; Nourani et al., 2006; Novoseler et al., 2005). The Spt2 protein has two High Mobility Group (HMG)-like domains, which are found in other characterized proteins with a role in chromatin structure and transcription (Kruger and Herskowitz, 1991; Lefebvre and Smith, 1993). Additionally, Spt2 represses transcription, although the mechanism is unclear (Roeder et al., 1985; Nourani et al., 2006). Pairwise comparison reveals that AN5995 and *S. cerevisiae* Spt2 have 23.8% identity and 33.3% similarity and significant similarity within the two HMG domains (Novoseler et al., 2005).

AN5995 was deleted from *A. nidulans* through DNA-mediated transformation of the *AN5995Δ::Afp_{pyrG}+* knockout construct obtained from the Fungal Genetics Stock Center into MH11068 (*veA1*, *pyrG89*, *pyroA4*, *nkuAΔ::Bar amdA7*) to generate a *AN5995Δ* mutant strain. While we were able to confirm the gene replacement through diagnostic PCR, we did not observe a phenotype that was different from wild type (data not shown).

While investigating the unique SNPs of the spontaneous sectors, we observed a pattern in SNP distribution. 140 of the 267 SNPs were located within 200 kilobases of the telomeres. The sub-telomeric regions of *A. nidulans* makes up about 10% of the genome. The SNPs were also unevenly distributed in the sub-telomeric regions (Figure 4.2). Overall, over 50% of the SNPs were found in only 10% of the *A. nidulans* genome, a significant bias towards the sub-telomeric regions. We hypothesize that extreme stress conditions, such as a lack of leucine supplementation for a BCAA auxotroph, can force a higher mutation rate in the sub-telomeric region of the genome.

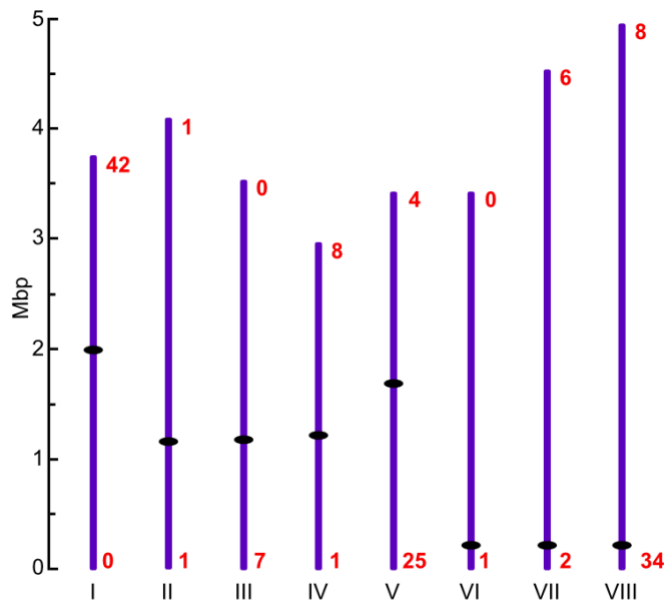


Figure 4.2 SNP Distribution across chromosomes.

Visual depiction of how many sub-telomeric SNPs were observed on each chromosome and on which arm of the chromosome. Black ovals represent locations of the centromeres.

4.5 Discussion

4.5.1 De-repression of transcription during oxygen rich conditions

Spt2 activity cycles with the yeast metabolic cycle, having the greatest activity during the oxidative phase (Rao and Pellegrini, 2011). It is possible that Spt2 responds to the changing oxygen concentrations during the cell cycle, repressing transcription during normoxic conditions and de-repressing during hypoxic conditions. The SNPs occurred within the SPT2 domain of AN5995, which could have disrupted the DNA binding or the oxygen sensing functions of this domain. Either case could potentially relieve repression by AN5995. The branched chain amino acid pathway is induced during hypoxia; *batB*, *batC* (*atnH*), *batD* (*atnJ*) and *batE* are expressed, with *batE* being the most highly expressed (Shimizu et al., 2010). While *batA* and *batB* were the most highly expressed *bat* genes under normoxic biosynthetic and catabolic conditions (Section 3.5.4 and 3.5.6), they are expressed at lower levels than *batC*, *batD*, or *batE* during hypoxia (Shimizu et al., 2010). One possibility is that AN5995 represses *batC*, *batD*, or *batE* expression during normoxic conditions but during

hypoxia, AN5995-dependent repression is inactive and *batC*, *batD*, or *batE* are de-repressed. This is consistent with the unaltered expression levels of *batE* during biosynthetic and catabolic conditions during normoxia (Section 3.5.4 and 3.5.6). In the case of the sector mutants, AN5995 may be unable to sense the oxygen concentrations or properly regulate its gene targets. This would allow genes normally repressed under normoxic conditions to be expressed, such as *batC*, *batD*, or *batE*. Up-regulation of *batE*, for example, may compensate for the loss of *batA* and *batB* for BCAA biosynthesis. As *batC* and *batD* are thought to catalyze biosynthesis of unusual branched chain amino acids that are precursors for aspercryptins biosynthesis (Chiang et al., 2016; Henke et al., 2016), up-regulation of these two genes is unlikely to provide the molecular basis for the suppressor phenotype.

The spontaneous mutants were selected as leucine auxotrophy revertants, on media supplemented with isoleucine and valine but lacking leucine. However, when subsequently tested, the mutants were found to be partial BCAA auxotroph revertants, showing partial growth without BCAA supplementation. Spontaneous sectors were not observed on media containing leucine. One explanation for the lack of suppressors is that leucine may inhibit the initial growth of spontaneous mutants of the *batA* Δ *batB* Δ double mutant arising on plates supplemented with leucine due to feedback inhibition. Leucine inhibits the α -IPM synthetase LeuC as part of a negative feedback loop. Exogenous leucine inhibits α -IPM production, which modulates the activity of the transcription factor LeuB and causes LeuB to act as a repressor of the BCAA biosynthesis genes (Kohlhaw, 2003; Polotnianka et al., 2004; Steyer et al., 2021; Downes et al., 2013). When supplementing with isoleucine and valine, there is no exogenous leucine inhibiting α -IPM synthetase, which causes α -IPM production and its interaction with LeuB, and thus LeuB is acting as an activator for gene expression (Sze et al., 1992; Downes et al., 2013). In our case of spontaneous mutants, the lack of leucine (and consequent LeuB activation of the BCAA pathway) combined with mutation in the possible causative gene AN5995 may allow for gene expression including *batE* (Steyer et al., 2021). The catalytic ability of *batE* could compensate the loss of *batA* and *batB* for BCAA production, thus accounting for the subsequent BCAA

auxotrophy.

4.5.2 Mutation Rate Bias

In filamentous fungal pathogens, genome architecture has evolved with the essential genes towards the centromere whereas the sub-telomeric regions are more gene sparse and contain non-essential genes such as genes encoding effector proteins or secreted secondary metabolites (Dong et al., 2015; Sperschneider et al., 2015). The difference in mutation rate in these two regions has led to the 'two-speed' genome concept. A two-speed genome has been shown in several filamentous fungal pathogens for effector protein genes (Dong et al., 2015; Faino et al., 2016; Sperschneider et al., 2015). However, the two-speed genome has not been shown for non-pathogenic filamentous fungi, such as *A. nidulans*. While *A. nidulans* does not encode effector proteins, secondary metabolite biosynthetic gene clusters are generally found in the sub-telomeric regions. Secondary metabolites are non-essential compounds that can confer a selective advantage for the producing organism when expressed (Gressler et al., 2015; Inglis et al., 2013; Keller et al., 2005; Sanchez et al., 2012; Tamiya et al., 2015). These biosynthetic gene clusters are silent in laboratory conditions and loss of these clusters does not impact primary metabolism.

4.6 Future Directions

Determining how the *batA*Δ *batB*Δ double mutant suppressor overcame the BCAA auxotrophy starts with confirming the causative gene. One approach to determine if AN5995 is the causative gene is to transform a wild type copy of AN5995 into the spontaneous sector mutants and test whether they regain the BCAA auxotrophy of the *batA*Δ *batB*Δ double mutant due to complementation or repair of the mutation. Additionally, the wild type AN5995 gene could be disrupted with a knockout construct in a *batA*Δ *batB*Δ background to determine if the *AN5995*Δ *batA*Δ *batB*Δ triple mutant had the same phenotype as the spontaneous suppressor mutant. If AN5995 is the causative suppressor gene, we would next need to determine how the disruption of AN5995 function causes the suppressor phenotype. One of the four

remaining *bat* genes, *batC*, *batD*, *batE*, or *batF* could be compensating for the loss of the primary *bat* genes *batA* and *batB*. Determining if de-repression of one of these *bat* genes is the molecular basis for overcoming the BCAA auxotrophy needs to be assessed. To do this, RT-qPCR would be used to compare expression of *batC*, *batD*, *batE*, and *batF* in the parental *batA*Δ *batB*Δ double deletion strain with selected suppressor mutants and the *AN5995*Δ *batA*Δ *batB*Δ triple mutant. We hypothesize that *batE* would be more highly expressed in the suppressor mutants than the *batA*Δ *batB*Δ double mutant as it has the highest expression during hypoxia and has not been characterized to be involved in making other types of BCAAs.

The potential of a two-speed genome in *A. nidulans* needs further investigation. Two-speed genomes are generally considered over evolutionary time scales, however, the sectors selected here arose within days. We hypothesize that extreme nutrient starvation stress causes this mutation bias. One method to test this would be to sequence other spontaneous mutants selected under a different extreme stress condition, such as growth on the toxic pyrimidine precursor 5-Fluoroorotic acid (5-FOA). By sequencing the parental strain in addition to these mutants, we could determine the distribution of mutations and the relative mutation rate for the core genome and the sub-telomeric region. Copy number variation analysis and examination of potential transposon position effects are needed to narrow our conclusions to the two-speed genome hypothesis.

Chapter 5 - Redundancy and Divergence of the Roles of Paralogous Leucine Biosynthesis Transcription Factors

5.1 Abstract

Mammals are incapable of synthesizing the branched chain amino acids leucine, isoleucine, and valine and must obtain them from their diets. However, fungi, plants, and bacteria are capable of de novo synthesis of these amino acids. The branched chain amino acid (BCAA) biosynthesis pathway has been characterized in the yeast *Saccharomyces cerevisiae* and recently in the filamentous fungi *Aspergillus nidulans* and *Aspergillus fumigatus*. Regulation of the BCAA biosynthesis genes occurs through the Zn(II)2Cys6 transcription factors, LEU3 in *S. cerevisiae* and LeuB in *Aspergilli*. Using sequence searches of the *A. nidulans* genome, two additional Zn(II)2Cys6 transcription factors, LeuR and AN7596, which are paralogs of LeuB, were identified. We show that in contrast to the *leuB* Δ mutant, which is a leaky leucine auxotroph, LeuR is dispensable for leucine biosynthesis. However, the *leuB* Δ *leuR* Δ double mutant is a tight leucine auxotroph. This indicates redundancy of these two transcription factors in regulating leucine biosynthesis. We characterize the regulatory roles of LeuB and LeuR in BCAA biosynthesis gene expression using RNA-Seq. Additionally, we demonstrate a role for LeuR in regulation of the NADP-dependent glutamate dehydrogenase gene, *gdhA*, and have identified two sites-of-action of LeuR in the *gdhA* promotor. Using leucine biosynthesis mutants to alter α -isopropylmalate (α -IPM) levels, it was found that α -IPM induces gene expression via LeuB but not LeuR.

5.2 Contribution Statement

Dr. Damien J. Downes made and confirmed the original *leuR* Δ deletion mutant. Cameron C. Hunter and Dr. Richard B. Todd constructed, confirmed, and conducted preliminary phenotypic analysis of the *leuB* Δ *leuR* Δ double mutant. The rest of this work was performed by Joel T. Steyer as part of this dissertation work.

Data presented in Section 5.5.1 was included in Steyer, J.T.,⁼ Downes, D.J.,⁼ Hunter, C.C., Migeon, P.A., Todd, R.B. (2021) Duplication and functional divergence of branched-chain amino acid biosynthesis genes in *Aspergillus nidulans*. *mBio*. **12**(3): e00768-21. <https://doi.org/10.1128/mBio.00768-21> [KAES contribution: 20-058-J] ⁼ denotes equal contribution.

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5.3 Introduction

The branched chain amino acids (BCAAs) isoleucine, leucine, and valine and are considered essential amino acids for mammals as they must obtain these amino acids from their diets. Fungi produce these amino acids *de novo* for protein biosynthesis, and as secondary metabolite precursors, and they are involved in virulence in the human fungal pathogens *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus*, and also in the fungal plant pathogens *Magnaporthe oryzae*, and *Fusarium graminearum* (Do et al., 2015; Kingsbury et al., 2004; Kingsbury and McCusker, 2010; Long et al., 2018; Oliver et al., 2012; Orasch et al., 2019).

The leucine biosynthesis pathway is primarily regulated by the Zn(II)₂Cys₆ transcription factors Leu3p in *S. cerevisiae* and LeuB in *Aspergillus nidulans* and *Aspergillus fumigatus* (Kohlhaw, 2003; Polotnianka et al., 2004; Steyer et al., 2021; Downes et al., 2013; Long et al., 2018). Both Leu3p and LeuB regulate gene expression by acting in gene promoters at the CCGN₄CGG consensus sequence while LeuB additionally acts at a non-consensus sequence, CCGN₅CGG (Downes et al., 2013; Downes et al., 2014b; Long et al., 2018). Leu3p and LeuB are components of a negative feedback loop that is controlled by the concentration of free leucine (Brisco and Kohlhaw, 1990; Downes et al., 2013; Polotnianka et al., 2004). When leucine levels are low, the leucine biosynthesis pathway intermediate α -isopropylmalate (α -IPM) concentrations are high. The α -IPM then interacts with Leu3p and LeuB, causing the transcription factors to switch from repressors to activators

(Sze et al., 1992; Downes et al., 2013). The leucine biosynthesis pathway is then activated and α -IPM is converted to leucine. In *S. cerevisiae*, it has been shown that as leucine concentrations increase, leucine acts as an inhibitor of α -IPM synthetase, Leu4p, reducing the levels of intracellular α -IPM (López et al., 2015). A similar mechanism is thought to operate via the α -IPM synthetase LeuC in *A. nidulans* and *A. fumigatus* (Downes et al., 2013; Long et al., 2018). Through this combination of conversion to leucine and inhibition of α -IPM synthetase activity, α -IPM levels decrease and α -IPM does not interact with Leu3p and LeuB, switching them to repressors (Downes et al., 2013; Polotnianka et al., 2004; Sze et al., 1992).

LeuB and Leu3p regulate expression of their respective NADP-dependent glutamate dehydrogenase (NADP-GDH) genes, *gdhA* and *GDH1* (Downes et al., 2013; Hu et al., 1995; Long et al., 2018; Polotnianka et al., 2004). Nitrogen assimilation occurs through NADP-GDH, producing glutamate. LeuB acts as a repressor of *gdhA* when exogenous leucine is added and an activator when no additional leucine is added, as shown by *A. nidulans* *gdhA-lacZ* reporter gene expression (Downes et al., 2013). Previous experiments demonstrated that the *leuB* Δ mutant is a leaky leucine auxotroph and suggested that an additional unknown factor regulated *gdhA* expression (Downes et al., 2013). Examination of the leucine biosynthesis genes reveals gene duplication and redundancy in ascomycete fungi (see Chapter 3). Gene duplication can result in either genome robustness due to neofunctionalization or genome fragility (Diss G et al., 2017; Escalera-Fanjul et al., 2019; Wapinski et al., 2007).

Orthologs of LeuB in other *Aspergillus* species have been shown to also regulate leucine biosynthesis. These orthologs also regulated additional metabolic processes such as iron metabolism and iron acquisition gene expression (Long et al., 2018; Orasch et al., 2019). Proper regulation of iron metabolism has also been implicated in fungal pathogen virulence with disruptions in iron metabolism causing reduced virulence. (Haas et al., 2008; Long et al., 2018; Schrettl et al., 2004; Schrettl et al., 2007; Schrettl et al., 2010). LeuB orthologs cross-regulate two essential metabolic processes in fungal pathogenicity, making it a potential and effective target

for anti-fungal agents as mammals do not make leucine and are an iron depleted environment.

In this study, we identify a paralog of LeuB, a second Zn(II)₂Cys₆ transcription factor we name LeuR, as a previously unidentified positive regulator in *gdhA* expression. We also characterize the role of LeuR in leucine biosynthesis, *gdhA* regulation, and iron metabolism. Using different iron concentrations and growth tests, we show that LeuB and LeuR both have roles under iron starvation while LeuB is involved during iron toxic conditions. We show that in the absence of LeuB, LeuR acts to positively regulate leucine biosynthesis and *gdhA* expression. When LeuB and LeuR are both absent, *A. nidulans* is a tight leucine auxotroph with reduced *gdhA* expression. We show, using metabolic mutants, that altered α -IPM levels affect LeuB but not LeuR activity. A third LeuB paralog AN7596 appears to play a very minor role in regulation of leucine biosynthesis.

5.4 Materials & Methods

5.4.1 Strain Construction

Aspergillus nidulans transformation was performed as described in Downes et al., (2013). The *nkuA* Δ non-homologous integration-defective mutant was used for targeted integration (Nayak et al., 2006). Knockout constructs sourced from the FGSC (McCluskey et al., 2010; De Souza et al., 2013) were transformed into MH11068 (*pyroA4 pyrG89 nkuA* Δ ::*Bar*) and selected for uracil and uridine prototrophy to generate *leuR* Δ (AN4744; RT478: Δ +14 to +2,393 bp) and AN7596 Δ (RT873: Δ +28 to +2253 bp). Homologous integration of the knockout constructs at *leuR* or AN7596 respectively was confirmed by Southern blot or diagnostic PCR (data not shown). Meiotic crossing was used to generate the double and triple mutants and *gdhA-lacZ* strains. Repair of the *leuB* Δ and *leuR* Δ mutations in the *leuB* Δ *leuR* Δ double mutant (RT500) was achieved by introduction the *leuB* (-822 to +3,681) or *leuR* PCR product (-873 to +3,626) amplified from MH1 and selected for leucine prototrophy.

5.4.2 Strains Used

A. nidulans strains used in this chapter are described in Table 5.1

Table 5.1 *A. nidulans* strains used.

Strain	Origin	Genotype ^a
MH1	M.J. Hynes	<i>biA1</i>
MH10217	M.J. Hynes	<i>biA1 pyroA4 riboB2 nkuAΔ::Bar leuBΔ::riboB</i>
MH10523	GO105 X A770	<i>biA1 riboB2 luA1</i>
MH11068	R.B. Todd	<i>pyrG89 pyroA4 nkuAΔ::Bar</i>
MH11094	14WFBA10 X MH11068	<i>amdS::lacZ nkuAΔ::Bar pyroA4 niiA4</i>
MH11324	TMXWT X leuBKO217	<i>biA1 pyroA4 tamAΔ leuB::riboB</i>
MH12101	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-800bpgdhAp-lacZ@amdS-lacZ</i>
MH12102	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-300bpgdhAp-lacZ@amdS-lacZ</i>
MH12181	Downes et al., 2013	<i>leuBΔ::riboB+ amdS::AfpyroA-gdhA(-753 bp)-lacZ pyroA4 niiA4</i>
MH12609	MH10217 X MH11069	<i>yA1 leuBΔ::riboB nkuAΔ::Bar niiA4 pyroA4 pabaA1</i>
RT152	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT155	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT156	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT157	Transformant of MH11094	<i>niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT182	MH12101 X MH10523	<i>biA1 niiA4 luA1 nkuAΔ::Bar pyroA4 AfpyroA-800bpgdhAp-lacZ@amdS-lacZ</i>
RT195	MH11324 X RT152	<i>leuBΔ::riboB+ biA1niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT198	MH11324 X RT155	<i>leuBΔ::riboB+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT201	MH11324 X RT156	<i>leuBΔ::riboB+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT204	MH11324 X RT157	<i>leuBΔ::riboB+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT220	RT219 X MH12102	<i>pyroA4 niiA4 leuCΔ::AfpyroA riboB2 AfpyroA-753bp gdhAp-lacZ@amdS-lacZ nkuA::argB</i>
RT349	Transformant of MH11094	<i>AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT350	RT349 X MH11324	<i>biA1 leuBΔ::riboB+ AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>

Strain	Origin	Genotype ^a
RT352	Transformant of MH11094	<i>AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT353	RT352 X MH11324	<i>biA1 leuBΔ::riboB+ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT356	Transformant of MH11094	<i>AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 niiA4</i>
RT357	RT356 X MH11324	<i>biA1 leuBΔ::riboB+ AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT415	Transformant of MH11068	<i>pyroA4 pyrG89 nkuAΔ::Bar batAΔ::AfpyrG+</i>
RT416	RT415 X MH10865	<i>yA1 pyrG89 nkuAΔ::Bar pabaA1 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT440	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar batBΔ::pyrG+</i>
RT456	RT416 X RT440	<i>yA1 pyrG89 pabaA1 batAΔ::AfpyrG+ batBΔ::AfpyrG+</i>
RT478	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuRΔ::AfpyrG+</i>
RT499	RT453 X RT478	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ pyroA4 nkuAΔ::Bar A.f.pyroA-gdhA(-753 bp)-lacZ@amdS-lacZ niiA4</i>
RT500	RT453 X RT478	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpyrG+ leuBΔ::riboB+</i>
RT503	RT453 X RT478	<i>leuRΔ::AfpyrG+ pyroA4 pyrG89 nkuAΔ::Bar A.f.pyroA-gdhA(-753 bp)-lacZ@amdS-lacZ niiA4</i>
RT687	RT0478 X RT0198	<i>leuRΔ::AfpyrG+ niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT689	RT0478 X RT0195	<i>leuRΔ::AfpyrG+ niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT690	RT0478 X RT0195	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ pyrG89 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-431 to -422) gdhAp-lacZ@amdS-lacZ</i>
RT691	RT0478 X RT0204	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT692	RT0478 X RT0204	<i>leuRΔ::AfpyrG+ pyrG89 biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492 and Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT693	RT0478 X RT0350	<i>biA1 leuBΔ::riboB+ leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT694	RT0478 X RT0350	<i>biA1 leuRΔ::AfpyrG AfpyroA-753bp(Δ-501 to -492, Δ-431 to -422, Δ-250 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT695	RT0478 X RT0353	<i>leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4</i>
RT696	RT0478 X RT0353	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ AfpyroA-753bp(Δ-501 to -241) gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar pyroA4 biA1 niiA4 pyrG89</i>

Strain	Origin	Genotype ^a
RT705	RT0478 X RT0198	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-501 to -492) gdhAp-lacZ@amdS-lacZ</i>
RT750	RT0478 X RT0201	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT751	RT0478 X RT0201	<i>leuRΔ::AfpyrG+ biA1 niiA4 nkuAΔ::Bar pyroA4 AfpyroA-753bp(Δ-250 to -241) gdhAp-lacZ@amdS-lacZ</i>
RT753	RT0478 X RT0357	<i>leuBΔ::riboB+ leuRΔ::AfpyrG+ biA1 niiA4 pyroA4 AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar</i>
RT760	RT0478 X RT0357	<i>leuRΔ::AfpyrG+ pyroA4 AfpyroA-240bp gdhAp-lacZ@amdS-lacZ nkuAΔ::Bar</i>
RT795	RT415 X RT453	<i>leuBΔ::riboB+ batAΔ::AfpyrG+ pyroA4 pyrG89</i>
RT796	RT440 X RT453	<i>leuBΔ::riboB+ batBΔ::AfpyrG+ pyroA4 pyrG89</i>
RT808	RT456 X RT478	<i>yA1 pabaA1 leuRΔ::AfpyrG+ batAΔ::AfpyrG+</i>
RT809	RT456 X RT478	<i>yA1 pyroA4 leuRΔ::AfpyrG+ batbΔ::AfpyrG+</i>
RT814	Transformant of RT500	<i>pyroA4 pyrG89 nkuAΔ::Bar leuRΔ::AfpyrG+ leuB::[repaired by linear DNA]</i>
RT816	Transformant of RT500	<i>pyroA4 pyrG89 nkuAΔ::Bar leuR::[repaired by linear DNA]</i>
RT873	Transformant of MH11068	<i>leuBΔ::riboB+ veA1 pyrG89 pyroA4 (nkuAΔ::Bar) amdA7 AN7596Δ::AfpyrG+</i>
RT880	MH12181 X RT182	<i>niiA4 pyroA4 luA1 leuBΔ::riboB+ AfpyroA-800bp gdhAp-lacZ@amdS-lacZ</i>
RT884	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuBΔ::riboB+ AN7596Δ::AfpyrG+</i>
RT885	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuRΔ::AfpyrG+ AN7596Δ::AfpyrG+</i>
RT888	RT873 X RT499	<i>pyrG89 pyroA4 nkuAΔ::Bar niiA4 leuBΔ::riboB+ leuRΔ::AfpyrG+ AN7596Δ::AfpyrG+</i>
RT895	RT220 X RT452	<i>pyroA4 leuCΔ::AfpyroA leuBΔ::riboB+ AfpyroA-753 gdhA(p)-lacZ@amdS-lacZ</i>
RT899	RT220 X RT478	<i>pyroA4 niiA4 leuCΔ::AfpyroA leuRΔ::AfpyrG+ AfpyroA-753 gdhA(p)-lacZ@amdS-lacZ</i>

^a all strains carry *veA1*

5.4.3 Genomic and RT-qPCR Primers

Genomic primers used in this chapter are described in Table 5.2

Table 5.2 Genomic primer sets for leucine biosynthesis genes.

Target	Primer Name ^A	Sequence (5' → 3')
<i>leuB</i>	LeuB (AN4486) 5'F	ACAGCAGTAGGATTGTGATCCT
		TGTCACGTATCTCTCAGCCT
	LeuB (AN4486) 3'R	TGTCACGTATCTCTCAGCCT

Target	Primer Name ^A	Sequence (5' → 3')
<i>leuR</i>	<u>ANID_04486.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGGTCACAGCAGT AGGATTGTG
	<u>ANID_04486.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCTGTCAGTGTAT CTCTCAGCC
	An4744FGSC5'F	CTGGTCAGGAGAGTTGCTA
	An4744FGSC3'R	CTGGAGGAAGAGACAGAGAG
	<u>ANID_04744.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGCTGGTCAGGA GAGTTGCTA
	<u>ANID_04744.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCCTGGAGGAAGA GACAGAGAG
AN7596	AN7596_5'F	AGGGTCTATCGTAGAACACG
	AN7596_3'R	CTAGCACTGGGAACAGTATG
	<u>ANID_07596.1 5'F</u>	GTAACGCCAGGGTTTTCCCAGTCACGACGAGGGTCTATCG TAGAACACG
	<u>ANID_07596.1 3'R</u>	GCGGATAACAATTTACACAGGAAACAGCCTAGCACTGGG AACAGTATG
<i>riboB</i>	AnRiboBR	AACAGATACATTTGACGG
<i>batA</i>	An4323FGSC5'F	CTCAGACGAGCGAGATTAC
	An4323FGSC3'R	CTACGGATCCCTACCTAGAA
<i>batB</i>	An5957FGSC5'F	CTAGTGCAGCAAGAGATCC
	An5957FGSC3'R	AAGGACGACTTGAGGACTAC

^A Underlined primers were used to amplify the deletion constructs.

RT-qPCR primers used in this chapter are described in Table 5.3

Table 5.3 RT-qPCR primer sets for leucine biosynthesis genes.

Target	Primer Name	Sequence (5' → 3')
<i>benA</i>	benA_RT_F	CCTGCTCCGCTSTCTTCC
	benA_RT_R	GACTGTTCTTGCTCTGGAT
<i>leuC</i>	leuC_RT_F	TCAAAGATCCCTCTAAGAAATACA
	leuC_RT_R	TTGTTCCGCCATCCATAGG
<i>luA</i>	luA_RT_F	TCTTGATCTATATCGACAGACA
	luA_RT_R	TGACGAGGTAGGGATGTT
<i>leuD</i>	leuD_RT_F	ATTGGTGGCCCAGAATGG
	leuD_RT_R	AATTGCATGGTCGCAAGTTG
<i>leuE</i>	leuE_RT_F	AAATCTGAGACCGTGTTT
	leuE_RT_R	ATTCGTCCATTGCATAATC
<i>batA</i>	batA_RT_F	GCTTCAGTGCTACTCGTG
	batA_RT_R	TGAACATGTGATCGGTAAAG
<i>batB</i>	batB_RT_F	CGAAGTTGTTGAGAAGAC
	batB_RT_R	TGATGAAGAATGCAGTCC
<i>batE</i>	batE_RT_F	CTCAATCATTCCCTCCTCCTC
	batE_RT_R	CATGGCCGTTAACTTCGC
<i>batF</i>	batF_RT_F	AAGTTCCGGTAAATGGTCTG
	batF_RT_R	CTCCTGTCCGTAGTTGAA

5.4.5 Overlay Chrome Azurol-S Siderophore Assay

Overlay Chrome-Azurol-S (O-CAS) blue agar siderophore assay was adapted from Schwyn and Neilands (1987) and Pérez-Miranda et al., (2007). Solutions and protocol are listed for 500 mL, scaled as needed. The Chrome-Azurol-S -- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ solution was slowly combined with the HDTMA solution. The CAS- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ -HDTMA and PIPES solution were prepared and autoclaved separately. After cooling to $\sim 50^\circ\text{C}$ post-autoclave, the CAS- $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ -HDTMA solution was slowly added to the PIPES solution and mixed gently to make the final O-CAS solution. 0.9% agarose was added to the mixture, boiled to solubilize the agarose, and cooled to about 50°C before gently overlaying the O-CAS solution onto the agarose plate containing a single point inoculated strain. The overlay solution was allowed to solidify, and orange coloration was allowed to form at 25°C . Photographs of the plates were taken at 3h and 7h.

5.4.6 Phylogenetic Analysis

The Pfam database (Finn et al., 2014) was used to identify orthologs of the isocitrate/isopropylmalate dehydrogenase protein family (PF00180), the aminotransferase class IV protein family (PF01063), and fungal Zn(2)-Cys(6) binuclear cluster domain DNA-binding domain family (PF00172). Sequence alignment and phylogenetic trees were constructed using MAFFT (<https://mafft.cbrc.jp/alignment/software/>; (Kato et al., 2019)). The neighbor-joining method with 100 bootstraps were used to generate consensus unrooted phylogenetic trees for both protein families. Tree visualization and label editing employed the Interactive Tree Of Life (iTOL) v4 program (<https://itol.embl.de>; (Letunic and Bork, 2019)).

5.4.7 RNA-Seq Analysis

Wild type (MH12101), *leuB* Δ (MH12181), *leuR* Δ (RT503), and *leuB* Δ *leuR* Δ (RT499) were grown for 16h on supplemented liquid ANM with 10mM ammonium as the primary nitrogen source and 2mM leucine at 37°C at 160 rpm. After 16h, mycelia

were harvested, washed with supplemented ANM, and transferred to fresh supplemented ANM with 10mM ammonium, but lacked 2mM leucine. Mycelia were grown for an additional 4hrs before harvested and RNA isolated.

RNA-Seq analysis was performed through the Galaxy Platform (www.usegalaxy.org) (Afgan et al., 2018). FastQC tool was used to determine sequence read quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were aligned to the *A. nidulans* FGSC A4 genome (Aspnd5rm) (Adrian Tsang, Concordia University) using the HISAT2 tool (Kim et al., 2015) with default settings. Transcripts per gene were counted with the htseq-count tool with default parameters (Anders et al., 2015). Differential expression analysis was performed with the SeqMonk Mapped Sequence Data Analyser Program from the Babraham Institute (www.bioinformatics.babraham.ac.uk/projects/). Within SeqMonk, made Replicate Sets for comparisons before checking quality with the RNA-Seq QC Plot. DESeq2 analysis was performed using the internal RNA-Seq Analysis Pipeline. Differentially expressed gene list was then exported for subsequent analysis and interpretation.

5.4.8 Gene Ontology Analysis

Gene Ontology (GO) analysis was done through ShinyGO 0.76 (<http://bioinformatics.sdstate.edu/go/>) with the DESeq2 list of genes from the RNA-Seq Analysis (see above) (Ge et al., 2020). Parameters were as follows: Organisms was “*Aspergillus nidulans* STRINGdb”, Pathway database was either “GO Molecular Function” or “GO Biological Process”, and # pathways to show was set to 10. All other parameters of the gene list were left as default. Visualization of GO analysis was done under the Chart heading, with pathways sorted by Category name, X-axis changed to Fold enrichment, and Color as $-\log_{10}(\text{FDR})$. Chart type was changed to barplot and aspect ratio to 2. Charts were then exported as a PNG file for subsequent figure formation.

5.5 Results

5.5.1 Regulation of Leucine Biosynthesis Genes by LeuB

The *leuB* Δ mutant has a partial leucine auxotrophy when grown without exogenous leucine indicating that LeuB regulates leucine biosynthesis genes (Polotniianka et al., 2004). The role of LeuB in regulation of leucine biosynthesis gene expression was investigated using RT-qPCR. RNA was isolated from wild type mycelia grown with and without exogenous leucine, which modulates LeuB activity via feedback inhibition (Downes et al., 2013), and RNA from mycelia of the *leuB* Δ mutant, and relative expression of the leucine biosynthesis genes was determined (Figure 5.1). The six genes involved in leucine biosynthesis, the α -isopropylmalate (α -IPM) synthetase gene *leuC*, the α -IPM isomerase gene *luA*, β -IPM dehydrogenase genes *leuD* and *leuE*, and the branched chain amino acid amino transferase (BAT) genes *batA* and *batB*, in addition to the dispensable BAT gene *batE*, showed decreased expression in both the *leuB* Δ mutant and the exogenous leucine condition in comparison to wildtype (Figure 5.1A). The expression of the aspercryptins biosynthesis gene cluster branched chain amino acid aminotransferase genes *batC* and *batD* was not statistically different from wild type under all three conditions whereas *batF* was undetectable (Figure 5.1B).

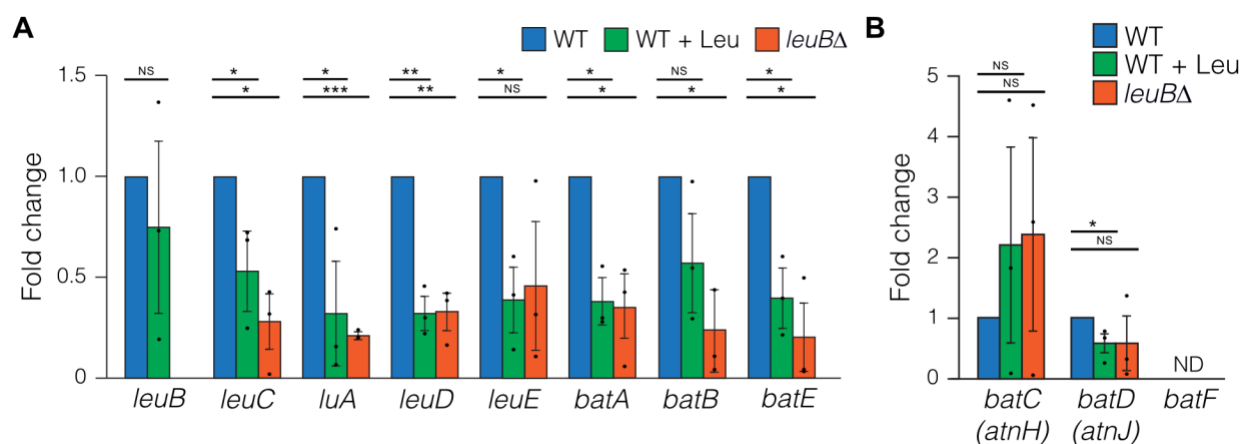


Figure 5.1 LeuB regulation of the leucine biosynthesis genes.

RT-qPCR of BCAA biosynthesis genes from wild-type (MH1) and *leuB*Δ (MH12609) strains grown for 16 h in supplemented liquid ANM-10 mM ammonium with or without 2 mM leucine (Leu). Expression is relative to the wild type. The means (bars) and individual results from three independent replicates (circles) are shown. *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$; NS, not significant, using two-tailed Student's t test with equal variance. **A**) Genes involved in BCAA biosynthesis, **B**) branched chain amino acid aminotransferase gene paralogs in *A. nidulans* not involved in BCAA biosynthesis.

5.5.2 Identification of LEU3-like transcription factors in *A. nidulans*

The *Aspergillus* genome is rich in transcription factors, particularly members of the fungal Zn(II)₂Cys₆ zinc binuclear cluster family (Todd et al., 2014). Three potential orthologs of the transcription factor Leu3p of *S. cerevisiae*, LeuB, AN4744 and AN7596, were found in the *A. nidulans* genome by using the LeuB protein sequence as the query. Reciprocal BLASTp searches confirmed that LeuB and AN4744 are reciprocally the closest paralogs in the genome. Protein sequence alignment revealed that LeuB and AN4744 show 45.8% identity/61.2% similarity while AN7596 showed 23.0% identity/43.7% similarity to Leu3p (Table 5.4). We named AN4744 *leuR*. Alignment of all three *Aspergillus* transcription factors to *S. cerevisiae* Leu3p showed strong conservation of the DNA binding domain, linker, and heptad repeats, particularly between LeuB and LeuR (Figure 5.2). Similarly, the Middle Homology Region was also strongly conserved between LeuB and LeuR. Conservation of the region directly C-terminal to the DNA binding motif may be significant as it is involved in sequence-specific DNA recognition and therefore suggests that these two transcription factors may bind similar target sequences (Reece and Ptashne, 1993). The masking region of Leu3p, involved in feedback regulation by α -IPM, is poorly conserved with LeuB, despite functional conservation of this mechanism. However, this region showed strong sequence conservation between LeuB and LeuR. LeuB is conserved widely throughout the Ascomycetes (Todd et al., 2014). Sequence database searches revealed that LeuR and AN7596 have a narrower distribution and are conserved in Eurotiales. Phylogenetic analysis showed that LeuB, LeuR, and AN7596, and their respective orthologs form separate clades from each other but LeuR and LeuB are more closely related to each other than AN7596 (Figure 5.3).

Table 5.4 Pairwise protein sequence comparison of LEU3, LeuB, LeuR and AN7596.

Protein	% Protein Identity/% Protein Similarity			
	LEU3	LeuB (AN4486)	LeuR (AN4744)	AN7596
Leu3p	100/100	25.2/47.2	25.9/44.7	16.4/29.5
LeuB (AN4486)		100/100	45.8/61.2	21.4/36.5
LeuR (AN4744)			100/100	19.6/34.8
AN7596				100/100

Pairwise protein sequences were aligned and percent identity/similarity was calculated using EMBOSS-Needle (EMBL-EBI) with default parameters.

```

LEU3 -----MEGRSDFVATSSQSSSEMSHS 20
LeuB M-----GGIGHTSIS--PAVASDGAGQARKRNAGMAGLD-----SSPSSIDDVE 42
LeuR MDLEYRGLAONGYPPHASLTAPPSPSDLRNPNRLPRP-LTGDFSVRSSEPS--DME 56
AN7596 -----ME 2

LEU3 ETRNRRTGMNARKRK*ACV*ECRQQS*KCD*AHERAPE*CTKCAKKNVPCILKRDFFRTYKRA 80
LeuB EVENRDEKRRQPVKRCACNECRQQLRCDVVDPPWTDCSRCRRLKLEQKIESNFKRVGKRS 102
LeuR KTPPVVTGKRRGGCKRCACNECRQQLRCDIVCTPADACSRCRRLNIEQKVEPSFKRISKRK 116
AN7596 DFHPLPVSKRS--KACACRCQQA*RCDA*YK--QP*CSRCEKLGIEQVLESEAFRRHKRQ 58

LEU3 RNEAIEKRFKELTRTLTNLTSDLEKKIEEEQEI VL----DNNFTEKEK----- 125
LeuB RNAEMEREIEELRKOIASVQSGSY---GQQQSHLP---SAQQTPKQ----- 142
LeuR RNAEMEREIAALRQRUSS-----EGSHAHVP---ENRASTKVS----- 151
AN7596 KYERLEKETDELRLKQLNAHTAS*HQIRESATTMMPRNPADNVKRNKATGVCIDANDAE 117

LEU3 -----VKQLRKSAFETTEIEPRSYKTLRGEPLSYSTNRRHTDSSPTTL 168
LeuB -----ENPSNQVSPARAYQTPSAMSADQFMGSH---EAVASLIDL 180
LeuR -----QSPEGICYGPDANVSRGRITLSAKL-EPQTLATPTM 187
AN7596 I SCNGAESLQLLFQDIASDGLPHLTAATSESPSSNGSKLPMPVPSI-YKNTASGQTRL 176

LEU3 LSSSTNFDPVHSTNVMDDQLKC-----LPSLGLDQVYLSSSDIAELFQEATKYHQ 219
LeuB RSG---YD--GS SYMRNGNHF-----KRIEDVAVPERVTELFDFLFTTYHP 223
LeuR HSE-----GSMLSQDDGV-----WRLQDVLSLRQVRLDFQFFKYHP 226
AN7596 QPL---QPPVGMGPVVSKKKLAAMAGVDPDRTLPRLGGVTLSGDEIDETFINFVROVWV 233

LEU3 FLRVVDLSKGAERIVHLSFCLFWVILLIGLRRKFGATDLMTRLISVLKSVLSEITISPII 279
LeuB FLRFLLDREQSPPEEYTVSPFLFWITISVGARRYQDPDNLNLSLA-----GPVT 271
LeuR FLPLLNPLKPPPEEYLRRCLOAWTICVASRRAPSEPGLLSALS-----GPFS 274
AN7596 FMCCLDTESSNLTLYEQSDFLFWTIGTASRTCAKNPTIFPALA---KHIIISMAFLSPIS 290

LEU3 RYTPSDKDEPVNIVASVYSVOAFLLYTFWPLTSSLSADTSWNTICAMFQALRVGLNC 338
LeuB RLWVST---LADVPQSYHVVKALCLCSWFFPTSSSTSDPTFMGLGLMIQTAMQLGLHR 327
LeuR RLWST---ITSVPQDVRVVKALCLCTWPLTTSQRTDQATFMGLGLMMQISMQLGLHR 330
AN7596 E-----SGAIVQGLLVNWRPFKADGGVDIIEPLTGLLLHVAMMYGMHN 337

LEU3 AGFSKEIASANSSEL-----VNEQRTWICCNVVSQTVASSFCFPAYVSFDVLYISSIRVP 393
LeuB PSHTQDPSKYIVELVEEELRDKVRTWATCNIVAGRVATSGYGGPPCTLYDWTLSSQES-- 384
LeuR PVQKEEETFTFMEGHGEA-VKDRLOTVVICNIVAGNVATSGYGGPPCTLYDWMALEPASL-- 387
AN7596 PVSSHEEFKTKQMPQVADISRSSELWAYTVITYGRACLMKGQPPRLPDMAHVDVNR-- 395

LEU3 NKSQVDIPNELLROMAQARFENQIVNTMNSTPASVTMVVSQEEKQPLHLVNLNOLSOLE 453
LeuB -MDPNFKLPESIRHRLDEKFCQKVTKALYTNRRDPVGLCGSOERSTLTSILSRDLDLE 443
LeuR -RDADYHPSDELRTRLLEKFCQDRTKSLYSSRPDPAEFI-SSEKLLIAOLLELREME 445
AN7596 -KTLYQNMSPHVRTRMKAQEI VTCGGAAVNEYGVLTLSAADEGGGLDLLKAYEVRVNDLH 454

LEU3 ISLEENNLDDRKFLLLVAKVHLTYFTDVTQSAGKSNGNIYEGSYSIMELDTSFETK 513
LeuB TQLKP-HNDYITDLYLRASNLHLHLSAFDDFTAKD-----Y-----R 480
LeuR LFGGR-DISSNMHILRAELRLRYFVFLGSNARS-----D----- 480
AN7596 ID---ATTWMARFDILVACLISLQGMHFLKNHTLYA-----D 487

LEU3 RGLVKVYNAAVNFIHANSMWEHDPTIKYFPGLFVLNIWQSACISKLIHSSLHSMIDV 573
LeuB ERLLSLFFAATTFLEAAMKLQTVQGMPLSNTPYYIYOMMVAGCCTLKLCKSFFASHIDM 540
LeuR -DITKLEIATTSFLGRVLDLETSPGELIGHATNYILQMITVSAAFALMKLLKSDFRRHIDF 539
AN7596 NRIKLEAAACAVIDAADMAELQSL-AVCPISQGWFGLLLAGSILRIIKGHDTDIDVF 546

LEU3 NSGKKAYNNAISLTFNASVLYKIDMAYRSSGIMRSIWSLFANMYDAWKNDQKEGGRLNND 633
LeuB DYAKGLFNRTIWAIRSVSISNDLPERLAELVLAQMWKLGSTSPKPPPDSEVM-----D 594
LeuR EHGLKLLFNGAISALRISVMDHDPVRLADILAQMNAGT-----PEDAGE----- 585
AN7596 AKARNYFMKSNLALMLTYDNADMPKIVTVLSHLYNSTKAFR---KADSEVM----- 596

LEU3 FNTGTTIKSRMSVNVVFFDCYILKEKCGMAKLERETKVSTAYNVDEEEEEDEDEEGEEEE 693
LeuB DSNMLKVRCRMMSMSLLFDSVWRVRE----- 619
LeuR DTELLKVRCRMMSMHYDTVWRVRH----- 610
AN7596 -NLNLRISRRLALCPIDAMWFEKD----- 619

LEU3 EEEELSSKVPENMDSQQLTRKFTNVRHPEKKARKIETIPLDPNPINASTSSGSSLT 753
LeuB -----DTRT-----KGRNIEAYLKNPTNPDNSADSSASSVGP 652
LeuR -----RFRP-----LKSTEDTQAAASNPNVSTATI 635
AN7596 -----EFEP----- 623

LEU3 PNSQVANTISYRGILNKMSPREQLNHANLDSVSTDIKDTEAVNEPLPIGRNAEHPANQP 813
LeuB P-----RTSPGLATGDPSLAPALL----- 671
LeuR P-----RPSDG-SLEDPDLMYS----- 651
AN7596 ----- 623

LEU3 PLSITQMCENT---LPATQANSLLLETPIVQSNPVTTTIKESPNSIMAGWDNWESDMV 869
LeuB PQSNMGVQSGNINIGSLPSGFMEPNY-EVF-----DPLN 703
LeuR PNFD---QDG---AFFNEAGFSEVF-----DSLNL 674
AN7596 PLPSTTTTEENT---EKPTFGPEGSAHLY-----DDGF 653

LEU3 WRDVIDILMNEFAFNPKV----- 886
LeuB WL-LDGLVD-LPYPSVSGMEAQGIA----- 727
LeuR WV-FDGFPTFPAPPAL----- 690
AN7596 WTDLEWALN-IPE----- 665

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Figure 5.2 Sequence comparison of the LeuR, LeuB, AN7596 and Leu3p proteins.

Clustal Omega alignment of *A. nidulans* LeuR LeuB, AN7596 and *S. cerevisiae* Leu3p. The middle homology region is boxed (green), as is the Leu3p α -IPM masking region (red). Cysteines that form the C6 DNA-binding motif are starred; the linker region is underlined (dashed) as are the heptad repeats (solid). Hydrophobic residues in the 1 and 4 positions of the heptad repeats are marked (arrow heads).

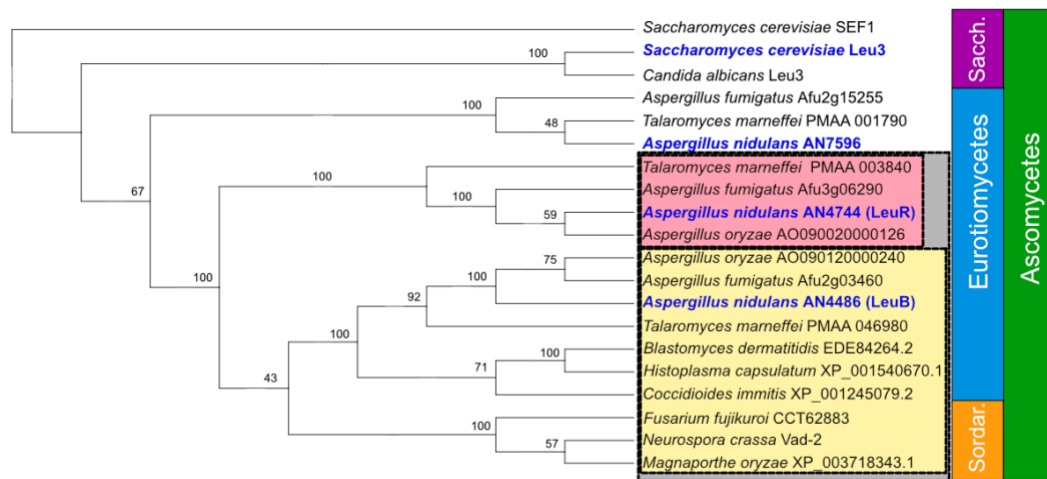


Figure 5.3 Phylogenetic analysis of LeuR, LeuB, and AN7596 orthologs.

Unrooted phylogeny of transcription factor LeuB, LeuR and AN7596 orthologs using Neighbor-Joining (NJ) method with Bootstrap support (100 replicates). Protein sequences were downloaded from FungiDB or NCBI. Ascomycota: *A. fumigatus* (1: Afu2g15255; 2: Afu3g06290; 3: Afu2g03460), *A. nidulans* (1: AN7596; 2: AN4744 (LeuR); 2: AN4486 (LeuB)), *A. oryzae* (1: AO090020000126; 2: AO090120000240), *Candida albicans* (LEU3), *Fusarium fujikuroi* (CCT62883), *M. oryzae* (XP_003718343.1), *Neurospora crassa* (Vad-2), *Saccharomyces cerevisiae* (SEF1; LEU3), *Talaromyces marneffeii* (1: PMAA 001790; 2: PMAA 003840; 3: PMAA 046980;), *Blastomyces dermatitidis* (EDE84264.2), *Histoplasma capsulatum* (XP_001540670.1), *Coccidioides immitis* (XP_001245079.2). The two distinct LeuB and LeuR clades are boxed. “Sacch.”: Saccharomycetes; “Sordar.”: Sordariomycetes.

5.5.3 Characterization of LeuB Paralogs

Since the *leuB* Δ mutant has a partial leucine auxotrophy, we hypothesized that the two LeuB paralogs may also regulate the leucine biosynthesis pathway. To determine their roles in leucine biosynthesis, we generated *leuR* Δ and *AN7596* Δ mutants by transformation of MH11068 (*pyroA4 pyrG89 nkuA* Δ) with the *AN4744* Δ ::*Afp_{pyrG}* and *AN7596* Δ ::*Afp_{pyrG}* knockout cassettes obtained from the Fungal Genetics Stock Center (De Souza et al., 2013) (Figure 5.4A). Transformants were selected for pyrimidine prototrophy. In contrast to the leaky leucine auxotrophy of the *leuB* Δ mutant (Polotnianka et al., 2004), the *leuR* Δ and *AN7596* Δ mutants showed no growth defects in the absence of exogenous leucine (Figure 5.4B). *leuB* Δ *leuR* Δ , *leuB* Δ *AN7596* Δ , and *leuR* Δ *AN7596* Δ double mutants, and the *leuB* Δ *leuR* Δ *AN7596* Δ triple mutants were generated by meiotic crossing to determine if LeuB, LeuR, or AN7596 have redundant functions. The *leuB* Δ *leuR* Δ double mutant is a tight leucine auxotroph (Figure 5.4B), indicating that LeuR participates in regulation of leucine biosynthesis genes. Additionally, the *leuB* Δ *AN7596* Δ mutant showed a slightly tighter leucine auxotrophy than the *leuB* Δ mutant, indicating that AN7596 has a minor role in leucine biosynthesis regulation. We complemented the tight leucine auxotrophy of the *leuB* Δ *leuR* Δ double mutant with a linear DNA fragment carrying the wild type copy of either *leuB* or *leuR* and directly selected transformants in the absence of leucine (Figure 5.5). Integration of either the *leuB* or *leuR* wild type gene conferred leucine prototrophy resembling the *leuR* Δ mutant or the leaky leucine auxotrophy resembling the *leuB* Δ , respectively.

To determine whether either or both of these transcription factors regulate BCAA catabolism, the single, double, and triple mutants were assessed for utilization of each BCAA as the predominant nitrogen source, with and without leucine supplementation (Figure 5.4C). The *leuB* Δ *leuR* Δ double mutant leucine auxotroph grew on leucine as a nitrogen source and on isoleucine as a nitrogen source in the presence of leucine, indicating that *leuB* and *leuR* were dispensable for leucine and isoleucine utilization. The *leuB* Δ *leuR* Δ double mutant showed slightly reduced growth compared with wild type on valine in the presence of leucine (Figure 5.4C). While this could be interpreted as a minor role in regulation of valine utilization, we instead

hypothesize that this may be the result of valine inhibition (see section 5.5.4). Additionally, the *leuB*Δ *AN7596*Δ and *leuR*Δ *AN7596*Δ double mutants were slightly weaker on media where isoleucine, leucine, and valine were the predominant nitrogen source, even with leucine supplementation for isoleucine and valine conditions, than *leuB*Δ, *leuR*Δ, and *AN7596*Δ single mutants and the *leuB*Δ *leuR*Δ *AN7596*Δ was the weakest. This suggests that loss of more than one transcription factor affects colony growth and that supplementation cannot completely restore wild type-growth. This may be due to reduced expression of key catabolic genes, such as *batA* and *batB*, to use the BCAAs as a nitrogen source.

Overall, our analysis of the single, double, and triple transcription factor mutants indicates that LeuB, LeuR, and AN7596 play roles in the regulation of leucine biosynthesis but to varying capacities. The addition of exogenous leucine to media restored growth for the auxotrophs. No major role for these transcription factors was detected for BCAA utilization.

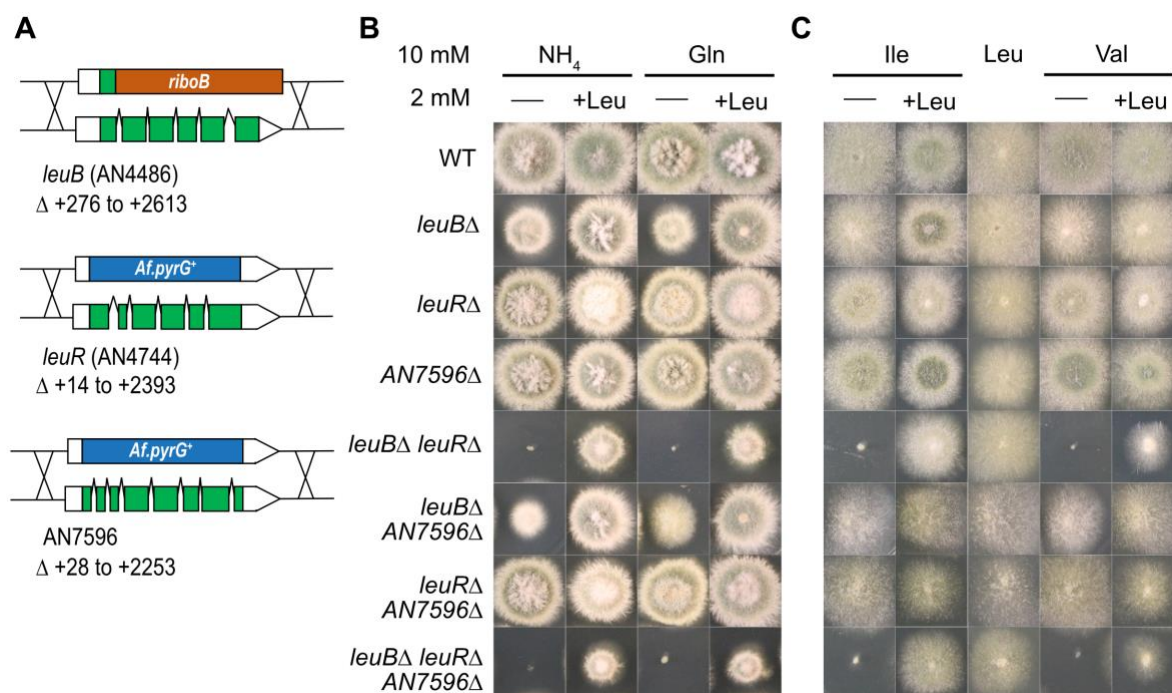


Figure 5.4 Deletions of *leuB*Δ, *leuR*Δ, and AN7596Δ confer varying leucine auxotrophies.

A) The *leuB* (AN4486), *leuR* (AN4744), and AN7596 knockout cassettes. **B-C)** Wild type (MH1), *leuB*Δ (RT452), *leuR*Δ (RT478), AN7596Δ (RT873), and *leuB*Δ *leuR*Δ (RT499), *leuB*Δ AN7596Δ (RT884), *leuR*Δ AN7596Δ (RT885), *leuB*Δ *leuR*Δ AN7596Δ (RT888), strains were grown on supplemented ANM solid media for 2 days with 10 mM ammonium (NH₄), glutamine (Gln), isoleucine (Ile), leucine (Leu), and valine (Val) as the predominant nitrogen source with or without 2 mM leucine (Leu) to supplement auxotrophies.

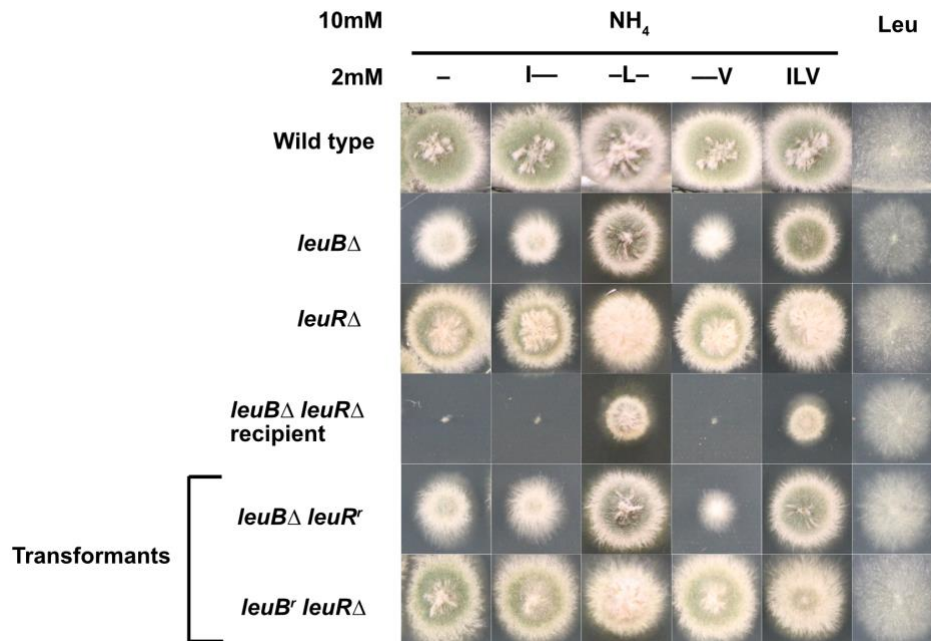


Figure 5.5 Complementation analysis of the transcription factor deletion genes.

Transformants of the *leuB*Δ *leuR*Δ mutant (RT500) with PCR-amplified wild type genomic DNA of either *leuB* or *leuR* genes. Transformants were grown at 37°C for 2 d on solid supplemented ANM with either 10mM ammonium (NH₄) or 10mM leucine (Leu) as the nitrogen source. Various combinations of 2mM isoleucine (I), leucine (L), and valine (V) were added to both types of media for supplementation. *leuB*^r and *leuR*^r indicate repaired wild type *leuB* or *leuR* by gene replacement of the *leuB*Δ::*riboB*⁺ or *leuR*Δ::*Afp_{pyrG}* construct, respectively.

We assessed whether a cryptic role for LeuR in regulating leucine or BCAA biosynthesis or catabolism might be revealed when either *batA* or *batB*, the reversible final enzymatic step of BCAA biosynthesis, were perturbed. We constructed *leuR* Δ *batA* Δ and *leuR* Δ *batB* Δ mutants and compared them to the single deletions and the *leuB* Δ *batA* Δ and *leuB* Δ *batB* Δ double mutants (Figure 5.6). The *leuB* Δ double mutants displayed the *leuB* Δ leaky leucine auxotrophy while the *leuR* Δ double mutants had a wild type phenotype. Thus, *leuB* Δ is epistatic to *batA* Δ and *batB* Δ , and there is no synthetic phenotype when *leuB* Δ or *leuR* Δ is combined with deletion of either BAT gene. BatA and BatB also catalyze the first step in BCAA utilization (Steyer et al., 2021). No synthetic BCAA utilization phenotype was observed for *leuB* Δ or *leuR* Δ mutations combined with either *batA* Δ or *batB* Δ .

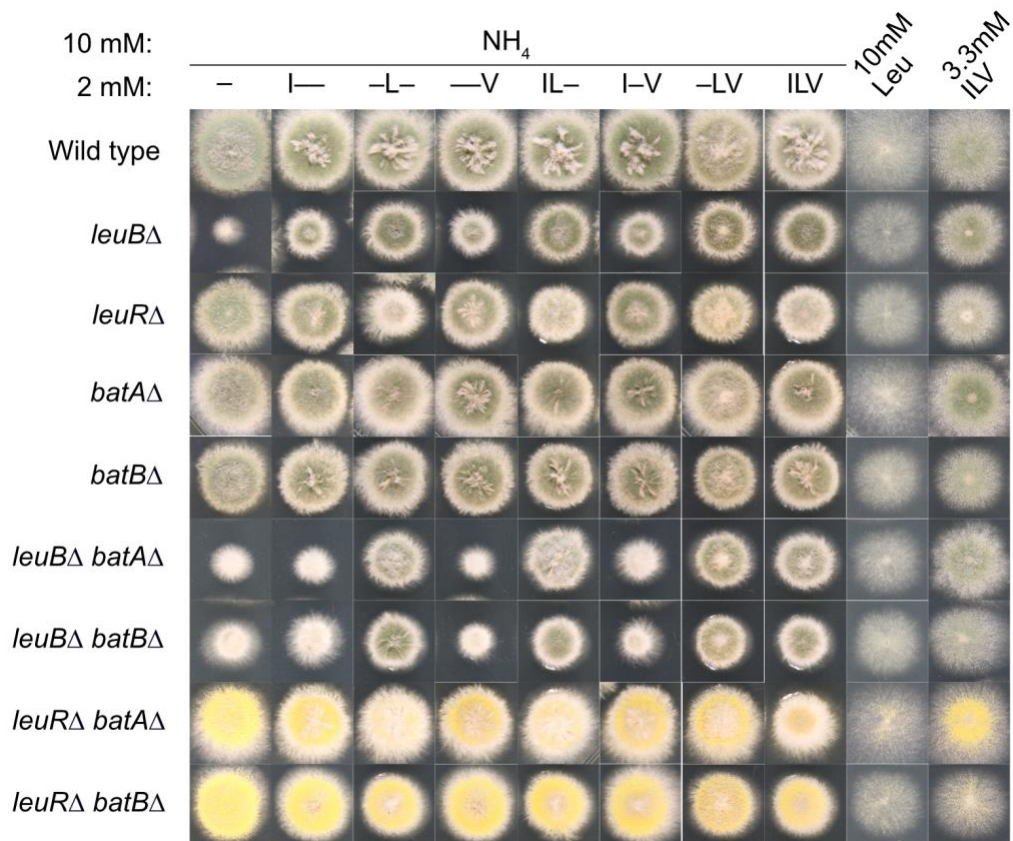


Figure 5.6 Growth analysis of *leuB* Δ and *leuR* Δ regulation of *batA* Δ and *batB* Δ . Wild type (MH1), *leuB* Δ (RT453), *leuR* Δ (RT478), *batA* Δ (RT415), *batB* Δ (RT440), *leuB* Δ *batA* Δ (RT795), *leuB* Δ *batB* Δ (RT796), *leuR* Δ *batA* Δ (RT808), and *leuR* Δ *batB* Δ (RT809) were grown at 37°C for 2 d on solid supplemented ANM with either 10 mM ammonium (NH_4), Leucine (Leu), or 3.3 mM each of isoleucine, leucine, or valine (ILV). Note that the yellow color of the *leuR* Δ *batA* Δ and *leuR* Δ *batB* Δ mutants is due to the *yA* conidial color mutation in these strains and is unrelated to *leuR* Δ , *batA* Δ or *batB* Δ .

5.5.4 Growth inhibition of *leuB* Δ and *leuB* Δ *leuR* Δ mutants by valine

While determining the phenotypes of the *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants with various combinations of BCAA supplementation, a reduced growth phenotype of the *leuB* Δ and *leuB* Δ *leuR* Δ mutants was observed when they were supplemented with valine (Figure 5.7). We considered the basis of a potential valine inhibition phenotype with knowledge of the BCAA biosynthesis pathway in the literature. Isoleucine, leucine, and valine share the first enzymatic steps for biosynthesis before diverging into amino acid specific pathways (Figure 5.8A). The first shared step is conversion of two pyruvate molecules to α -acetolactate by an acetohydroxy acid synthase (AHAS) dimer (Umbarger and Brown, 1958). This enzymatic dimer contains a catalytic subunit and a regulatory subunit. In *S. cerevisiae*, *ILV2* is the catalytic subunit gene and *ILV6* is the regulatory subunit gene. *A. nidulans* has a single ortholog of both genes, AN4956 and AN4430 respectively. Studies in bacteria, fungi, and plants have shown that the regulatory subunit is feedback inhibited by valine as part of a negative feedback loop while the catalytic subunit is largely unaffected (Cullin et al., 1996; Pang and Duggleby, 2001; Mifflin and Cave, 1972). Missense mutations affecting the valine-binding site within *Ilv6* in *S. cerevisiae* cause the regulatory subunit to be insensitive to valine, breaking the negative feedback loop (Kopecký et al., 1999; Wu et al., 1994; Bourgin et al., 1985; Park et al., 2007; Takagi, 2019; Takpho et al., 2018a). The conservation of the valine binding sites within the AN4430 protein sequence suggests that valine feedback inhibition of BCAA biosynthesis is likely conserved in *A. nidulans* and therefore valine feedback inhibition may account for the inhibitory effects of valine supplementation observed in the *leuB* Δ and *leuB* Δ *leuR* Δ mutants (Figure 5.8B).

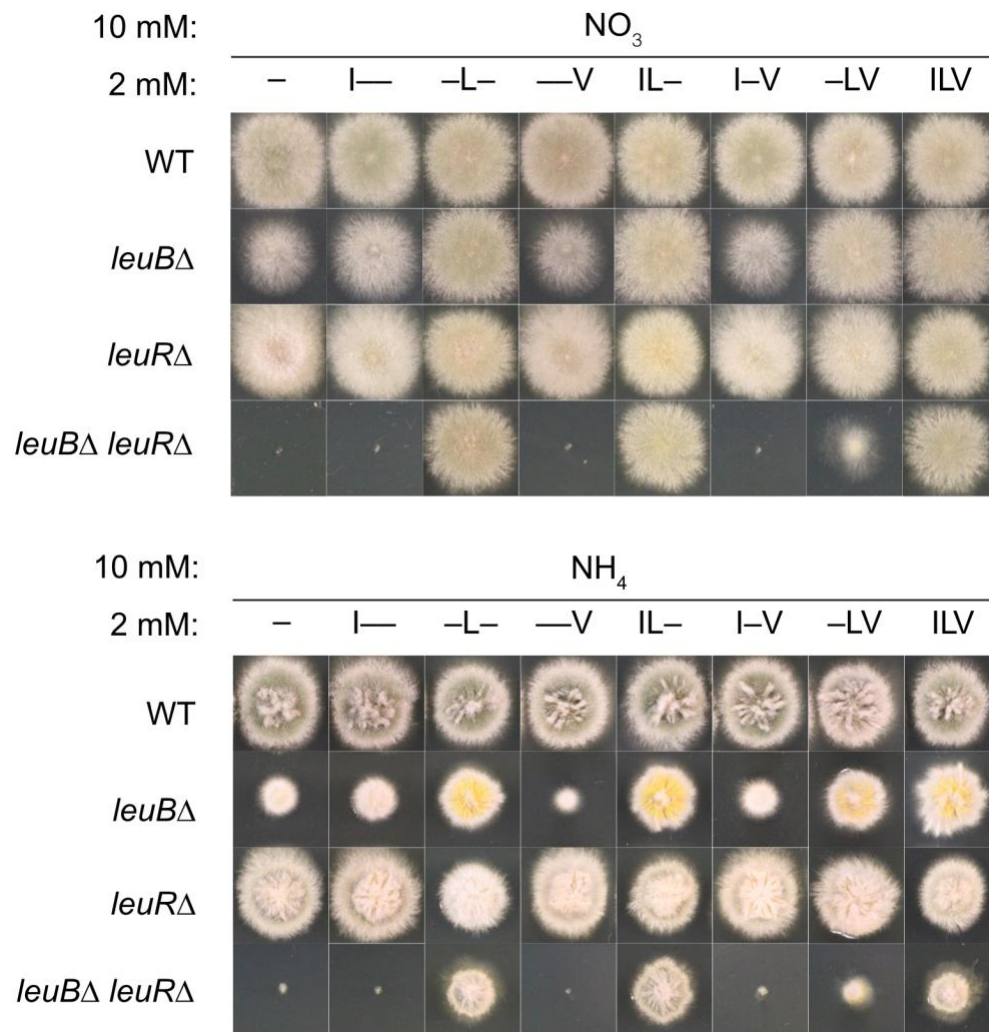


Figure 5.7 Inhibition of *leuB*Δ and *leuB*Δ *leuR*Δ mutants by valine.

Wild type (MH1), *leuB*Δ (MH10217, MH12609), *leuR*Δ (RT478) and *leuB*Δ *leuR*Δ (RT500) strains were grown on supplemented ANM solid media for 2 days at 37°C with 10 mM nitrate (NO₃) or ammonium (NH₄) as the predominant nitrogen source and combinations of 2 mM (each) isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. —, an omitted amino acid. Note that the yellow color of the *leuR*Δ *batA*Δ and *leuR*Δ *batB*Δ mutants is due to the *yA* conidial color mutation in these strains and is unrelated to *leuR*Δ, *batA*Δ or *batB*Δ.

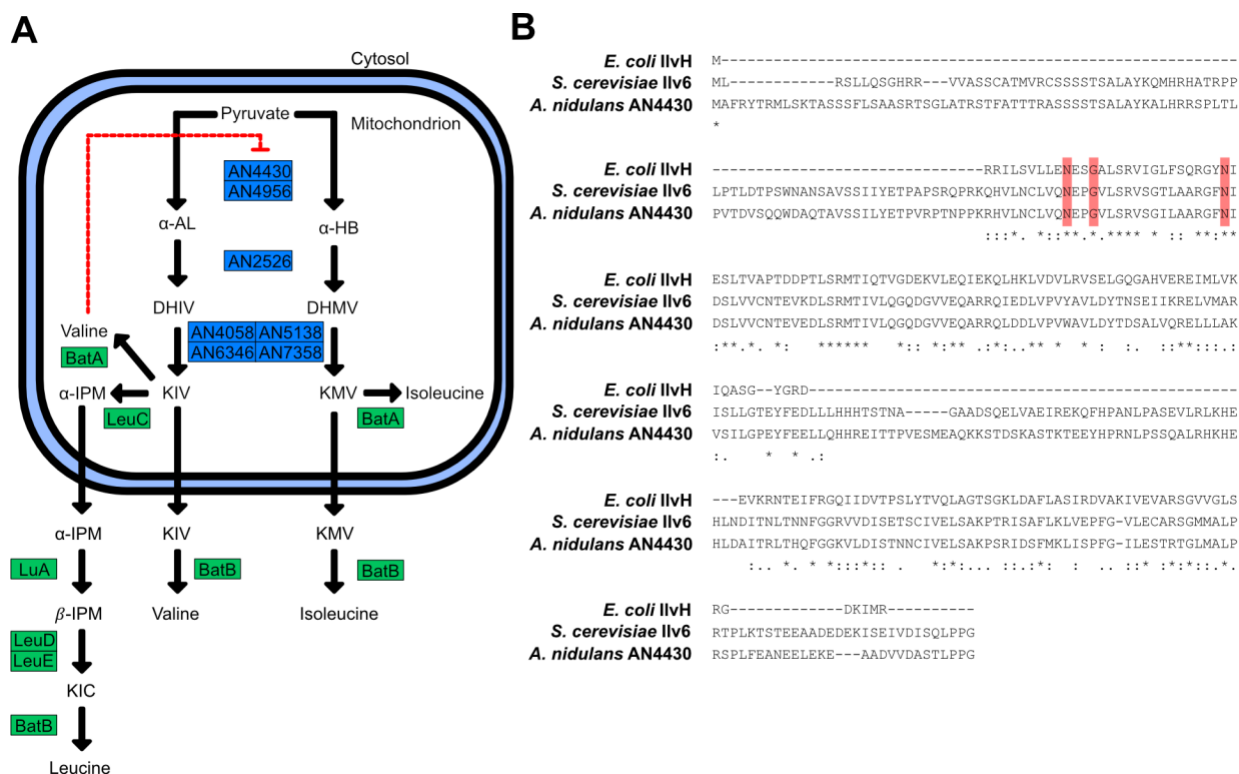


Figure 5.8 Valine inhibition of the extended BCAA pathway in *Aspergillus nidulans*.

A) Extended biosynthetic pathway of the BCAAs starting from pyruvate. Valine inhibition of the regulatory subunit of Ilv6 ortholog (AN4430) shown by dashed red line. Orthologues of *S. cerevisiae* enzymes are listed, as shown in the blue boxes. Previously characterized BCAA biosynthesis enzymes, as presented in green boxes, are shown as well. α -AL: α -acetolactate. α -HB: α -aceto- α -hydroxybutyrate. DHIV: α,β -dihydroxyisovalerate. DHMV: α,β -dihydroxy- β -methylvalerate. KIV: α -ketoisovalerate. KMV: α -keto- β -methylvalerate. **B)** Sequence alignment of *E. coli* IlvH, *S. cerevisiae* Ilv6, and *A. nidulans* AN4430 protein sequences. Residues involved in valine inhibition are highlighted with a red box.

The *leuB*Δ single mutant had a slight reduction in growth when supplemented with only valine compared with the unsupplemented *leuB*Δ mutant. This slightly reduced growth was rescued by addition of leucine. The *leuR*Δ single mutant had no phenotype when supplemented with only valine (Figure 5.7). With the valine negative feedback loop in mind, we determined the relative expression compared with wild type of both AN4956 and AN4430 in our RNA-seq data (See below Table 5.5 in Section 5.5.8). Both genes are downregulated in the *leuB*Δ and *leuR*Δ single mutants. The *leuB*Δ single mutant shows reduced expression of more downstream BCAA biosynthesis genes than the *leuR*Δ single mutant. Valine inhibition of AN4430 likely exacerbates the lack of BCAA biosynthesis gene expression of *leuB*Δ, causing the colony growth reduction. In the *leuR*Δ single mutant, fewer genes in the BCAA biosynthesis pathway are affected and the valine inhibition may not have as big of an effect on overall BCAA production, thus not causing a valine inhibition phenotype.

Since the *leuB*Δ *leuR*Δ double mutant is a leucine auxotroph, valine inhibition cannot be assessed without leucine supplementation. Valine inhibition was observed in the double mutant supplemented with both leucine and valine compared with leucine supplementation alone. In the presence of all three BCAAs, valine inhibition was not observed, indicating that isoleucine reversed the valine inhibition growth phenotype in the *leuB*Δ *leuR*Δ double mutant. Using the RNA-Seq data, we determined that both AN4956 and AN4430 genes are up-regulated in the *leuB*Δ *leuR*Δ double mutant. Although AHAS enzyme levels in the *leuB*Δ *leuR*Δ double mutant are transcriptionally up regulated, the enzyme is likely inhibited by the exogenous valine. Loss of both transcription factors lowers expression of several other steps involved in BCAA biosynthesis so lack of pyruvate conversion for BCAA production reduces overall BCAA production and colony growth.

We hypothesize that in the *leuB*Δ single mutant supplemented with valine or with isoleucine and valine, the reduced AN4430 expression levels coupled with valine inhibition of the reduced AN4430 protein concentration further decreases the production of all three BCAAs. This causes an overall tighter leucine auxotrophy of the *leuB*Δ mutant than on non-supplemented media. The *leuB*Δ *leuR*Δ double mutant is hypothesized to be affected similarly by exogenous valine when supplemented with

leucine and valine. We hypothesize that the addition of isoleucine to the leucine and valine supplementation to the media supplements an isoleucine auxotrophy in the *leuB* Δ *leuR* Δ double mutant, caused by the lack of both transcription factors that regulate BCAA biosynthesis genes and reduced AHAS activity due to valine inhibition of the AHAS regulatory subunit. Overall, while the *leuB* Δ *leuR* Δ double mutant is normally a leucine auxotroph, the outcome of valine supplementation and valine feedback inhibition is an additional leaky isoleucine auxotrophy.

5.5.5 Regulation of the nitrogen assimilation gene *gdhA* by LeuB and LeuR

LeuB regulates *gdhA* expression (Polotnianka et al., 2004), and two sites of action for LeuB were previously identified in the *gdhA* promoter (Downes et al., 2013). To determine whether LeuR regulates *gdhA*, we assayed extracts from mutants that contained the *gdhA-lacZ* reporter gene construct (Figure 5.9). The *leuR* Δ mutant showed similar levels of β -galactosidase to wild type. As observed previously (Downes et al., 2013), the *leuB* Δ mutant showed reduced *gdhA-lacZ* expression. The *leuB* Δ *leuR* Δ double mutant showed further reduced *gdhA-lacZ* expression compared with *leuB* Δ , indicating that LeuR acts positively to regulate *gdhA* in the absence of LeuB. Previous work revealed that LeuB acts through two different regions of the *gdhA* promoter, at the proximal CCGN₄CGG consensus binding site (-250 to -241) and a CCGN₅CGG non-consensus site (-431 to -422) (Downes et al., 2013). In that study, it was determined that there was another positive-acting factor in the absence of LeuB that acted at the proximal consensus binding site. To determine where LeuR acts strains carrying the *gdhA-lacZ* promoter deletions were constructed (Downes et al., 2013) and β -galactosidase levels in extracts from the *leuB* Δ mutant were compared with those from the *leuB* Δ *leuR* Δ double mutant (Figure 5.10). The effect of *leuR* Δ in when the proximal and non-consensus sites are intact but not the distal site in the *gdhA* promoter was additive indicating that LeuR acts through the same regions of the *gdhA* promoter as LeuB.

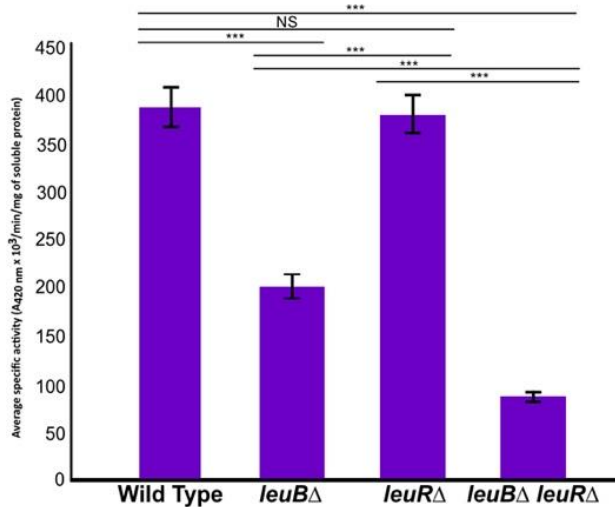


Figure 5.9 LeuB and LeuR regulate *gdhA* expression.

β -galactosidase specific activity levels expressed from the single copy *gdhA-lacZ* reporter gene (targeted at *amdS*) were assayed in soluble cell-free extracts from sixteen biological replicates of wild type (MH12101), *leuB*Δ (MH12181), *leuR*Δ (RT503), and *leuB*Δ *leuR*Δ (RT499) mycelia grown on supplemented 1% glucose minimal media with 2mM leucine and 10mM ammonium tartrate for 16h at 37°C. Error bars depict SEM (n=16). Statistical significance determined using a one-way ANOVA, as $P < 0.01$ (**), $P < 0.001$ (***), or not significant (NS).

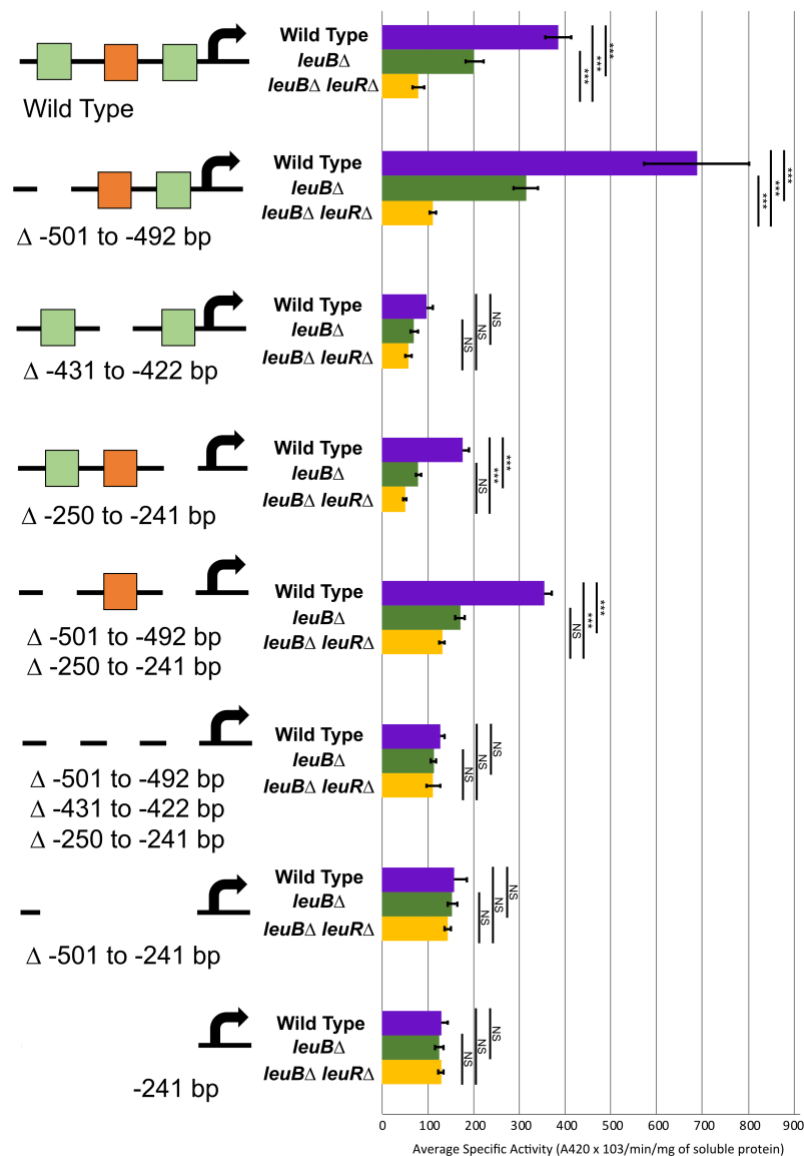


Figure 5.10 LeuR regulates *gdhA* expression through two sites of action.

β -galactosidase specific activity levels expressed from the single copy *gdhA-lacZ* reporter gene were assayed in soluble cell-free extracts from four biological replicates of wild type, *leuB*Δ, and *leuB*Δ *leuR*Δ mycelia grown on supplemented 1% glucose minimal media with 2mM leucine and 10mM ammonium tartrate for 16h at 37°C. Orange box indicates non-consensus binding sites while green boxes indicate consensus binding sites. Error bars depict SEM (n=4). Statistical significance

determined using a one-way ANOVA, as $P < 0.01$ (**), $P < 0.001$ (***), or not significant (NS).

5.5.6 α -IPM induction via LeuB, but not LeuR

Leu3p is modulated from repressor to activator by the concentration of α -IPM (Sze et al., 1992). Previous work in *A. nidulans* showed that α -IPM modulated expression of *gdhA-lacZ* (Downes et al., 2013). In the absence of knowledge that LeuR existed, it was assumed that α -IPM modulation occurred via LeuB. However, it is possible that the observed effect of α -IPM may occur via LeuR or both transcription factors. To determine whether α -IPM modulation occurs via LeuB or LeuR, we crossed the *leuB* Δ and *leuR* Δ mutants with the α -IPM isomerase point mutant, *luA1*, and α -IPM synthetase mutant, *leuC* Δ , to generate double mutants affected by one transcription factor gene and each metabolic gene (Cavalieri et al., 1999; Downes et al., 2014b; Polotnianka et al., 2004). Progeny were first scored for leucine auxotrophy for the *luA1* or *leuC* Δ mutation and then confirmed by PCR for *leuB* Δ or *leuR* Δ . The *leuR* Δ *luA1* mutant showed increased *gdhA-lacZ* expression due to higher levels of α -IPM compared with the *leuR* Δ and *leuR* Δ *leuC* Δ mutants. Therefore, α -IPM modulates LeuB activity. However, *gdhA-lacZ* expression in the *leuB* Δ mutants were unaffected by the loss of *luA1* or *leuC* Δ (Figure 5.11). This shows that, in contrast to Leu3p and LeuB, LeuR activity is not modulated by α -IPM concentrations.

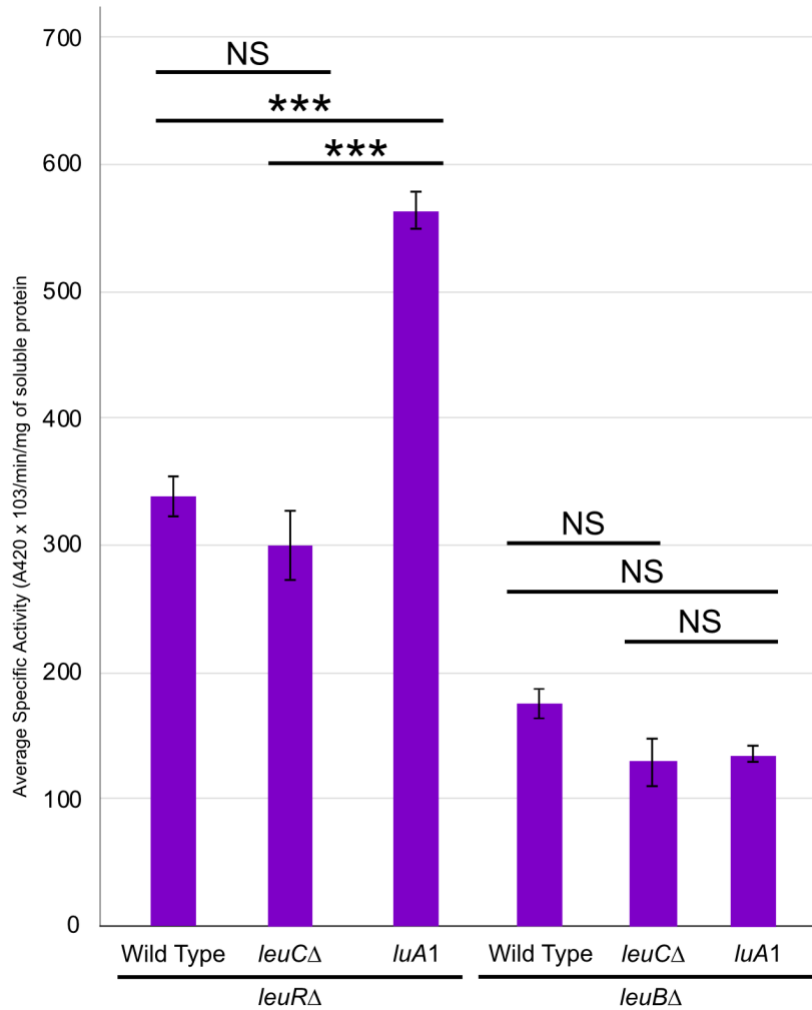


Figure 5.11 LeuB, but not LeuR, is modulated by α -IPM concentration to regulate *gdhA* expression.

β -galactosidase specific activity levels expressed from the single copy *gdhA-lacZ* reporter gene (targeted at *amdS*) were assayed in soluble cell-free extracts from four biological replicates of *leuBΔ* (MH12181), *leuRΔ* (RT499), *leuCΔ leuRΔ* (RT899), *luA1 leuRΔ* (RT877), *leuCΔ leuBΔ* (RT895), and *luA1 leuBΔ* (RT880) mycelia grown on supplemented 1% glucose minimal media with 2mM leucine and 10mM ammonium tartrate for 16h at 37°C. Error bars depict SEM (n=4). Statistical significance determined using a one-way ANOVA, as $P < 0.001$ (***), or not significant (NS).

5.5.7 Regulation of iron homeostasis by LeuB and LeuR

The LeuB ortholog in *Aspergillus fumigatus* has been shown to regulate iron homeostasis genes in addition to leucine biosynthesis genes and *gdhA*. In *A. fumigatus*, the *leuB* Δ mutants are sensitive to iron starvation but are unaffected by iron excess (Long et al., 2018; Orasch et al., 2019). To investigate whether the *A. nidulans* LeuB paralogs were also involved in iron homeostasis, the *leuB* Δ , *leuR* Δ , *AN7596* Δ , *leuB* Δ *leuR* Δ , *leuB* Δ *AN7596* Δ , *leuR* Δ *AN7596* Δ , and *leuB* Δ *leuR* Δ *AN7596* Δ mutants were growth tested under iron starved, iron replete, and iron excess conditions. Iron starvation media was made by addition of the iron chelator Bathophenanthroline disulfonic acid (BPS). Iron excess was obtained by adding 5mM or 10 mM Ferrous Sulfate to the iron replete media. The *leuB* Δ *leuR* Δ and *leuB* Δ *leuR* Δ *AN7596* Δ mutants showed reduced growth on iron starvation conditions compared with other mutants and wild type, indicating that both LeuB and LeuR have roles in regulating iron homeostasis during iron starvation. During iron excess, the *leuB* Δ mutants had reduced growth compared with the other mutants (Figure 5.10). Therefore, LeuB is involved in maintaining iron homeostasis during both iron starvation and excess conditions while LeuR is involved only in iron starvation. Additionally, we conclude that AN7596 is dispensable for maintaining iron homeostasis.

Another aspect of iron homeostasis is the regulation of siderophore production. Siderophores are low-molecular mass iron chelators that are produced and secreted to obtain iron from the environment (Haas, 2003). *A. nidulans* produces intracellular siderophore, ferricrocin, and extracellular siderophores, fusarine C and triacetylfusarine C (Haas, 2003; Charlang et al., 1981; Oberegger et al., 2001). Siderophore production has been shown to be necessary for virulence in *A. fumigatus* infection (Moore, 2013; Schrettl et al., 2004; Schrettl et al., 2007). As iron can also be toxic at high concentrations, iron acquisition is a highly controlled process. We assessed the *leuB* Δ , *leuR* Δ , *AN7596* Δ , *leuB* Δ *leuR* Δ , *leuB* Δ *AN7596* Δ , *leuR* Δ *AN7596* Δ , and *leuB* Δ *leuR* Δ *AN7596* Δ mutants using an O-CAS assay to understand if loss of one or more of the transcription factors affected siderophore regulation (Pérez-Miranda et al., 2007; Schwyn and Neilands, 1987) (Figure 5.12). Chrome

Azurol-S (CAS) is converted from blue to orange when exposed to extracellular hydroxamate siderophores, such as the TAFC siderophore from *A. nidulans*. The wild type shows a tight orange ring around the edge of the colony while the rest of the media is blue. This shows that the colony has excreted siderophores to scavenge iron from the media but siderophore production and excretion is tightly regulated. When compared to wild type, loss of either *leuB* Δ , *leuR* Δ , or *AN7596* Δ causes a dysregulation of siderophore production and excretion, as seen by the more diffuse orange ring around the edge of the colony and less blue media observed. We conclude that LeuB, LeuR, and AN7596 all participate in the regulation of siderophore production to some capacity.

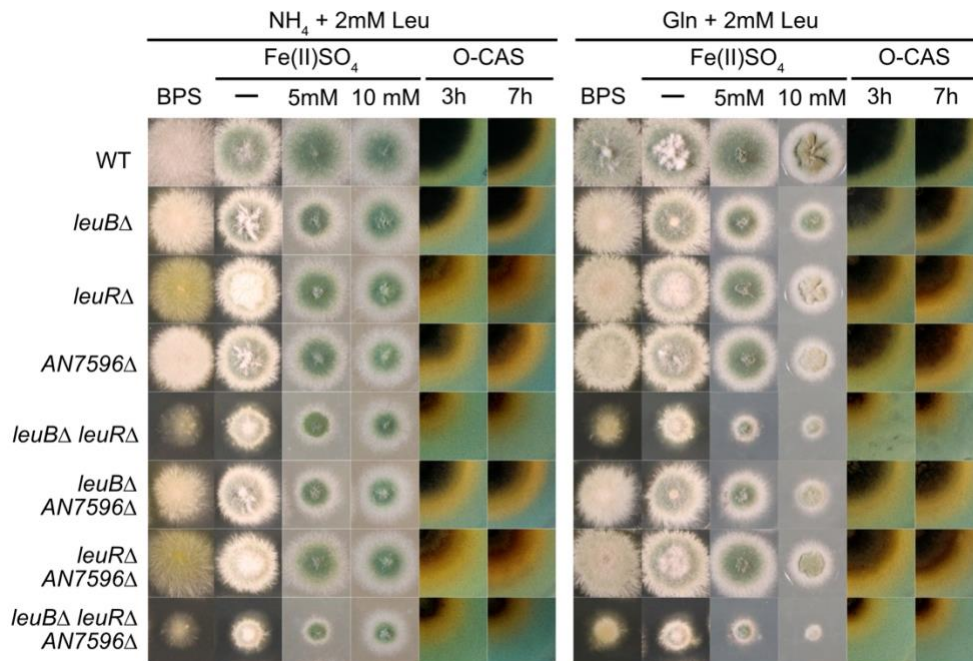


Figure 5.12 Growth analysis of *leuB*Δ, *leuR*Δ, and *AN7596*Δ mutants on different iron conditions.

Wild type (MH1), *leuB*Δ (RT452), *leuR*Δ (RT478), *AN7596*Δ (RT873), *leuB*Δ *leuR*Δ (RT500), *leuB*Δ *AN7596*Δ (RT884), *leuR*Δ *AN7596*Δ (RT885), and *leuB*Δ *leuR*Δ *AN7596*Δ (RT888) were grown at 37°C for 2 d on solid supplemented ANM with either 10 mM ammonium (NH₄) or Glutamine (Gln) as the nitrogen source with 2mM Leucine (Leu) for supplementation. Bathophenanthroline disulfonic acid (BPS) is an iron chelator, causing iron starvation conditions when added. Iron replete minimal media (-) contains 6μM Fe(III)PO₄. 5mM and 10mM exogenous ferrous sulfate was added to minimal media to generate iron excess conditions. Strains were grown 37°C for 5 d on solid supplemented ANM with either 10 mM ammonium (NH₄) or Glutamine (Gln) as the nitrogen source with 2mM Leucine (Leu) for supplementation before overlayed with O-CAS agar. Plates were photographed at 3h and 7h following overlay with one fourth of the colony edge shown.

5.5.8 Genome-wide Regulation by LeuR and LeuB

To investigate the genome-wide gene targets of LeuB and LeuR, we prepared RNA from wild type, and the *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants for RNA-Seq analysis. We also wanted to determine which steps of the leucine biosynthesis pathway were regulated specifically by LeuB and LeuR. The *leuB* Δ *leuR* Δ mutant is a leucine auxotroph but exogenous leucine is also a repressor of the leucine biosynthesis pathway. All samples were grown for 16h with leucine to supplement the auxotrophy and then the mycelia was harvested, washed and transferred for 4h to media without leucine where the leucine biosynthetic pathway would be active. At a genome wide level in comparison to wild type without leucine supplementation, 1,865 genes were significantly differentially expressed in the *leuB* Δ mutant while 3,042 genes were significantly differentially expressed in the *leuR* Δ mutant in comparison to wild type (Figure 5.13A). 1,073 gene targets, including *gdhA* and many of the same enzymes of the leucine biosynthesis pathway (Table 5.5), were differentially expressed in both mutants (Figure 5.13A). Looking at the upregulated genes in comparison to wild type, 445 unique gene targets were observed in the *leuB* Δ mutant and 1,083 unique gene targets were observed in the *leuR* Δ mutant (Figure 5.13B). They shared 603 gene targets. For downregulated genes, 362 unique gene targets were observed in the *leuB* Δ mutant, 901 unique gene targets were observed in the *leuR* Δ mutant, and they shared 455 gene targets (Figure 5.13C). In the *leuB* Δ *leuR* Δ double mutant a total of 2,953 genes were significantly differentially expressed compared with wild type, with 1,517 genes down-regulated and 1,436 genes up-regulated. There are shared and unique gene targets between the *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants for total, upregulated, and downregulated genes (Figure 5.13).

Table 5.5 Fold change of leucine biosynthesis, iron metabolism, and nitrogen assimilation genes.

	<i>leuB</i> Δ	<i>leuR</i> Δ	<i>leuB</i> Δ <i>leuR</i> Δ
Leucine Biosynthesis Genes			
<i>luA</i>			-1.29
<i>leuC</i>	-0.44		0.40
<i>leuD</i>	-0.94	-0.39	-1.50
<i>leuE</i>			
<i>batA</i>	-0.45	-0.60	0.58
<i>batB</i>	-0.84	-0.89	2.27
<i>batC</i>			
<i>batD</i>			
<i>batE</i>	-1.54		-2.02
<i>batF</i>			
<i>leuB</i>	N/A		N/A
<i>leuR</i>		N/A	N/A
AN7596			
<i>cpcA</i>		0.19	0.68
AN4430	-0.38	-0.55	0.65
AN4956	-0.38		
AN6526	-1.67	-1.58	-1.49
AN3865	-0.27		
AN3702	-0.36		0.51
AN10393	-0.20		
AN0705			1.44
AN10195			1.12

Iron Metabolic Genes			
<i>hapX</i>	-0.75		
<i>sidA</i>	-0.74		-1.19
<i>sidC</i>	-0.10		-0.21
<i>sidD</i>	-1.04	-0.57	-1.39
<i>sidF</i>	-1.24	-0.83	-1.51
<i>sidG</i>	-0.17	-0.18	-0.41
<i>sidH</i>	-1.17	-0.91	-1.29
<i>sreA</i>			
<i>mirA</i>			-1.75
<i>mirB</i>	-0.81	-0.65	-2.74
<i>mirC</i>			-0.51
<i>amc-1</i>			
<i>npgA</i>			

Nitrogen Assimilation Genes			
<i>gdhA</i>	-1.54	-0.37	-2.46
<i>gdhB</i>		0.74	
<i>glnA</i>	0.23		-0.23
<i>gltA</i>	-1.07		

Nitrogen Regulation			
<i>areA</i>		1.04	
<i>areB</i>	-0.24	-0.52	
<i>nmrA</i>	-0.41		-0.59
<i>tamA</i>		0.42	
<i>xprG</i>	0.84	0.89	
<i>pnmB</i>	0.38	0.51	-1.28

Genes with significantly increased (green) and decreased (orange) expression in wild type as compared to mutant strains using corrected shrunk log2 fold change values. Mycelia were grown for 16h at 37°C on ammonium-minimal media with 2mM leucine, then transferred to media lacking leucine for 4h.

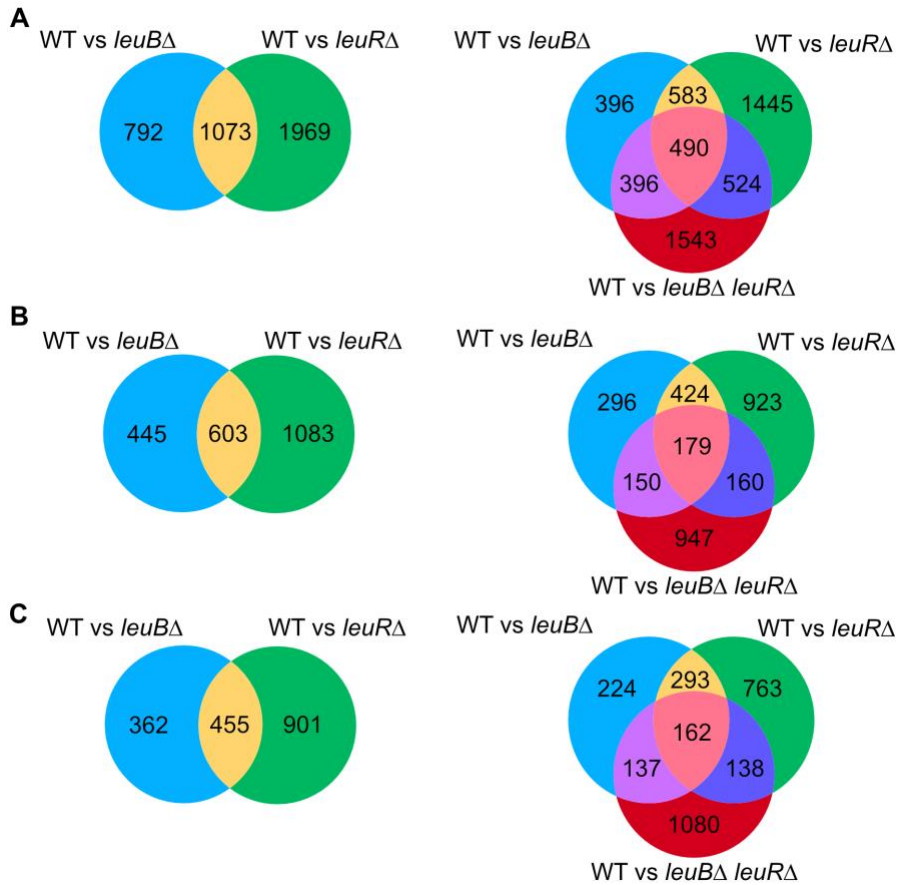


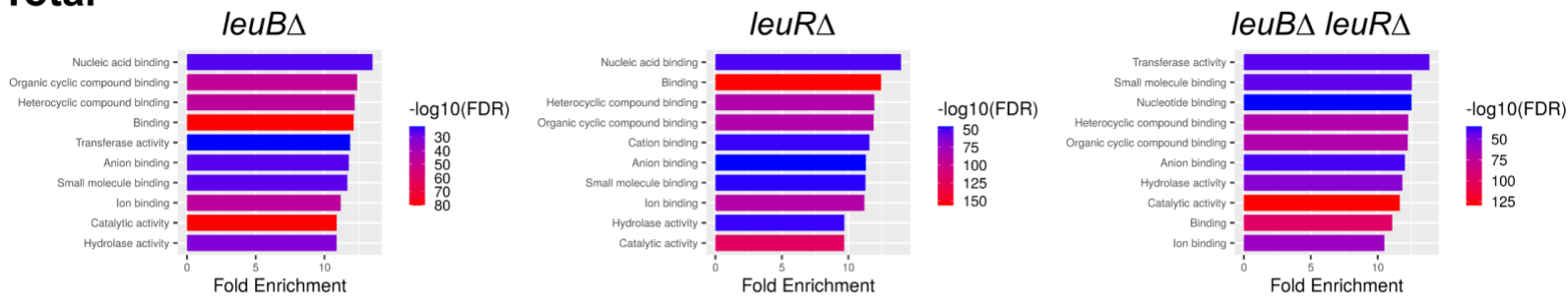
Figure 5.13 Venn Diagrams of shared and unique differentially expressed genes in the *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants in comparison to wild type.

Venn diagrams depict **A**) overall shared and unique differentially expressed genes in *leuB* Δ and *leuR* Δ mutants compared with wild type on the left followed by *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants in comparison to wild type on the right **B**) shared and unique upregulated genes in *leuB* Δ and *leuR* Δ mutants compared with wild type followed by *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants in comparison to wild type on the right, and **C**) shared and unique down-regulated genes in *leuB* Δ and *leuR* Δ mutants compared with wild type followed by *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants in comparison to wild type on the right.

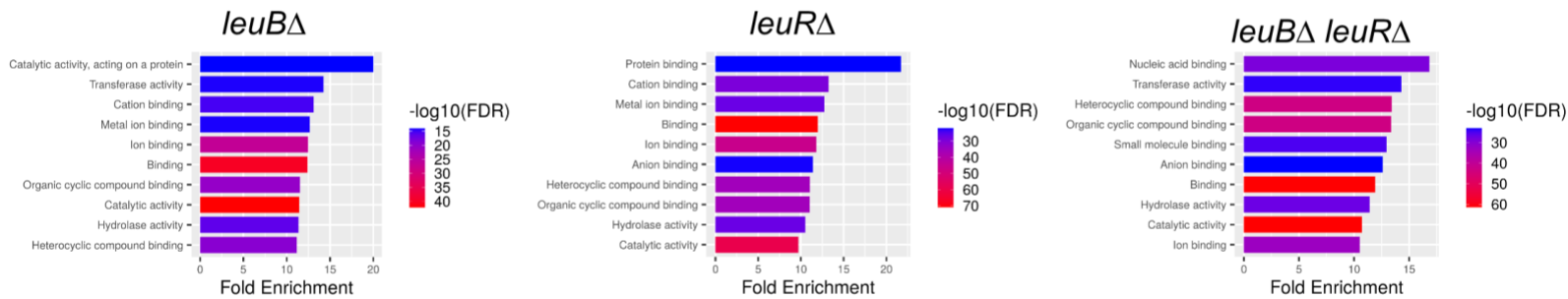
We analyzed the total, up-regulated, and down-regulated differentially expressed genes based on molecular function and biological process gene ontology (GO) terms (Figure 5.14). The up-regulated genes and down-regulated genes indicate where LeuB and LeuR would normally act directly or indirectly as repressors and activators, respectively. In the molecular function analysis, *leuB* Δ and *leuR* Δ regulated genes categorized within many 'binding' GO terms, which include various types of enzymes or metabolic processes (10.9-13.5-fold and 9.7-14.0-fold enrichment, respectively) (Figure 5.14A). LeuB represses genes involved directly with catalytic steps, as seen in the 'catalytic activity, acting on a protein', 'transferase activity', and 'hydrolase activity' (11.2-20-fold enrichment). LeuR represses genes involved with binding of compounds and ions (9.7-21.7-fold enrichment). In the down-regulated genes, the top hit for LeuB is translation initiation factor activity (67.5-fold enrichment). The LeuR down-regulated genes are involved with binding as well, but the GO terms are nucleotide binding instead of compound or ion binding (9.7-27.5-fold enrichment).

A
Total

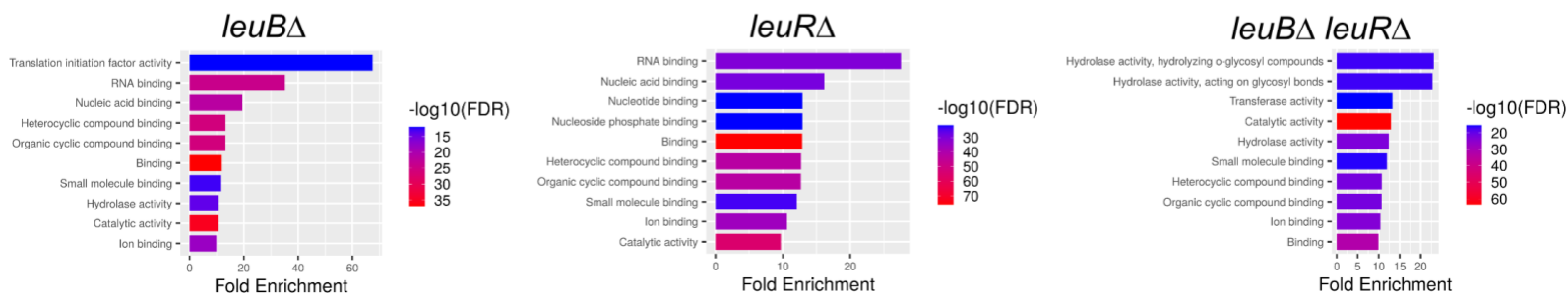
Molecular Function



Up Regulated



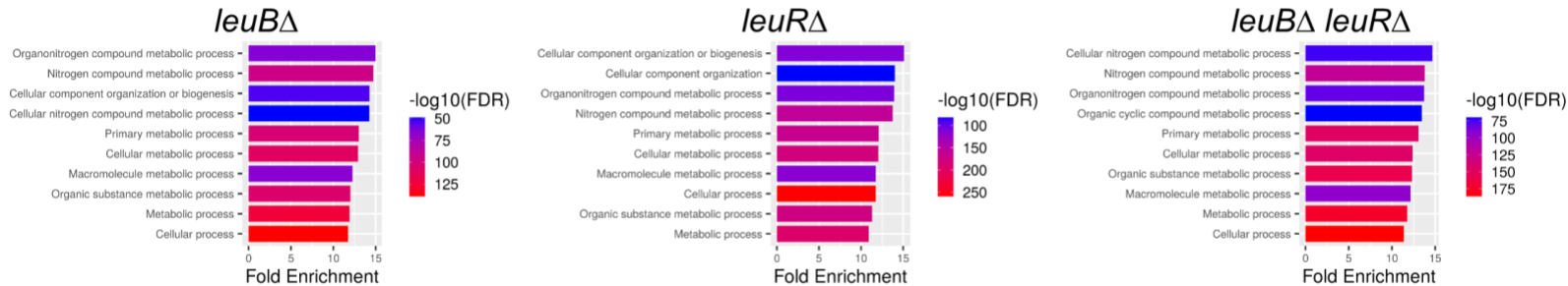
Down Regulated



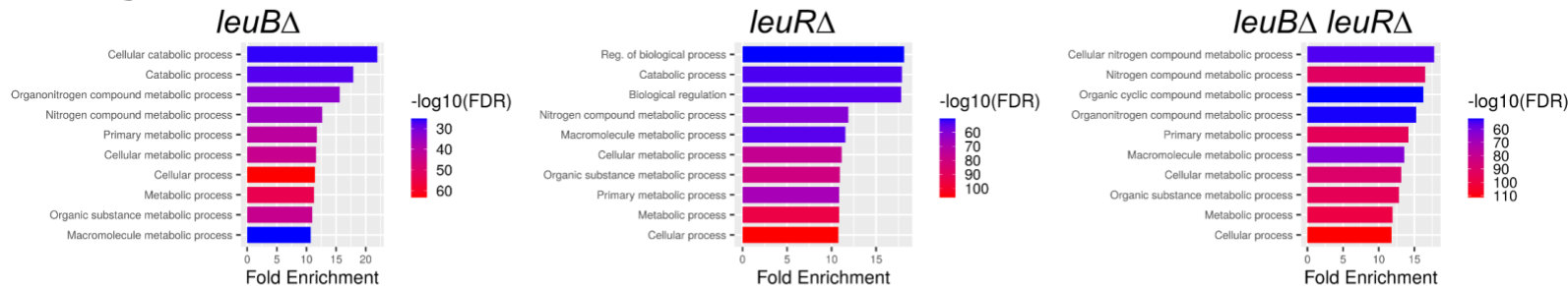
B

Total

Biological Process



Up Regulated



Down Regulated

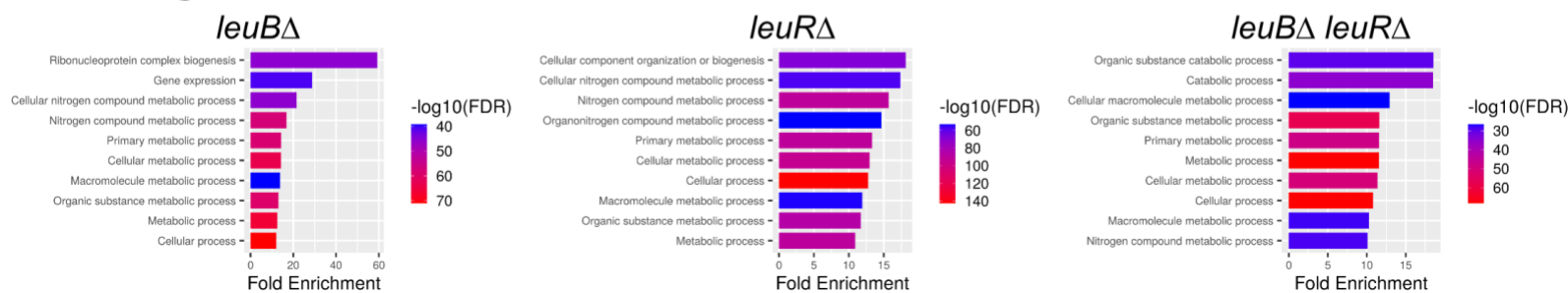


Figure 5.14 GO Analysis of differentially expressed genes in *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants compared with wild type.

Gene Ontology Analysis of significant differentially expressed genes in the *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ mutants compared with WT. Panels **A)** Molecular Function and **B)** Biological Process were generated with total differentially expressed genes, upregulated genes, and down regulated genes, as shown. Category Names are shown on the y-axis, fold enrichment values on the x-axis, and bar colors as False Discovery Rate (FDR). Note the difference in the fold enrichment scales.

Analysis of the GO terms for biological process for total differentially expressed genes reveals that nine of the top ten terms, which are metabolic process terms, are shared between the *leuB* Δ and *leuR* Δ mutants, with 'Cellular nitrogen compound metabolic process' unique for *leuB* Δ and 'Cellular component organization' unique for *leuR* Δ . Analysis of the GO terms for biological process for down-regulated genes, reveals that both *leuB* Δ and *leuR* Δ mutants share eight of the top ten GO terms and regulate genes categorized in multiple metabolic processes GO terms (~12.1-59.3-fold and 10.9-18.2-fold enrichment, respectively) (Figure 5.14B). The two biological process GO terms unique to *leuB* Δ are 'Ribonucleoprotein complex biogenesis' (59.3-fold enrichment) and 'Gene expression' (28.7-fold enrichment), which show the greatest enrichment, a level of enrichment greater than for any of the GO terms for *leuR* Δ (maximum enrichment for any class 18.2-fold). This suggests that LeuB plays a role in transcriptional activation of translation and protein biosynthesis, which is not shared with LeuR. The two biological process GO terms unique to *leuR* Δ are 'Cellular component organization or biogenesis' and 'Organonitrogen compound metabolic process'. The latter term is found in the *leuB* Δ up-regulated list, suggesting that LeuB and LeuR may play opposing roles in regulation of this biological process. The former biological process GO term 'Cellular component organization or biogenesis' is, however, shared with similar levels of enrichment for *leuB* Δ and *leuR* Δ mutants in their total differentially expressed gene GO analysis.

We next analyzed the top down-regulated and up-regulated genes to determine common gene targets and potential metabolic pathways each of the transcription factors regulate. About half of the ten most down-regulated genes were shared between *leuB* Δ and *leuR* Δ (AN7331, AN11015, AN6525, AN5918, and AN7764). These genes have a range of functions including cyanate hydratase (AN7331), formate dehydrogenase *aciA* (AN6525), a catalase gene *catC* (AN5918), among other metabolic functions. Comparing *leuB* Δ to the *leuB* Δ *leuR* Δ , there was only one gene shared in the top ten down regulated genes, AN1566, which is currently uncharacterized (Table 5.6). When we analyzed the ten most up-regulated genes of each of the mutants, we found that the top ten of each mutant were unique (Table 5.7). We conclude that while LeuB and LeuR have redundancy in what genes and

processes are regulated, there is strong evidence for divergence in the gene targets for each.

Table 5.6 Top ten differentially expressed down regulated genes.

Top Down Regulated Genes						
	<i>leuB</i> Δ		<i>leuR</i> Δ		<i>leuB</i> Δ <i>leuR</i> Δ	
1	AN1566	-4.1158519	AN7764	-4.3021264	AN3520	-7.07193
2	AN7764	-4.0952344	AN7331	-3.6154907	AN12477	-6.75007
3	AN6525	-3.8237269	AN6525	-3.5788798	AN7867	-6.1045804
4	AN7331	-3.6812377	AN11015	-3.219711	AN9444	-6.080645
5	AN8063	-3.4014266	AN5918	-2.3058167	AN4111	-5.5857725
6	AN7382	-3.1400526	AN8782	-2.0358658	AN0404	-5.5105033
7	AN11015	-3.0161917	AN6526	-1.5832093	AN10402	-5.408087
8	AN1730	-3.0005507	AN7953	-1.4383006	AN7392	-5.348803
9	AN2077	-2.8516998	AN9474	-1.4351572	AN9348	-5.319801
10	AN5918	-2.6448674	AN8099	-1.3910317	AN1566	-5.3139873

Corrected shrunk fold change of top ten down-regulated genes in wild type as compared to each mutant. Mycelia were grown for 16h at 37°C on ammonium-minimal media with 2mM leucine, then transferred to media lacking leucine for 4h. Highlighted genes indicated shared between two of the mutants.

Table 5.7 Top ten differentially expressed up regulated genes.

Top Up Regulated Genes						
	<i>leuB</i> Δ		<i>leuR</i> Δ		<i>leuB</i> Δ <i>leuR</i> Δ	
1	AN3200	3.07239437	AN8900	3.4513652	AN5453	8.074599
2	AN3305	2.0547359	AN4180	3.3943164	AN0903	6.7911315
3	AN0670	2.0244689	AN7348	2.2959588	AN10958	5.524486
4	AN8980	1.85953033	AN3491	2.0069299	AN2038	5.502272
5	AN5083	1.85753226	AN11725	1.5832082	AN3970	5.347991
6	AN8986	1.51794112	AN10288	1.5671064	AN9131	5.2572184
7	AN6227	1.50035012	AN12239	1.5430802	AN10432	5.1279755
8	AN4914	1.40958607	AN9510	1.3709551	AN2349	5.0889287
9	AN7762	1.39002764	AN1547	1.3007883	AN9108	5.0380464
10	AN3585	1.38101029	AN6523	1.2146289	AN4136	4.669564

Corrected shrunk fold change of top ten up-regulated genes in wild type as compared to each mutant. Mycelia were grown for 16h at 37°C on ammonium-minimal media with 2mM leucine, then transferred to media lacking leucine for 4h.

The RNA-seq data was used to assess regulation by LeuB and LeuR of genes comprising the BCAA biosynthetic pathway and involved in iron metabolism, nitrogen assimilation and nitrogen regulation. Both LeuB and LeuR, based on comparison of each single mutant with wild type, regulated the acetohydroxy acid synthase regulatory subunit AN4430, the acetohydroxy acid reductoisomerase AN2526, the major β -IPM dehydrogenase *leuD* and the two major branched chain amino acid aminotransferase (BAT) genes *batA* and *batB* (Figure 5.14, Table 5.5). LeuB regulates the α -IPM synthetase *leuC* and a third BAT enzyme *batE* (Figure 5.15, Table 5.5). LeuB also regulated the putative leucyl-tRNA ligases (leucine-tRNA synthetases) AN6526, AN3865, AN3702, and the putative isoleucine-tRNA synthetase AN10393 (Table 5.5). In the *leuB* Δ *leuR* Δ double mutant, *luA* is downregulated but downregulation of *luA* is not observed in either of the single mutants. *batE* expression was reduced in the *leuB* Δ mutant, but not the *leuR* Δ mutant, and was further reduced in the double mutant. Additionally, *leuD* expression is lower in the double mutant than either single mutant and surprisingly *leuC* and *batA* and *batB* are up regulated in the double mutant. This may indicate that LeuB and LeuR are redundant in regulating *luA* and *batE*, and additive in regulating *leuD*. For the genes that are upregulated in the double mutant, an additional transcription factor may regulate those genes in the absence of LeuB and LeuR. The *leuB* Δ *leuR* Δ mutant also revealed regulation of the putative isoleucine-tRNA synthetase AN0705 and valine-tRNA synthetase AN10195. LeuB and LeuR both also regulate multiple steps in the intracellular and extracellular iron siderophore biosynthesis pathway in the *leuB* Δ and *leuR* Δ mutants (Figure 5.16). For the siderophore gene *sidL*, expression is unaltered in either of the single mutants but lowered in the double mutant. Additionally, they both regulate several major nitrogen assimilation genes, including *gdhA*, and transcriptional regulators of nitrogen metabolism (Table 5.5). LeuB and LeuR are also independently regulated, meaning the loss of one does not affect the expression of the other (Table 5.5). Since these transcription factors are redundant in many metabolic processes and for many gene targets, they may compensate for each other when one of them is absent.

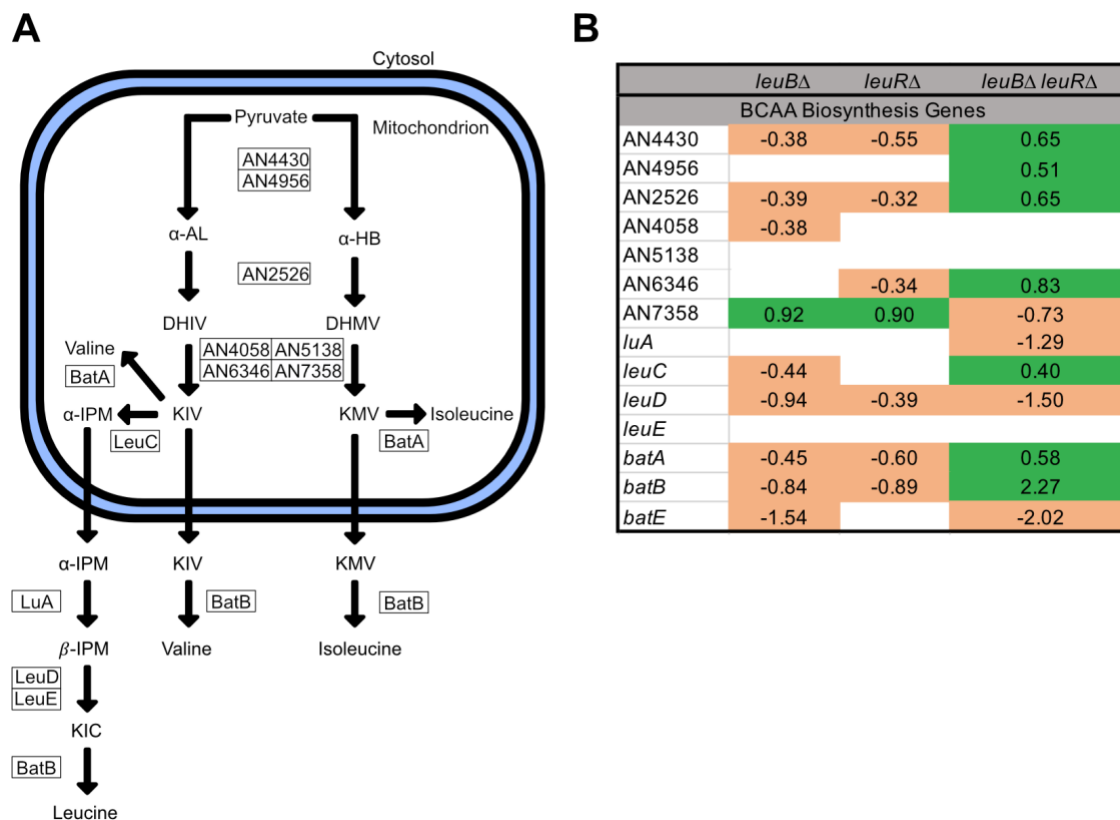


Figure 5.15 Visualization of the regulation of the BCAA pathway by LeuB and LeuR.

A) Extended BCAA biosynthesis pathway starting at pyruvate and ending with either isoleucine, leucine, or valine. The *A. nidulans* genes encoding the enzymes for each step are listed. **B)** Fold change values of LeuB and LeuR regulation of the extended BCAA biosynthesis pathway. All values are $p < 0.05$.

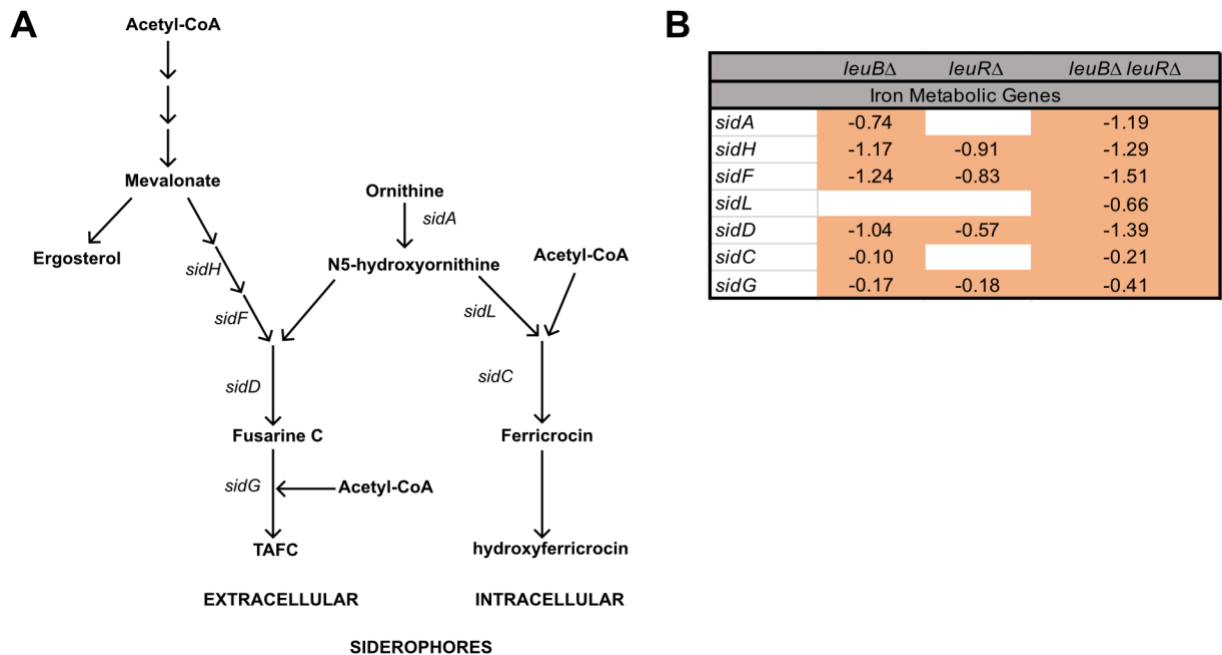


Figure 5.16 Visualization of the regulation of the iron siderophore pathway by LeuB and LeuR.

A) Siderophore biosynthesis pathway starting at acetyl-CoA. The *A. nidulans* genes encoding the enzymes for each step are listed. **B)** Fold change values of LeuB and LeuR regulation of the extended BCAA biosynthesis pathway. All values are $p < 0.05$.

5.6 Discussion

This study has characterized the role of the Zn(II)2Cys6 transcription factors LeuB, LeuR, and AN7596 in the regulation of leucine biosynthesis genes, the NADP-dependent glutamate dehydrogenase gene *gdhA*, and iron metabolism genes. Sequence analysis of LeuR revealed strong similarity to LeuB, particularly in the DNA-binding domain. The *leuB* Δ mutant is a leaky leucine auxotroph that is rescued by exogenous leucine (Polotnianska et al., 2004). Deletion of *leuR* conferred no obvious growth defects in the absence or presence of leucine. However, the *leuB* Δ *leuR* Δ double mutant is a tight leucine auxotroph, indicating that LeuB and LeuR have redundant functions in regulating leucine biosynthesis genes. Additionally, the *leuB* Δ *AN7596* Δ double mutant is a slightly tighter leucine auxotroph than the *leuB* Δ single mutant, indicating that all three transcription factors have at least some redundant functions in regulating leucine biosynthesis, albeit a very minor role for AN7596. Plate testing showed that the *leuB* Δ *leuR* Δ double mutant had a reduction in colony growth size compared with the *leuB* Δ or *leuR* Δ single mutant when supplemented with leucine. One explanation is that under biosynthetic conditions leucine does not fully supplement to wild type growth size even though it does supplement for conidiation. The *leuB* Δ *AN7596* Δ , *leuR* Δ *AN7596* Δ , and *leuB* Δ *leuR* Δ *AN7596* Δ mutants also had a reduced growth phenotype, indicating that AN7596 may play a minor role in both leucine biosynthesis and catabolism.

We showed that LeuB and LeuR have shared sites-of-action in regulating *gdhA* expression. The *leuB* Δ mutant shows reduced *gdhA* expression while the *leuR* Δ mutant shows no difference from wild type. The *leuB* Δ *leuR* Δ double mutant has even greater reduction in *gdhA* expression than the single *leuB* Δ mutant, indicating that the two transcription factors show redundancy in *gdhA* regulation. Therefore, the regulation of *gdhA* by the LeuB and LeuR paralogs provides a robustness for expression of the critical nitrogen assimilation gene *gdhA* by allowing activation and nitrogen nutrients to be assimilated if either paralog is lost.

We further defined where LeuR acted in the *gdhA* promoter by testing the wildtype, *leuB* Δ , *leuR* Δ , and *leuB* Δ *leuR* Δ strains that contained deletions in the promoter of a *gdhA-lacZ* reporter gene. The promoter contained combinations of three

sequence deletions, two that lacked the consensus promoter sequence, CCGN₄CGG, and one lacking a non-consensus sequence, CCGN₅CGG, for LeuB (Downes et al., 2013). Since LeuR has strong sequence similarity to LeuB, we hypothesized that LeuR would act at the same sites as LeuB. We showed that LeuR also acts through the proximal consensus site and the non-consensus site to regulate *gdhA* in the absence of LeuB.

In *S. cerevisiae*, Leu3p activity is modulated by the leucine biosynthetic pathway intermediate α -IPM (Sze et al., 1992). Previous work showed that *gdhA-lacZ* expression was modulated in mutants with altered α -IPM levels (Downes et al., 2013), and this effect was thought to occur via LeuB. However, the existence of LeuR and its redundancy with LeuB raised the possibility that α -IPM modulation could occur via LeuR or both LeuB and LeuR. We found, using *gdhA-lacZ* reporter assays and mutations in α -IPM synthase (*leuC*) and α -IPM isomerase (*luA*) to artificially alter the α -IPM concentrations, combined with *leuB* Δ or *leuR* Δ mutations, that α -IPM modulated the activity of LeuB but not LeuR. Therefore, the functional aspect of the α -IPM masking region is not present for LeuR.

We began to characterize the role of LeuB and LeuR in iron homeostasis. Iron homeostasis is tightly regulated, as both lack and excess of iron is detrimental and iron metabolism has been shown to be important in infection and pathogenicity in numerous fungal pathogens (Chen et al., 2011; Haas, 2003; Long et al., 2018; Orasch et al., 2019; Schrettl et al., 2004; Gressler et al., 2015; López-Berges et al., 2012). Both LeuB and LeuR have redundant roles during iron starvation, as indicated by the sensitivity of *leuB* Δ *leuR* Δ strains on plate tests. Under iron excess conditions, the *leuB* Δ mutants were the most sensitive while the *leuR* Δ mutants were unaffected. The *AN7596* Δ mutant was unaffected in either iron condition. The siderophore pathway was examined, as siderophores are the main component of siderophore-mediated iron acquisition (Haas, 2003; Moore, 2013). Using an O-CAS assay, we were able to determine that loss of either LeuB, LeuR, or *AN7596* caused a dysregulation of siderophore production and excretion as visualized by the diffuse orange ring surrounding the fungal colony. In *A. fumigatus*, the *leuB* Δ mutant was unable to grow

under iron starvation conditions and had further reduction of colony growth on excess iron conditions in the *leuBΔ hapXΔ* double mutant (Long et al., 2018).

Lastly, we determined the direct or indirect gene targets of LeuB and LeuR using RNA-Seq under leucine biosynthesis conditions to provide an overall picture of the physiology regulated by these two transcription factors. Chromatin immunoprecipitation of the Leu3p transcription factor shows that Leu3p regulates gene expression directly through the CCGN₄CGG DNA motif and indirectly through other transcription factor binding partners (Liu et al., 2006; Tang et al., 2006). LeuB and LeuR regulate many of the same genes within the leucine biosynthesis pathway, but also some genes known to encode enzymes in the nitrogen assimilation pathway and iron metabolic pathway. However, there is divergence in the gene targets between LeuB and LeuR, which could be attributed to the neofunctionalization of LeuB and LeuR. LeuB had been shown to regulate each step of the leucine biosynthesis pathway (Steyer et al., 2021). We now show that LeuR also regulates the final steps of the leucine biosynthesis pathway, the β -IPM dehydrogenase *leuD*, and the branched chain amino acid aminotransferase genes *batA* and *batB*. LeuB, but not LeuR, cross-regulates the iron metabolism transcription factor HapX (Gsaller et al., 2014; Schrettl et al., 2010). LeuB and LeuR may compensate for the loss of the other in regulating gene expression of shared genes targets. Genes with lowered expression in one of single mutant and the double mutant may indicate that there is compensation by the remaining transcription factor. This was observed for genes such as *luA*, *batE*, *sidA*, and *sidL* where, compared with wild type, the *leuRΔ* mutant has no difference in gene expression, but the double mutant has a lowered gene expression than the *leuBΔ* single mutant. Other compensation mechanisms are observed for some genes where expression is reduced in the single mutants but up-regulated in the absence of both LeuB and LeuR and may include the general amino acid transcription factor CpcA, the Gcn4 ortholog, which activates genes in response to amino acid starvation (Hoffmann et al., 2001; Magee and Robichon-Szulmajster, 1968; Wanke et al., 1997; Xiao and Rank, 1988).

GO analysis provided insight into the regulatory roles of LeuB and LeuR across the genome. The molecular function GO terms showed that both LeuB and LeuR

regulated genes involved with catalytic or binding activity while the biological process GO terms showed that they were involved in many types of metabolic process. An interesting result was that both transcription factors are involved in cellular component organization or biogenesis and LeuB specifically in ribonucleoprotein complex biogenesis. These terms are broadly defined as “a process that results in the biosynthesis of constituent macromolecules, assembly, or arrangement of constituent parts”. This would indicate that LeuB and LeuR have a significant role in regulating translation, specifically where the amino acids are ligated to their respective tRNAs for incorporation into growing peptide chains.

The lack of leucine biosynthesis in mammals and low iron availability within mammalian hosts, make LeuB and LeuR strong candidates for anti-fungal targets as they are a junction between two essential metabolic processes in fungal pathogenesis (Do et al., 2015; Kingsbury et al., 2004; Kingsbury and McCusker, 2010; Long et al., 2018; Oliver et al., 2012; Orasch et al., 2019). Additionally, LeuB and LeuR regulate nitrogen assimilation, a necessary component for fungal growth (Downes et al., 2013; Polotnianka et al., 2004). This study indicates that targeting both transcription factors would prevent expression of crucial metabolic pathways in infection and disease.

Overall, this work has characterized the redundancy and divergence of the *A. nidulans* transcription factors LeuB, LeuR, and AN7596 (Figure 5.17). All three transcription factors regulate leucine biosynthesis and siderophore regulation. LeuB and LeuR both regulate *gdhA* regulation. Yet, they diverge in the gene targets that they regulate within the leucine and iron metabolic pathways. LeuB and LeuR also have more unique gene targets than shared gene targets and GO analysis reveals that they regulate similar biological functions and metabolic processes. Redundancy of the transcription factors allows for a more robust gene expression regulation but divergence between LeuB and LeuR gene targets indicates that they have undergone a level of neofunctionalization in *A. nidulans*.

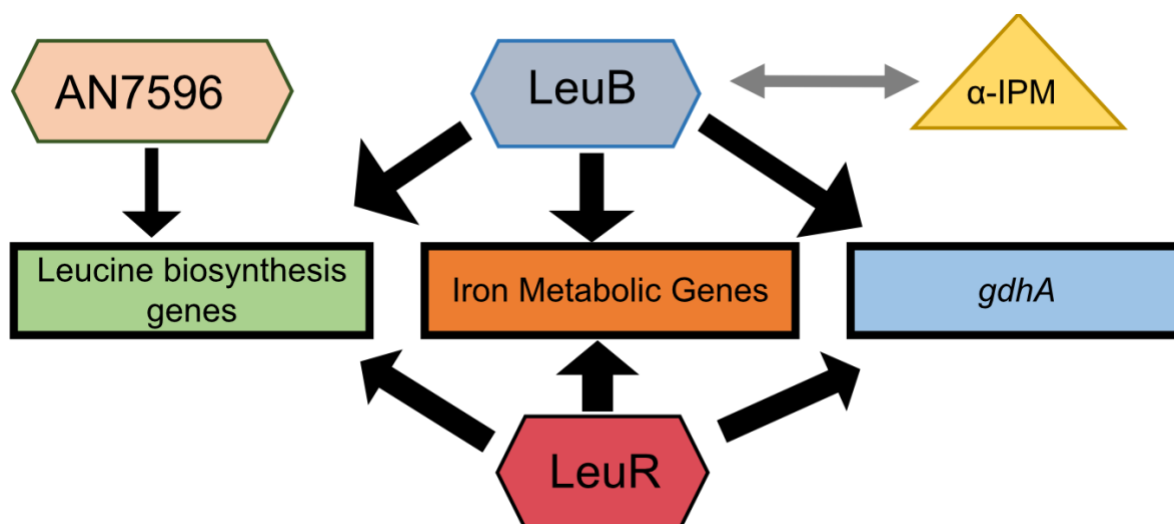


Figure 5.17 Model of the three paralogous transcription factors LeuB, LeuR, and AN7596.

Visualization of the relationship between LeuB, LeuR, and AN7596 in regulating leucine biosynthesis genes, iron metabolic genes, and the nitrogen assimilation gene *gdhA*.

5.7 Future Directions

This research has characterized the roles of LeuB, LeuR, and AN7596 in leucine biosynthesis, nitrogen assimilation, and iron metabolism but there is much remaining to be discovered. We have not determined a phenotype for the *leuR*Δ mutant or the *AN7596*Δ mutant. Continued analysis of the *leuR*Δ RNA-Seq data may identify pathways regulated by LeuR and not LeuB, and this information may be used to develop plate tests that might reveal a growth phenotype. Additionally, while we have found a minor role for AN7596 in leucine biosynthesis, it is unclear which steps AN7596 regulates and to what extent. Other aspects of future work would be to understand the interplay between LeuB, LeuR, and the iron homeostasis regulator HapX by generating *hapX*Δ *leuB*Δ, *hapX*Δ *leuR*Δ, and *hapX*Δ *leuB*Δ *leuR*Δ mutants and plate test on different iron concentrations to determine auxotrophy as the *leuB*Δ single mutant is a leaky auxotroph and *leuR*Δ mutant is a prototroph. Finally, it would be important to understand how LeuB and LeuR interact with each other and other transcription factors to regulate metabolic processes. While outside the scope of this

current research, it would be beneficial to know if LeuB and LeuR heterodimerize. We could then determine if a LeuB-LeuB homodimer, LeuR-LeuR homodimer, or a LeuB-LeuR heterodimer regulate the same gene targets or if different combinations cause up or down regulation of these processes.

Chapter 6 - Discussion and Future Directions

6.1 Dissertation Discussion

Fungi, plants, and bacteria can produce the three branched chain amino acids (BCAAs), isoleucine, leucine, and valine de novo, while mammals obtain these amino acids from their diets. The BCAAs share the first steps in biosynthesis but diverge later for amino acid specific steps. The yeast *Saccharomyces cerevisiae* has a well characterized BCAA biosynthesis pathway and the transcription factor, Leu3, has been shown to regulate the BCAA biosynthesis pathway (Kohlhaw, 2003). In this work, we characterized the branched chain amino acid (BCAA) biosynthetic pathway in the model eukaryotic filamentous fungus *Aspergillus nidulans*, focusing on the final step of the pathway encoded by the branched chain amino acid aminotransferase (BAT) genes. We also characterized the Leu3 orthologous transcription factors LeuB and LeuR and show that they both regulate leucine biosynthesis, nitrogen metabolism, and iron homeostasis. However, we show that there is divergence in what genes they regulate in those within those metabolic processes, the overall role they play, and in the total number of genes that they regulate across the genome.

In Chapter 3, the final two steps of the leucine biosynthesis pathway were characterized, with emphasis on the BCAA aminotransferase (BAT) genes, the final step shared for biosynthesis of all three BCAAs. *A. nidulans* encodes six putative *bat* genes. This work showed that *batA* and *batB* are the primary BAT genes, and one or other of these two genes is necessary for BCAA biosynthesis. Although loss of either *batA* or *batB* did not cause BCAA auxotrophy, loss of both genes conferred tight BCAA auxotrophy (Section 3.5.5 and 3.5.6). This indicated that either *batA* and *batB* can functionally compensate for the loss of the other without any change in expression of the remaining one of these two *bat* genes (Section 3.5.5 and 3.5.6). Further characterization using RT-qPCR and plate testing showed that *batB* is primarily catabolic while *batA* is primarily biosynthetic. Two other BAT genes, *batE* and *batF*, did not have an apparent function in BCAA biosynthesis or catabolism. *batE* is expressed at low levels during both biosynthetic and catabolic condition but cannot compensate for the loss of *batA* and *batB* (Section 3.5.4 and 3.5.6). *batF* expression

was not detectable under any of the conditions tested. The terriquinone A biosynthetic gene cluster is located near *batF* but the full biosynthetic pathway has been determined in terriquinone A biosynthesis (Inglis et al., 2013; Baichwal et al., 1983; Bouhired et al., 2007; Kelly et al., 2009; Balibar et al., 2007). *batF* may be involved in making an alternative secondary metabolite using the *tdi* cluster or unlinked secondary metabolism genes. The last two BAT genes, *batC* and *batD*, were discovered by others to be a part of the aspercryptins biosynthetic gene cluster and named *atnH* and *atnJ*, respectively (Chiang et al., 2016; Henke et al., 2016).

Gene duplication led to genome robustness in this case in *A. nidulans* as loss of either β -IPM dehydrogenase genes (*leuD* and *leuE*) or BAT genes (*batA* and *batB*) did not cause BCAA auxotrophy (Diss et al., 2017; Escalera-Fanjul et al., 2019; Wapinski et al., 2007). Paralogous genes can also acquire new functions, known as neofunctionalization, when both are present and expressed in the genome. *batA* and *batB* may be one case of neofunctionalization as *batA* is primarily biosynthetic while *batB* is primarily catabolic but other ascomycete species, such as *Kluyveromyces lactis* that only have one BAT enzyme, do not have separate biosynthetic and catabolic roles (Colón et al., 2011). Neofunctionalization may also account for the other four BAT enzymes functioning in other metabolic processes, such as BatC and BatD in the aspercryptins biosynthetic gene cluster and BatE under hypoxia (Chiang et al., 2016; Henke et al., 2016; Shimizu et al., 2010).

In Chapter 4, spontaneous suppressor mutants of the BCAA auxotrophy of the *batA* Δ *batB* Δ double mutant were isolated, and candidate causative genes were identified using whole genome sequencing and single nucleotide polymorphism (SNP) analysis. All ten sequenced spontaneous mutants contained shared nonsynonymous SNPs compared with the parent in six genes. (Section 4.4.3). One of these genes, the uncharacterized gene AN5995 is a strong candidate for harboring the causative mutation. The predicted AN5995 protein contains an SPT2 domain sequence and has similarity with other Spt2 proteins. Spt2 proteins have been implicated in chromatin remodeling, transcriptional regulation, and genome stability (Kruger and Herskowitz, 1991; Nourani et al., 2006; Novoseler et al., 2005; Roeder et al., 1985). Spt2 also has a cyclic expression in the *S. cerevisiae* metabolic cycle, having the greatest level of

expression during the oxidative phase (Rao and Pellegrini, 2011). Considering that *batE* expression is induced under hypoxic conditions, one hypothesis is that the mutated AN5995 in the mutants is unable to repress *batE* expression during normoxic conditions. *batE* is normally expressed at low levels, but that level of expression is not enough to support growth. De-repression by AN5995 would allow *batE* to be expressed at elevated levels, potentially overcoming the BCAA auxotrophy of the *batA* Δ *batB* Δ mutant.

Also in Chapter 4, the concept of a two-speed genome was introduced (Section 4.5.2). The core region of the genome contains the essential genes and generally has a lower mutation rate than the sub-telomeric region, which contains effector proteins, secondary metabolite biosynthetic gene clusters and transposons, and has a higher mutation rate (Dong et al., 2015; Sperschneider et al., 2015; Croll and McDonald, 2012). Two-speed genomes have been shown in filamentous fungal pathogens but not in the non-pathogenic species of *A. nidulans* (Dong et al., 2015; Faino et al., 2016; Sperschneider et al., 2015; Plissonneau et al., 2018). While investigating SNPs in the spontaneous mutants, a pattern emerged in SNP distribution across the genome. Over 50% of the identified SNPs were in the sub-telomeric region of the genome that comprises about 10% of the whole genome. This indicates a significant mutation rate bias in the sub-telomeric regions of the genome of a non-pathogenic filamentous fungus.

Chapter 5 characterized the functional redundancy and divergence of the paralogous transcription factors LeuB and LeuR. The *leuB* Δ mutant is a leaky leucine auxotroph and regulates the nitrogen assimilation gene, *gdhA* (Polotnianska et al., 2004; Downes et al., 2013). Sequence searches of the *A. nidulans* genome revealed two additional transcription factors similar to *leuB*, named *leuR* and AN7596 (Section 5.4.3). LeuB was more broadly conserved in the Ascomycetes and orthologs were found in the human pathogen *Aspergillus fumigatus* and plant pathogen *Magnaporthe oryzae*. LeuB orthologs in *M. oryzae* were tight leucine auxotrophs (Que et al., 2020) and a slightly leaky leucine auxotroph in *A. fumigatus* (Long et al., 2018). We did not find a LeuR ortholog in *M. oryzae* but did identify one in *A. fumigatus*. Growth testing revealed that the *leuR* Δ and AN7596 Δ mutants were leucine prototrophs but the *leuB* Δ

leuR Δ mutant was a tight auxotroph and the *leuB* Δ *AN7596* Δ mutant was slightly tighter than the *leuB* Δ single mutant. This indicated that all three transcription factors regulated leucine biosynthesis in varying capacities, with LeuB the major regulator, LeuR playing a redundant role, and AN7596 playing only a minor role. We show that there is redundancy in leucine biosynthesis regulation in *A. nidulans* and the LeuR ortholog may explain the leaky leucine auxotrophy of the *leuB* Δ mutant in *A. fumigatus*.

Isoleucine, leucine, and valine share the first few steps of biosynthesis before divergence into amino acid specific steps. LeuB, LeuR, and AN7596 are leucine, but not isoleucine or valine, auxotrophs but regulate many of the shared and valine and isoleucine pathway-specific steps. One explanation could be that other transcription factors may regulate those pathway specific steps. CpcA is the Gcn4 ortholog in *A. nidulans* and may regulate isoleucine and valine biosynthesis in the *leuB* Δ single mutant and *leuB* Δ *leuR* Δ double mutant (Hoffmann et al., 2001; Magee and Robichon-Szulmajster, 1968; Wanke et al., 1997; Xiao and Rank, 1988). CpcA regulates amino acid biosynthesis during amino acid starvation and could be compensating for the loss of LeuB and LeuR in response to leucine starvation in the *leuB* Δ *leuR* Δ double mutant leucine auxotroph. There may also be isoleucine and valine specific transcription factors that have not been characterized yet and may not be affected by the loss of LeuB or LeuR. In addition to regulating leucine biosynthesis, LeuR regulates *gdhA* expression in the absence of LeuB (Section 5.5.4). LeuB and LeuR also act at the same sites in the *gdhA* promoter (Downes et al., 2013) (Section 5.5.5). Glutamate, the product of GdhA, provides the amino group for the BAT enzymes during BCAA biosynthesis. Cross regulation of *gdhA* and leucine biosynthesis by both LeuB and LeuR thus ensures that there is enough nitrogen being assimilated to produce isoleucine, leucine, and valine.

Here is where the functions of LeuB and LeuR started to diverge. LeuB activity is modulated by the leucine pathway intermediate α -IPM while LeuR is unaffected by α -IPM concentrations (Section 5.5.6). The *S. cerevisiae* ortholog Leu3p is also modulated by α -IPM (Brisco and Kohlhaw, 1990; Downes et al., 2013; Polotnianka et al., 2004). This means that LeuB is a component of the leucine feedback inhibition loop while LeuR is insensitive to it. LeuR could regulate the BCAA pathway when LeuB

is not active, providing a level of transcription to continue making amino acids for protein synthesis and secondary metabolite production. This insensitivity would also expand to other metabolic processes that LeuB and LeuR co-regulate, such as nitrogen assimilation through *gdhA* and iron metabolism.

The roles of LeuB and LeuR in iron metabolism are also different, where the *leuB* Δ mutants are more sensitive to iron excess conditions and the *leuB* Δ *leuR* Δ mutants were more sensitive to iron starvation (Section 5.5.7) (Long et al., 2018; Orasch et al., 2019). LeuB and LeuR have unique but overlapping transcriptomes as well. RNA-Seq confirmed that both transcription factors regulate *gdhA* and many steps of the leucine biosynthesis pathway and the iron metabolic pathway. LeuB regulated 1,833 genes while LeuR regulated 3,008 genes in comparison to wild type. They share 1,064 gene targets, including *gdhA* and many of the same enzymes of the leucine biosynthesis pathway. Looking at the upregulated genes in comparison to wild type, LeuB has 424 unique gene targets and LeuR has 1,082 unique gene targets. They shared 598 gene targets. For downregulated genes, LeuB has 359 unique gene targets, LeuR has 881 unique gene targets, and they share 451 gene targets. Using GO analysis, we further characterized the functional divergence between these groups of genes (Section 5.5.8). We determined that the loss of *leuB* results in differential expression of genes enriched in translation initiation factor activity while *leuR* Δ results in differential expression of genes enriched is involved in nucleic acid binding. For biological process GO terms, we show that *leuB* Δ results in differential expression of genes enriched in ribonucleoprotein complex biogenesis while *leuR* Δ results in differential expression of genes enriched in carbon and nitrogen compound metabolism.

This redundancy of the transcription factors provides transcriptional regulation robustness but the divergence provides additional transcriptional control of metabolic processes. Leu3p acts as a dimer when bound to DNA in *S. cerevisiae* (Remboutsika and Kohlhaw, 1994). It has yet to be shown if LeuB or LeuR form hetero- or homodimers with each other but we hypothesize that they may dimerize based on the inverted triplet of the DNA binding sequence. Transcriptional regulation may be different between a LeuB homodimer, and LeuB-LeuR heterodimer, and a LeuR

homodimer. The modulation of LeuB, but not LeuR, by α -IPM provides additional transcriptional control. Gene regulation and expression may also be modulated between highly α -IPM -sensitive, α -IPM-sensitive, and α -IPM-insensitive dimers.

Overall, this work has characterized the last step in the BCAA biosynthetic pathway and first step in BCAA catabolism, completing the annotation of the leucine biosynthesis genes. Furthermore, candidate suppressor mutants of BCAA auxotrophy were isolated and Whole Genome Sequencing and SNP analysis was used to identify potential causative mutations. While the causative mutation(s) have not yet been identified, AN5995 has emerged as a potential candidate. Lastly, the roles of the paralogous transcription factors LeuB and LeuR were determined for leucine biosynthesis, *gdhA* regulation, and iron metabolism. This work highlights the importance of thorough genome analysis to identify paralogs of enzymes encoding genes and their regulators and determine their roles when considering potential anti-fungal treatments and targets. There is divergence in the number of genes encoding enzymes across yeast and fungal species for each enzymatic step of the BCAA biosynthesis pathway (MacDonald et al., 1974; Oliver et al., 2012; Polotnianka et al., 2004; Williams et al., 1996; Shimizu et al., 2010). Loss of a single gene may not cause complete auxotrophy, as shown in both the *batA* and *batB* single deletion mutants and *leuD* and *leuE* single deletion mutants of the enzymes or the *leuB* and *leuR* single deletion mutants of the transcription factors in *A. nidulans*. Complete characterization of a metabolic pathway and its regulators will provide a fuller understanding of the metabolic pathway and the potential for anti-fungal targets for human and plant fungal pathogens.

6.2 Future Directions

This work builds a framework to determine and test potential anti-fungal targets and treatments. Within the BCAA biosynthetic pathway, the *luA* and *leuC* steps are strong candidates as there are no paralogs of these genes to compensate their loss or inactivity. The final two steps have duplicated genes and loss of one gene in a step does not confer auxotrophy. Disrupting both the β -IPM dehydrogenase genes, *leuD* and *leuE*, does cause auxotrophy. A similar outcome occurs with the six branched

chain amino acid aminotransferase (BAT) genes. Here, the loss of the two highly expressed bat genes, *batA* and *batB*, does cause a BCAA auxotrophy. Targeting both genes or enzymes through potential drug treatment or RNA interference would be more effective than targeting just one of them. However, targeting both genes or enzymes does not mean that it would be an effective anti-fungal treatment as we have shown that *A. nidulans* can acquire spontaneous suppressor mutants to overcome auxotrophies. The spontaneous suppressor mutants in the *batA* Δ *batB* Δ double mutants show that *A. nidulans* can overcome the loss of the primary BAT genes under strong enough selection. Identifying the causative suppressor mutation(s) will help our understanding of the mechanisms that can overcome a BCAA auxotrophy and provide additional genes, enzymes, and strategies for developing anti-fungal targets. Lastly, as loss of both *leuB* Δ and *leuR* Δ causes a leucine auxotrophy, one could target both the enzymes and the transcriptional regulators to confer auxotrophy.

In addition to the characterization of potential anti-fungal targets, there are many aspects of the BCAA biosynthetic pathway and its regulation that should be explored. One important experiment is to determine if LeuB and LeuR dimerize and if different dimerization partners change what genes are regulated or to what extent. This will be insightful for understanding the regulation roles of both LeuB and LeuR in different metabolic processes. It would also be beneficial to continue to analyze the RNA-Seq data and find a phenotype of the *leuR* Δ single mutant. Currently, the phenotypic effect of the loss of LeuR is only observed when *leuB* is knocked out.

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Appendix A - Steyer et al. 2021

Data presented as part of this dissertation in Chapters 3 and 5 was published as Steyer, J.T.,⁼ Downes, D.J.,⁼ Hunter, C.C., Migeon, P.A., Todd, R.B. (2021) Duplication and functional divergence of branched-chain amino acid biosynthesis genes in *Aspergillus nidulans*. **mBio.** **12**(3): e00768-21. <https://doi.org/10.1128/mBio.00768-21> [KAES contribution: 20-058-J] ⁼ denotes equal contribution.

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Duplication and Functional Divergence of Branched-Chain Amino Acid Biosynthesis Genes in *Aspergillus nidulans*

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Joel T. Steyer and Damien J. Downes made equal contributions to the manuscript. Author order was determined at the suggestion of Damien J. Downes and by mutual agreement.

ABSTRACT Fungi, bacteria, and plants, but not animals, synthesize the branched-chain amino acids: leucine, isoleucine, and valine. While branched-chain amino acid (BCAA) biosynthesis has been well characterized in the yeast *Saccharomyces cerevisiae*, it is incompletely understood in filamentous fungi. The three BCAAs share several early biosynthesis steps before divergence into specific pathways. In *Aspergillus nidulans*, the genes for the first two dedicated steps in leucine biosynthesis have been characterized, but the final two have not. We used sequence searches of the *A. nidulans* genome to identify two genes encoding β -isopropylmalate dehydrogenase, which catalyzes the penultimate step of leucine biosynthesis, and six genes encoding BCAA aminotransferase, which catalyzes the final step in biosynthesis of all three BCAA. We have used combinations of gene knockouts to determine the relative contribution of each of these genes to BCAA biosynthesis. While both β -isopropylmalate dehydrogenase genes act in leucine biosynthesis, the two most highly expressed BCAA aminotransferases are responsible for BCAA biosynthesis. We have also characterized the expression of leucine biosynthesis genes using reverse transcriptase-quantitative PCR and found regulation in response to leucine availability is mediated through the Zn(II)₂Cys₆ transcription factor LeuB.

IMPORTANCE Branched-chain amino acid (BCAA) biosynthesis is important for pathogenic fungi to successfully cause disease in human and plant hosts. The enzymes for their production are absent from humans and, therefore, provide potential antifungal targets. While BCAA biosynthesis is well characterized in yeasts, it is poorly understood in filamentous fungal pathogens. Developing a thorough understanding of both the genes encoding the metabolic enzymes for BCAA biosynthesis and how their expression is regulated will inform target selection for antifungal drug development.

KEYWORDS LEU3, amino acid biosynthesis, branched-chain amino acid metabolism, filamentous fungi, gene regulation, isopropylmalate, leucine, primary metabolism, transcription factors, valine

The branched-chain amino acids (BCAA) leucine, isoleucine, and valine are essential dietary amino acids in mammals. Leucine levels provide an acute signal for nutrient availability to control the protein kinase mTORC1 (mammalian Target of Rapamycin Complex 1), which is a pleiotropic regulator of many cellular processes, including cell growth, protein biosynthesis, the response to nutrient availability, and autophagy (1, 2). Unlike mammals, fungi synthesize BCAA for use in protein biosynthesis and as precursors for secondary metabolites (3). BCAA biosynthesis genes also play important roles during infection for fungal pathogens. BCAA auxotrophs in the opportunistic human fungal pathogens *Cryptococcus neoformans*, *Candida albicans*, and *Aspergillus fumigatus* show decreased pathogenicity (4–9), and the plant pathogens *Magnaporthe oryzae* and *Fusarium graminearum* require BCAA biosynthesis genes for full virulence

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(10–15). Therefore, the enzymes for BCAA biosynthesis are potential drug and antifungal agent targets.

Synthesis of the three BCAAs occurs via a dichotomous biochemical pathway and is well characterized in *Saccharomyces cerevisiae* (16). Studies of BCAA biosynthesis in *A. fumigatus*, *Aspergillus niger*, and *Aspergillus nidulans* have revealed both divergence from and similarity to *S. cerevisiae* (5, 17–20). For example, both *A. nidulans* and *S. cerevisiae* encode a single α -isopropylmalate isomerase (19, 21). *A. fumigatus* encodes two functional dihydroxyacid dehydratases (5), whereas *S. cerevisiae* encodes only one (22), whereas the production of α -isopropylmalate (α -IPM) in *S. cerevisiae* is carried out by two α -IPM synthetases, encoded by *LEU4* and *LEU9* (23–26), but only a single gene in *A. nidulans*, *leuC*, encodes a functional *LEU4/LEU9* ortholog (27). The final two steps in leucine biosynthesis are catalyzed sequentially by β -isopropylmalate (β -IPM) dehydrogenase and the bidirectional BCAA aminotransferase (BAT), which also produces isoleucine and valine and catalyzes the first step in BCAA catabolism (28). Although the genes encoding these enzymes have been characterized in *S. cerevisiae*, their *A. nidulans* orthologs are unknown.

Leucine biosynthesis in *A. nidulans* is thought to be regulated by the $\text{Zn(II)}_2\text{Cys}_6$ transcription factor LeuB (19). LeuB regulates target genes through either consensus CCGN₄CGG DNA-binding sites, like its *S. cerevisiae* counterpart Leu3p, or a nonconsensus CCGN₅CGG motif, which is also the target of TamA (27, 29). Regulation by LeuB and Leu3p is controlled by feedback inhibition through intracellular levels of free leucine (19, 27, 30). When leucine is abundant, it interacts with the α -IPM synthetase Leu4p to inhibit its activity and decrease production of the leucine biosynthesis pathway intermediate α -IPM (31). Leu3p acts as a repressor when α -IPM levels are low but is converted to an activator by binding of α -IPM (32). *A. nidulans* leucine biosynthesis loss-of-function mutants *luA1* (affecting α -IPM isomerase) and *leuCΔ* (affecting α -IPM synthetase), which are predicted to have increased or decreased α -IPM levels, respectively, show that LeuB responds similarly to Leu3p (27). This mechanism is conserved in *A. fumigatus* LeuB, which regulates leucine biosynthesis and iron acquisition genes (9, 33).

In addition to regulating leucine biosynthesis genes, Leu3p and LeuB regulate expression of their respective NADP-dependent glutamate dehydrogenase (NADP-GDH)-encoding genes, *GDH1* and *gdhA* (9, 19, 33, 34). Consistent with feedback inhibition of leucine biosynthesis through LeuB, exogenous leucine also negatively affects *A. nidulans* *gdhA-lacZ* reporter gene expression (27). NADP-GDH assimilates nitrogen nutrients producing glutamate, which is the amino donor in the final step of leucine biosynthesis. Coregulation of NADP-GDH production by the leucine pathway transcription factor is thought to ensure glutamate levels sufficient to sustain leucine production (16). It has been suggested that, through the feedback mechanisms provided by leucine levels and the coregulation of NADP-GDH expression, leucine, which is one of the most common protein-incorporated amino acids and one of the least abundant free cellular amino acids, acts as a general sensor for amino acid abundance (16).

The *A. nidulans* leucine biosynthesis pathway genes encoding α -IPM synthase (*leuC*) and α -IPM isomerase (*luA*) have been characterized previously (19, 27). In this study, we characterize the two genes encoding β -IPM dehydrogenases and six genes predicted to encode branched-chain amino acid aminotransferases, which together constitute the final two steps of the leucine biosynthesis pathway in *A. nidulans*. We demonstrate roles for both β -IPM dehydrogenase genes and reveal that only two of the six branched-chain amino acid aminotransferases are major contributors to BCAA production. We have also investigated the regulation of these genes by LeuB and leucine.

RESULTS

Identification of the two *A. nidulans* β -isopropylmalate dehydrogenase genes.

The penultimate step in leucine biosynthesis is catalyzed by β -IPM dehydrogenase (Fig. 1). A single gene in yeast, *LEU2*, encodes β -IPM dehydrogenase (35, 36), whereas in *A. niger*, two enzymes, Leu2A and Leu2B, encoded by separate genes, carry out this

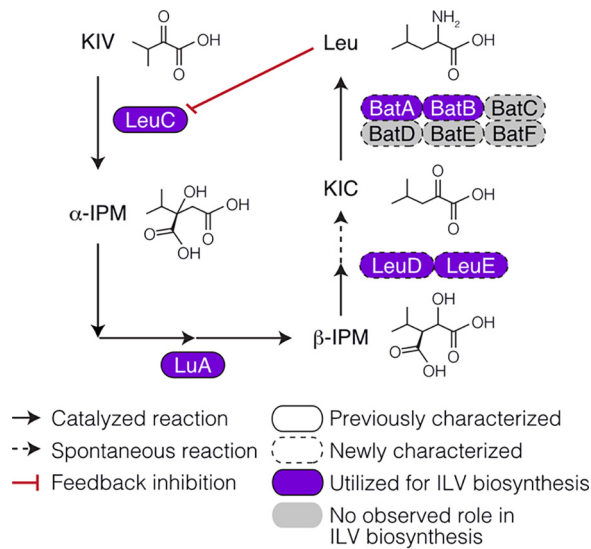


FIG 1 Leucine biosynthesis in *Aspergillus nidulans*. Pathway of committed leucine (Leu) biosynthesis enzymes (rounded rectangles). The stages involving generation of α -isopropylmalate (α -IPM) from α -ketoisovalerate (KIV) by α -IPM synthetase (LeuC) and subsequent conversion to β -isopropylmalate (β -IPM) by α -IPM isomerase (LuA) have previously been characterized. The two β -IPM dehydrogenase enzymes (LeuD and LeuE), which generate α -ketoisocaproate (KIC), and two BCAA aminotransferases (BatA and BatB), which also function in isoleucine and valine biosynthesis and isoleucine, leucine, and valine (ILV) catabolism, were characterized in this work from eight candidate coding genes.

role (18). Two *A. nidulans* β -IPM dehydrogenase enzymes, encoded by AN0912 and AN2793, were identified in BLASTp searches with *S. cerevisiae* Leu2p as the query. AN0912 and AN2793 showed high levels of similarity and identity with Leu2p, Leu2A, and Leu2B, with AN0912 most similar to Leu2A and AN2793 most similar to Leu2B (Table 1). AN0912 and AN2793 showed 50.5% identity and 67.3% similarity with each other. Alignment of the five proteins revealed strong conservation throughout the protein, including in the substrate binding loop and NAD binding motif (see Fig. S1 in the supplemental material). We designated AN0912 *leuD* and AN2793 *leuE*. *leuD* is found on chromosome VIII in a region of highly conserved gene colinearity in all 27 *Aspergillus* species genomes available at FungiDB (Fig. S2A). In contrast, *leuE* is located on chromosome VI and lacks colinearity with its 24 orthologs in the 27 *Aspergillus* species (Fig. S2B).

We investigated the relationships of the two *A. nidulans* β -IPM dehydrogenase genes through construction of a phylogenetic tree (Fig. 2). LeuD and LeuE formed distinct clades with their respective *Aspergillus* orthologs. The LeuD clade is consistent with the position of *A. nidulans* in the fungal evolutionary tree (37), whereas the LeuE clade lies between the Ascomycota and the Basidiomycota clades.

***leuD* and *leuE* both function in leucine biosynthesis.** To determine whether *leuD* and *leuE* are functional genes, we generated deletion mutants by gene replacement (Fig. S3A; see Materials and Methods). Deletion of genes required for leucine biosynthesis results in leucine auxotrophy (19, 27), yet neither *leuD* Δ nor *leuE* Δ strain conferred strict

TABLE 1 Pairwise protein sequence comparisons of β -IPM dehydrogenases

Protein ^a	Systematic name	Leu2p		Leu2A		Leu2B	
		% Identity	% Similarity	% Identity	% Similarity	% Identity	% Similarity
Leu2p	YCL018W	100	100	ND ^b	ND	ND	ND
LeuD	AN0912	62.8	79.6	87.7	94.5	50.7	66.3
LeuE	AN2793	50.1	64.8	53.3	67.7	84.9	92.2

^a*A. nidulans* LeuD and LeuE β -IPM dehydrogenase full-length protein sequences were aligned pairwise and compared with *S. cerevisiae* Leu2p and *A. niger* Leu2A and Leu2B.

^bND, not determined.

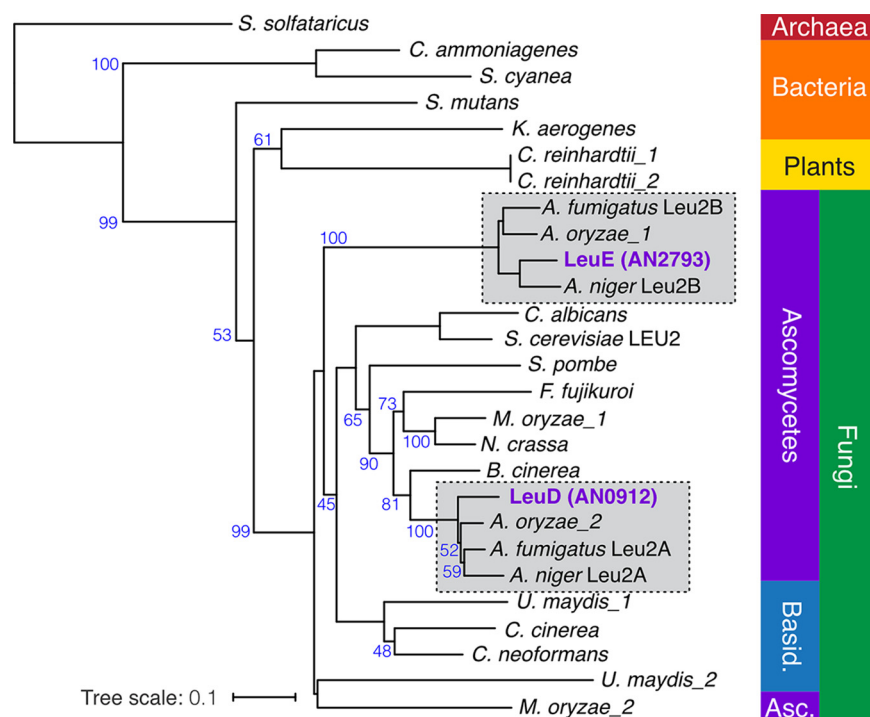


FIG 2 Phylogenetic analysis of β -IPM dehydrogenases. Unrooted phylogeny of β -IPM dehydrogenases is shown. Bootstrap support (100 replicates) greater than 40% is shown. Protein sequences for *A. nidulans* were downloaded from AspGD, sequences for *S. cerevisiae* were downloaded from SGD, and all other sequences came from Pfam or NCBI. Archaea, *Sulfolobus solfataricus* (Q9UXB2.1); Bacteria, *Corynebacterium ammoniagenes* (D5NZR1.1), *Klebsiella aerogenes* (WP_077203698.1), *Streptococcus mutans* (Q8DTG3.1), *Saccharomonospora cyanea* (H5XNC6.1); Basidiomycota (Basid.), *Coprinopsis cinerea* (A8NYJ8.1), *Cryptococcus neoformans* (Q5KP37.1), *Ustilago maydis* (1, XP_011387179.1; 2, XP_011391948.1); Ascomycota (Asc.), *A. fumigatus* Leu2A (Q4WRM6.1), Leu2B (Q4WLG7.1), *A. nidulans* LeuD (AN0912), LeuE (AN2793), *A. niger* Leu2A (P87256.1), Leu2B (P87257.1), *A. oryzae* (1, Q2TYA5.1; 2, Q877A9.1), *Botrytis cinerea* (XP_001546815.1) *Candida albicans* (C4YTB1.1), *Fusarium fujikuroi* (C1L3C2.1), *M. oryzae* (1, G4N5B0.1; 2, G4NIK0.1), *Neurospora crassa* (P34738.2), *Saccharomyces cerevisiae* Leu2p (YCL018W), *Schizosaccharomyces pombe* (P18869.1); Planta, *Chlamydomonas reinhardtii* (1, A8I7N4.1; 2, A8I7N8.1). The scale bar corresponds to the branch length for an expected number of 0.1 substitutions per site. The two distinct *Aspergillus* clades are boxed.

leucine auxotrophy (Fig. 3A). However, while the *leuEΔ* strain grew similarly to the wild type in the absence of leucine, the *leuDΔ* mutant showed reduced growth compared with the wild type unless supplemented with exogenous leucine. Transformation of the *leuD* gene into the *leuDΔ* mutant restored leucine prototrophy (Fig. S4A). To determine whether the leaky nature of the *leuDΔ* leucine auxotrophy resulted from LeuE activity, we constructed a *leuDΔ leuEΔ* double mutant by meiotic crossing and found that the double mutant was a strict auxotroph, showing growth only when supplemented with exogenous leucine (Fig. 3A). Leucine supplementation of a *C. neoformans* auxotroph lacking α -IPM isomerase is possible when glutamine or asparagine, but not ammonium, is the nitrogen source (6). In contrast, the *leuCΔ* mutant lacking α -IPM isomerase can be supplemented on ammonium (27). Likewise, the *leuDΔ leuEΔ* leucine auxotrophy could be supplemented on the preferred nitrogen sources ammonium and glutamine and on the alternative nitrogen source nitrate (Fig. 3A). Therefore, regulation of leucine uptake in *A. nidulans* is not regulated by nitrogen metabolite repression.

To complement the tight leucine auxotrophy of the *leuDΔ leuEΔ* double mutant, we introduced a plasmid carrying the wild-type *leuE* gene and directly selected transformants in the absence of leucine (Fig. S4B to D). Single-copy integration conferred partial leucine auxotrophy that resembled the *leuDΔ* single mutant, whereas multicopy transformants showed stronger growth, indicating that additional copies of the *leuE* gene partially suppress the *leuDΔ* phenotype. We next considered whether levels of expression were the source of the different degrees of effect of *leuDΔ* and *leuEΔ*. We

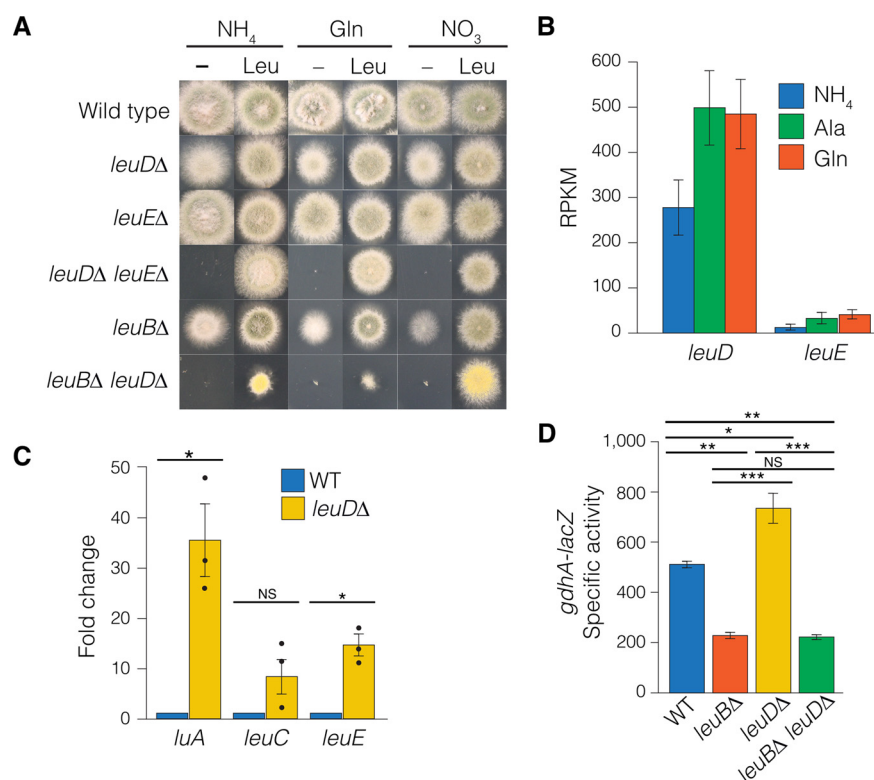


FIG 3 *leuD* encodes the major β -IPM dehydrogenase. (A) Wild-type (MH1), *leuDΔ* (RT411), *leuEΔ* (RT413), *leuDΔ leuEΔ* (RT444), *leuBΔ* (RT452), and *leuBΔ leuDΔ* (RT460) strains were grown at 37°C for 2 days on solid supplemented ANM with or without 2 mM leucine (Leu) and with 10 mM ammonium (NH₄), glutamine (Gln), and nitrate (NO₃) as the nitrogen source. Note that the yellow colony color of RT460 is due to the *yA1* conidial color mutation and is unrelated to the *leuBΔ leuDΔ* phenotype. (B) Mean reads per kilobase per million mapped reads (RPKM) from RNA-seq of MH1 grown at 37°C for 16 h in supplemented liquid ANM with 10 mM ammonium (NH₄), glutamine (Gln), and alanine (Ala). (C) RT-qPCR quantification of mean fold change in transcript expression in *leuDΔ* (RT411) strain compared to the wild type (MH1) grown at 37°C for 16 h in supplemented liquid ANM–10 mM ammonium and 2 mM leucine. Bars indicate the mean fold change from three independent biological replicates (circles). *, $P \leq 0.05$. NS, not significant using two-tailed Student's *t* test with equal distribution. (D) LacZ specific activity for wild-type (MH12101), *leuBΔ* (MH12181), *leuDΔ* (RT458), and *leuBΔ leuDΔ* (RT460) strains, which contain the –753 bp *gdhA-lacZ* reporter construct. Strains were grown at 37°C for 16 h in supplemented liquid ANM with 10 mM ammonium and 2 mM leucine ($n = 3$). *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$; NS, not significant; using one-way ANOVA. For panels B to D, error bars depict standard error of the mean ($N = 3$).

found, using reverse transcription-quantitative PCR (RT-qPCR), that *leuD* had ~64-fold higher expression than *leuE* after 16 h of growth in 10 mM ammonium-minimal medium. In transcriptome sequencing (RNA-seq) data from wild-type mycelia, *leuD* showed higher expression than *leuE* when grown on ammonium (35-fold), alanine (12-fold), and glutamine (13-fold) (Fig. 3B). As leucine production is regulated by feedback inhibition, we examined the effect of the *leuDΔ* mutation on expression of *leuE* and two other leucine biosynthesis genes, *luA* and *leuC*, by RT-qPCR, and *gdhA*, which is coregulated with leucine biosynthesis, using enzyme activity of LacZ expressed from the *gdhA-lacZ* translational fusion reporter gene (19, 27). For all three leucine biosynthesis genes, and for *gdhA-lacZ*, we found that *leuDΔ* resulted in increased expression over wild-type levels (Fig. 3C and D). Therefore, reduced leucine production as a result of *leuDΔ* results in compensation by upregulation of *leuE* and the other leucine biosynthesis genes as well as *gdhA*.

As *leuEΔ* had no effect on growth and *leuE* upregulation in the *leuDΔ* deletion mutant is expected to be LeuB dependent, we constructed a *leuBΔ leuDΔ* double mutant (Fig. 3A). In contrast to the *leuBΔ* and *leuDΔ* single mutants, which are leaky leucine auxotrophs, the *leuBΔ leuDΔ* double mutant is a strict leucine auxotroph, suggesting

TABLE 2 Pairwise protein sequence comparisons of BATs

Protein ^a	Systematic name	Bat1p		Bat2p	
		% Identity	% Similarity	% Identity	% Similarity
Bat1p	YHR208W	100	100	73.5	81.2
Bat2p	YJR148W	73.5	81.2	100	100
BatA	AN4323	49.3	59.7	49.0	59.2
BatB	AN5957	40.5	54.4	43.4	58.6
BatC	AN7878	44.9	62.9	45.8	59.9
BatD	AN7876	24.3	41.3	23.8	41.4
BatE	AN0385	24.2	36.6	24.9	39.0
BatF	AN8511	21.7	31.8	25.1	35.8

^aA. *nidulans* BatA, BatB, BatC, BatD, BatE, and BatF branched-chain amino acid aminotransferase full-length protein sequences were aligned pairwise and compared with *S. cerevisiae* Bat1p and Bat2p.

that LeuB regulation of *leuE* is required for leucine biosynthesis in the absence of *leuD*. We assayed *gdhA-lacZ* reporter gene expression in the double mutant (Fig. 3D). Unlike the single *leuDΔ* mutant, there was no increase in expression above *leuBΔ* levels in the double mutant, consistent with the *leuDΔ*-induced upregulation of leucine biosynthesis genes occurring through LeuB.

Identification of six branched-chain amino acid aminotransferase genes. The final step in leucine biosynthesis, catalyzed by the BCAA aminotransferase (BAT), is common to isoleucine and valine biosynthesis (Fig. 1). In *S. cerevisiae*, BAT enzymes are encoded by two genes, *BAT1* and *BAT2* (38, 39). Six BAT enzymes predicted to catalyze this step have been previously identified in *A. nidulans* (20). We confirmed the identity of these six BATs, and their coding genes, using BLASTP analysis and designated them BatA (AN4323), BatB (AN5957), BatC (AN7878), BatD (AN7876), BatE (AN0385), and BatF (AN8511). Pairwise protein sequence comparisons with Bat1p and Bat2p revealed >21% identity and >31% similarity to both proteins (Table 2). Alignment of these eight proteins showed strong conservation of NAD cofactor binding residues and absolute conservation of the catalytic lysine residue (Fig. S5). The two *S. cerevisiae* BATs function in different subcellular compartments. Bat1p is primarily targeted to mitochondria, whereas Bat2p is cytoplasmic (39). To predict the subcellular location of the six *A. nidulans* BAT enzymes, we used DeepLoc-1.0, TargetP v1.1, and Predotar targeting signal predictions (40–43). For all three algorithms, BatA and BatC, like Bat1p, were predicted to be predominantly mitochondrial, and the remaining BAT enzymes were predicted by DeepLoc-1.0 to localize in the cytoplasm (Data Set S1). The BAT protein alignment revealed that Bat1p, BatA, and BatC have extended N termini containing a predicted mitochondrial targeting signal (Fig. S5).

We examined the colinearity of genes surrounding each of the six *A. nidulans* BAT-encoding genes to identify orthologous genes (Fig. S6). *batA* and *batB* orthologs are conserved in regions of high colinearity in all 27 species. *batE* orthologs are found in a region of moderate colinearity in 13 species. In contrast, *batC*, *batD*, and *batF* were located in regions lacking colinearity. *batD* only had orthologs in *A. niger* and *A. oryzae*, whereas *batC* and *batF* have no predicted ortholog. Interestingly, two of the BAT-encoding genes, *batC* and *batD*, are separated by just 2 kbp within the aspercryptins secondary metabolite gene cluster (44–47). The tight physical linkage of these two genes suggests that they arose from gene duplication by unequal crossover and, therefore, would show high sequence homology. However, the proteins encoded by these genes are highly diverged, showing only 28.9% protein sequence identity.

To determine the relationship of the six *A. nidulans* BATs, we performed phylogenetic analysis (Fig. 4). The BATs formed two distinct groups within the fungi. Group I, the larger group containing 37 out of 52 of the fungal BATs, included BatA, BatB, BatC, and *S. cerevisiae* Bat1p and Bat2p, as well as at least one protein from every other fungus examined. Group II was a smaller group, with only 15 of the 52 proteins, and was almost entirely composed of BAT enzymes from Pezizomycotina genera (*Aspergillus*,

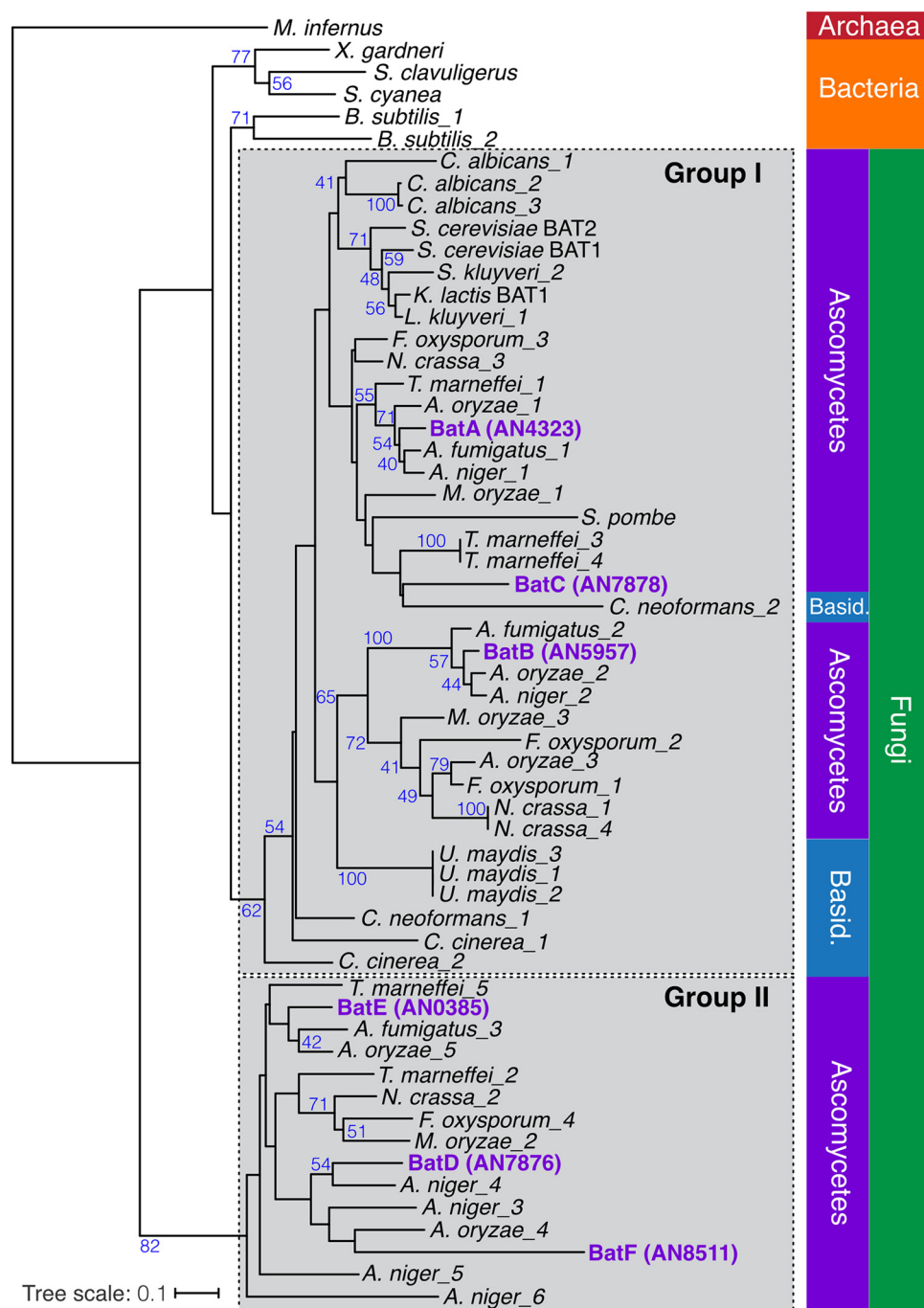


FIG 4 Phylogeny of BCAA aminotransferases. Unrooted phylogeny of BCAA aminotransferases. Bootstrap support (100 replicates) greater than 40% is shown. Protein sequences for aspergilli were downloaded from Aspergillus Genome Database (ASGD), sequences for *S. cerevisiae* were downloaded from SGD, and all other sequences came from Pfam or NCBI. Archaea, *Methanocaldococcus infernus* (D5VS26.1); Bacteria, *Bacillus subtilis* (1, O31461.1; 2, P39576.5), *Streptomyces clavuligerus* (B5H0M8.1), *S. cyanea* (H5XQ56.1), *Xanthomonas gardneri* (F0C966.1); Basidiomycota (Basid.), *C. cinerea* (1, A8N0B4.2; 2, A8N0V2.2), *C. neoformans* (1, Q5K761.1; 2, Q5KD20.1), *U. maydis* (1, XP_011386074.1; 2, NC_026478.1:289079-290305; 3, CM003140.1:289079-290305); Ascomycota, *A. fumigatus* (1, Afu4g06160; 2, Afu2g10420; 3, Afu1g01680), *A. nidulans* (BatA, AN4323; BatB, AN5957; BatC, AN7878; BatD, AN7876; BatE, AN0385; BatF, AN8511), *A. niger* (1, An04g00430; 2, An02g06150; 3, An09g01990; 4, An10g00620; 5, An01g06530; 6, An05g01100), *A. oryzae* (1, AO090023000977; 2, AO090011000598; 3, AO090023000123; 4, AO090011000044; 5, AO090005000936), *C. albicans* (1, Q59Y59.1; 2, Q5AHJ9.1; 3, Q5AHX4.1), *Fusarium oxysporum* (1, F9FH16.1; 2, F9FH71.1; 3, F9FL84.1; 4, F9FPH4.1), *M. oryzae* (1, G4MK83.1; 2, G4MNR9.1; 3, G4NDD5.1), *N. crassa* (1, Q9HEB7.2; 2, Q7SFT9.2; 3, Q7S699.1; 4, Q1K779.1), *S. cerevisiae* (Bat1p, YHR208W; Bat2p, YJR148), *S. pombe* (O14370.2), *Kluyveromyces lactis* (XP_451451.1), *Saccharomyces kluyveri* (1, CM000688.1:1070225-1071418; 2, CM000688.1:1071418-1070225), and *Talaromyces marneffei* (1, XP_002144420.1; 2, XP_002147519.1; 3, XP_002148544.1; 4, XP_002148548.1; 5, XP_002152979.1). The scale bar corresponds to the branch length for an expected number of 0.1 substitutions per site. The two distinct fungal BAT clades are boxed.

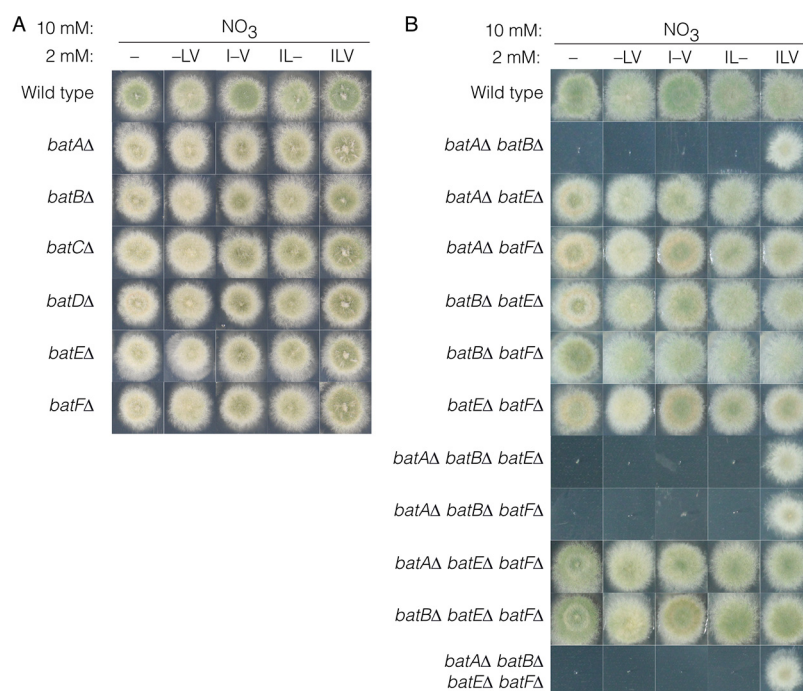


FIG 5 Single and combinatorial deletion analysis of BAT genes. (A) Wild-type (MH1), *batA*Δ (RT415), *batB*Δ (RT440), *batC*Δ (RT475), *batD*Δ (RT419), *batE*Δ (RT417), and *batF*Δ (RT441) strains were grown on supplemented ANM solid media for 2 days with 10 mM nitrate as the predominant nitrogen source and combinations of 2 mM (each) isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. –, an omitted amino acid. (B) Wild-type (MH1), *batA*Δ *batB*Δ (RT457), *batA*Δ *batE*Δ (RT648), *batA*Δ *batF*Δ (RT645), *batB*Δ *batE*Δ (RT636), *batB*Δ *batF*Δ (RT526), *batE*Δ *batF*Δ (RT466), *batA*Δ *batB*Δ *batE*Δ (RT520), *batA*Δ *batB*Δ *batF*Δ (RT523), *batA*Δ *batE*Δ *batF*Δ (RT647), *batB*Δ *batE*Δ *batF*Δ (RT531), and *batA*Δ *batB*Δ *batE*Δ *batF*Δ (RT642) strains grown on supplemented ANM solid media for 2 days with 10 mM nitrate (NO_3) as the predominant nitrogen source and combinations of 2 mM each isoleucine (I), leucine (L), and valine (V) to supplement potential auxotrophies. –, an omitted amino acid.

Penicillium, *Fusarium*, *Neurospora*, *Magnaporthe*) and lacked any Saccharomycotina genera (*Saccharomyces*, *Candida*). Notably, BatC is in group I and BatD is in group II, consistent with separate recruitment to the aspercryptins cluster.

Genetic analysis of six *A. nidulans* BATs. The expansion of the number of BAT-encoding genes in *A. nidulans* indicates specialization for the production of isoleucine, leucine, or valine by specific BATs or the evolution of completely new roles. To determine which BAT-encoding genes were required for BCAA biosynthesis, we constructed individual knockout mutants of each of the six BATs (Fig. S3B; see Materials and Methods). Growth tests of the six individual *bat* knockout mutants showed none were BCAA auxotrophs (Fig. 5A). Therefore, each of the six BATs is dispensable for BCAA biosynthesis. During this study, the two BAT genes found in the aspercryptins gene cluster *batC* (AN7878) and *batD* (AN7876) were published by others as *atnH* and *atnJ*, respectively, and are thought to be involved in biosynthesis of 2-aminocaprylic acid, 2-aminododecanoic acid, and 2-aminodecanoic acid, three unusual BCAAs that are components of aspercryptins (46, 47).

Analysis of RNA-seq expression data from wild-type mycelia grown on ammonium, alanine, or glutamine (Fig. 6A) showed that *batA* has the highest expression under all three conditions. *batB* was the next most highly expressed and showed increased expression on alanine and glutamine compared to ammonium. *batC*, *batD*, and *batE* all showed intermediate expression levels, whereas *batF* was not expressed under these conditions. As *batC* and *batD* are involved in biosynthesis of unusual BCAAs (46, 47), we focused on the other four BAT genes. We measured expression of *batA*, *batB*, *batE*, and *batF* using RT-qPCR of RNA prepared from samples grown on ammonium, alanine, or nitrate. *batA*, *batB*, and *batE* expression did not substantially change under these conditions (Fig. 6B).

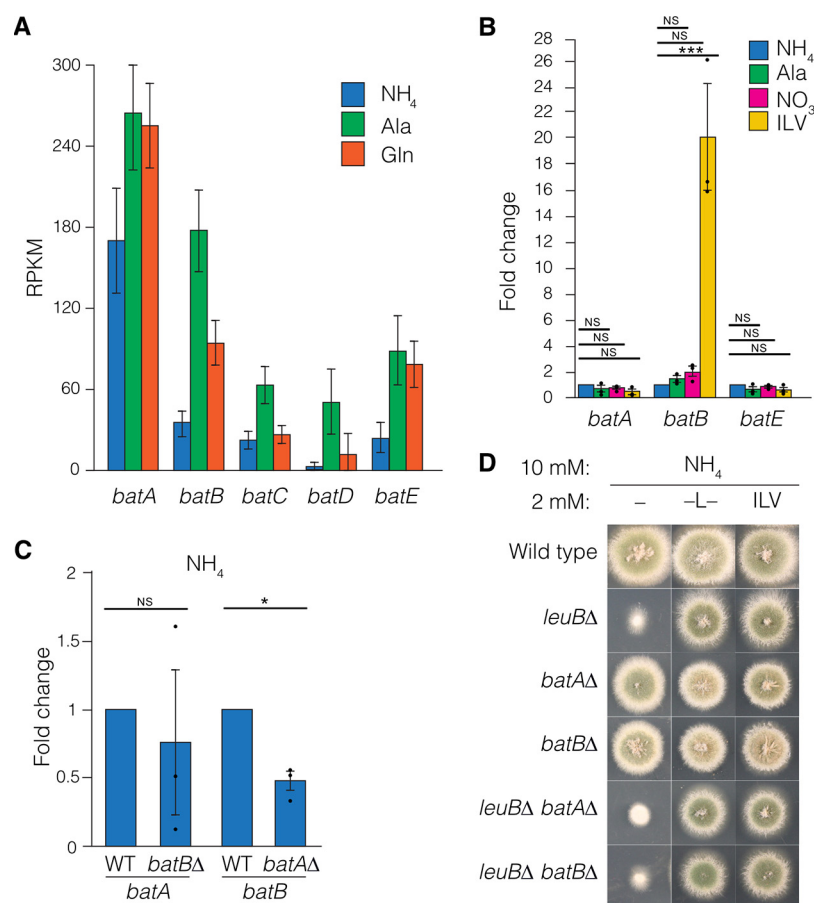


FIG 6 Expression analysis of BAT genes. (A) Mean reads per kilobase per million mapped reads (RPKM) from RNA-seq of MH1 grown at 37°C for 16 h in supplemented liquid ANM with 10 mM ammonium (NH₄), glutamine (Gln), and alanine (Ala). Error bars depict SEM (N=3). (B) RT-qPCR to measure expression levels of *batA*, *batB*, and *batE* under anabolic conditions compared with catabolic conditions. The wild type (MH1) was grown for 16 h in supplemented liquid ANM with 10 mM ammonium (NH₄), nitrate (NO₃), or alanine (Ala) (anabolic conditions) or 3.3 mM (each) ILV (catabolic conditions). Mean fold change (bars) in expression is shown relative to the wild type on 10 mM ammonium for three independent replicates (circles). ***, $P \leq 0.0001$; NS, not significant, using a two-tailed Student's *t* test with equal variance. *batF* was not detected by either RNA-seq or RT-qPCR. (C) RT-qPCR of *batA* and *batB* in the wild-type (MH1), *batA*Δ (RT415), or *batB*Δ (RT440) strains grown for 16 h in supplemented liquid ANM with 10 mM ammonium. Mean fold change in expression (bars) relative to the wild type for three independent replicates (circles) is shown. *, $P \leq 0.05$; NS, not significant, using a two-tailed Student's *t* test with equal variance. (D) Wild-type (MH1), *batA*Δ (RT415), *batB*Δ (RT440), *leuB*Δ (RT453), *leuB*Δ *batA*Δ (RT793), and *leuB*Δ *batB*Δ (RT794) strains were grown on supplemented ANM solid media for 2 days with 10 mM ammonium as the predominant nitrogen source with (ILV) or without (–) 2 mM (each) isoleucine, leucine, and valine or with 2 mM leucine (L).

batF was not expressed under these conditions, consistent with it being undetectable by RNA-seq. We constructed double, triple, and quadruple mutants combining *batA*Δ, *batB*Δ, *batE*Δ, and *batF*Δ by meiotic crossing. The *batA*Δ *batB*Δ double mutant, which combined deletions of the two most related and highly expressed genes, was a strict BCAA auxotroph and could only grow if supplemented with all three BCAAs (Fig. 5B). Therefore, BatA and BatB are the major BAT enzymes for isoleucine, leucine, and valine (ILV) biosynthesis. The *batA*Δ *batB*Δ *batE*Δ and *batA*Δ *batB*Δ *batF*Δ triple mutants and the *batA*Δ *batB*Δ *batE*Δ *batF*Δ quadruple mutant showed BCAA auxotrophy identical to that of the *batA*Δ *batB*Δ double mutant. In contrast, all of the other double and triple mutants constructed, which contained a wild-type copy of either *batA* or *batB*, were BCAA prototrophs. We confirmed that introduction of either the *batA* or *batB* gene into the *batA*Δ *batB*Δ mutant restored BCAA prototrophy (Fig. S4E). We investigated whether

loss of either *batA* or *batB* would cause a compensatory increase in expression of *batB* or *batA*, respectively. However, on ammonium, *batA* expression was not upregulated in the *batBΔ* mutant and *batB* expression was not upregulated in the *batAΔ* mutant (Fig. 6C). This indicates that the expression levels of either one of the major *bat* genes for BCAA biosynthesis is sufficient for prototrophy. We constructed *leuBΔ batAΔ* and *leuBΔ batBΔ* double mutants. These two double mutants showed leaky leucine auxotrophy similar to that of the *leuBΔ* single mutant, indicating that *leuBΔ* is epistatic to *batAΔ* and *batBΔ* (Fig. 6D).

In addition to their role in BCAA biosynthesis, BATs also form the first step in ILV catabolism (28). We examined expression of *batA*, *batB*, *batE*, and *batF* with ILV as the sole nitrogen source to determine their expression pattern during catabolic conditions (Fig. 6B). For both *batA* and *batE*, expression levels were similar under anabolic and catabolic conditions. However, *batB* levels were elevated substantially during ILV catabolism compared with biosynthetic growth conditions, suggesting that BatB is the predominant catabolic enzyme. *batF* expression was undetectable. During BCAA catabolic growth, neither *batA* nor *batB* expression showed compensatory upregulation in the *batBΔ* or *batAΔ* strain, respectively (Fig. 7A). We assessed whether mutants carrying single or multiple BAT gene deletions could utilize each BCAA as the predominant nitrogen source in the presence of lower levels of the other two BCAAs to supplement the auxotrophy (Fig. 7B). All six single BAT mutants could utilize the three BCAAs. Mutants lacking *batB* but not *batA* showed slightly reduced colony morphology compared with *batB*⁺ strains. Notably, mutants lacking both *batA* and *batB* showed severely reduced growth on each of the BCAAs as a predominant nitrogen source, and the reduction in growth was greater on isoleucine and valine than on leucine. We also examined growth of the *batAΔ* and *batBΔ* single and double mutants on increasing concentrations of equimolar ILV and found that *batBΔ* shows reduced colony morphology compared with both wild-type and *batAΔ* strains but stronger growth than the *batAΔ batBΔ* double mutant (Fig. 7C). Therefore, BatA and BatB are the major BAT enzymes in *A. nidulans* for both BCAA biosynthesis and utilization. We did not observe a phenotype for *batEΔ* or *batFΔ* mutant in BCAA catabolism. Transformation analysis of the *batA* or *batB* gene into the *batAΔ batBΔ* recipient repaired BCAA utilization to the wild-type phenotype (Fig. S4F).

Regulation of leucine biosynthesis pathway gene expression by LeuB. The transcription factor LeuB is thought to regulate leucine biosynthesis genes because the *leuBΔ* mutant is a leaky leucine auxotroph (19). To determine whether LeuB regulates these genes in response to leucine levels, we performed RT-qPCR on RNA isolated from mycelia grown with exogenous leucine, which represses LeuB activation (27), and in a *leuBΔ* strain (Fig. 8). *leuB* expression was not altered in response to leucine. The six genes we demonstrated to function in leucine biosynthesis, *leuC*, *luA*, *leuD*, *leuE*, *batA*, and *batB*, as well as *batE*, showed decreased expression in response to exogenous leucine and/or in the *leuBΔ* mutant compared to the wild type.

DISCUSSION

We have completed annotation of the *A. nidulans* leucine biosynthesis pathway and characterized the genes encoding enzymes for the final two steps. Our analysis has revealed divergence between aspergilli and yeast in the number of genes encoding the enzymes for each step. In *S. cerevisiae*, ketoisovalerate is converted to α -IPM by two α -IPM synthetases, Leu4p and Leu9p, which form homodimers and heterodimers that show differential sensitivity to leucine feedback inhibition (23–26, 31). In contrast, a single α -IPM synthetase gene exists in *A. nidulans* (27). α -IPM is converted to β -IPM by the isopropylmalate isomerase, which is encoded by a single gene in both *S. cerevisiae* (*LEU1*) and *A. nidulans* (*luA*) (19, 21). β -IPM is then converted to ketoisocaproate by a single β -IPM dehydrogenase in *S. cerevisiae*, Leu2p (35, 36), but two enzymes, LeuD and LeuE, in *A. nidulans*. The final step in BCAA biosynthesis is catalyzed by BCAA aminotransferase (BAT). *S. cerevisiae* has two BAT genes (38, 39). *A. nidulans* carries six BAT

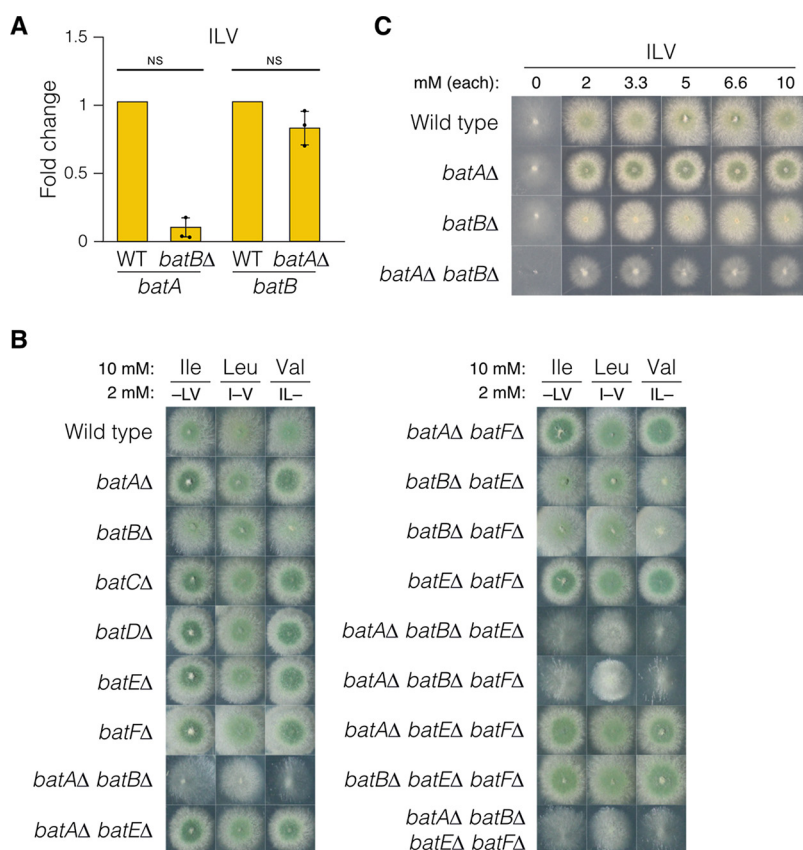


FIG 7 Combinatorial analysis of BAT genes during catabolic growth. (A) RT-qPCR of *batA* and *batB* in the wild-type (MH1), *batA*Δ (RT415), or *batB*Δ (RT440) strains grown for 16 h in supplemented liquid ANM with 3.3 mM (each) isoleucine (I), leucine (L), and valine (V), i.e., catabolic conditions. Mean fold change in expression (bars) relative to the wild type for three independent replicates (circles) is shown. NS, not significant using two-tailed Student's *t* test with equal variance. (B) Wild-type (MH1), *batA*Δ (RT415), *batB*Δ (RT440), *batC*Δ (RT475), *batD*Δ (RT419), *batE*Δ (RT417), *batF*Δ (RT441), *batA*Δ *batB*Δ (RT457), *batA*Δ *batE*Δ (RT648), *batA*Δ *batF*Δ (RT645), *batB*Δ *batE*Δ (RT636), *batB*Δ *batF*Δ (RT526), *batE*Δ *batF*Δ (RT466), *batA*Δ *batB*Δ *batE*Δ (RT520), *batA*Δ *batB*Δ *batF*Δ (RT523), *batA*Δ *batE*Δ *batF*Δ (RT647), *batB*Δ *batE*Δ *batF*Δ (RT531), and *batA*Δ *batB*Δ *batE*Δ *batF*Δ (RT642) strains were grown on supplemented ANM solid media for 2 days with 10 mM isoleucine (Ile), leucine (Leu), or valine (Val) as the predominant nitrogen source and combinations of 2 mM each isoleucine (I), leucine (L), and valine (V) to supplement auxotrophies. –, an omitted amino acid. (C) Wild-type (MH1), *batA*Δ (RT415), *batB*Δ (RT440), and *batA*Δ *batB*Δ (RT457) strains were grown on supplemented ANM solid media for 2 days at 37°C with increasing equimolar concentrations of isoleucine (I), leucine (L), and valine (V).

genes; however, primarily two, BatA and BatB, play major roles in ILV biosynthesis. Interestingly, the genes encoding the dimeric enzymes in the pathway, α -IPM synthetase (31), β -IPM dehydrogenase (48, 49), and BAT (50), differ in copy number, whereas the nonduplicated gene for α -IPM isomerase is monomeric (51). The *LEU4/LEU9* and *BAT1/BAT2* gene duplications resulted from the ancestral whole-genome duplication (WGD) and exhibit functional diversification associated with the acquisition of fermentative metabolism (52).

The *Aspergillus* lineage did not experience an ancestral WGD, but alternative mechanisms have mediated gene duplication within the leucine biosynthesis pathway. The acquisition of additional copies of genes often leads to robustness via the evolution of new functions but in some cases confers fragility (52–54). We found that both *leuD* and *leuE* function in leucine biosynthesis, although *leuE* plays a lesser role based upon its low expression, the prototrophy of the *leuE*Δ mutant, and the leaky leucine auxotrophy conferred by deletion of *leuD*. This gene duplication provides robustness in the form of redundancy, as perturbation of leucine biosynthesis by deletion of *leuD* resulted in LeuB-dependent upregulation of *leuE* and partial compensation of the leucine auxotrophy. Our functional analysis showed

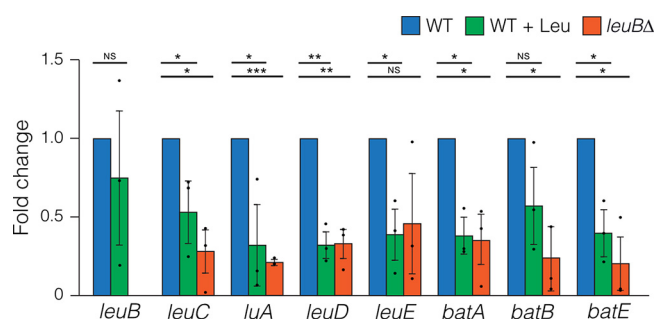


FIG 8 *LeuB* regulation of the leucine biosynthesis genes. RT-qPCR of BCAA biosynthesis genes from wild-type (MH1) and *leuBΔ* (MH12609) strains grown for 16 h in supplemented liquid ANM-10 mM ammonium with or without 2 mM leucine (Leu). Expression is relative to the wild type. The means (bars) and individual results from three independent replicates (circles) are shown. *, $P \leq 0.05$; **, $P \leq 0.001$; ***, $P \leq 0.0001$; NS, not significant, using two-tailed Student's *t* test with equal variance.

that each of the six *A. nidulans* BATs are dispensable. Combining BAT gene deletions, however, revealed that BatA and BatB are the major enzymes in both BCAA biosynthesis and utilization. BatA contains a mitochondrial targeting signal and shows higher biosynthetic expression, while the likely cytoplasmic BatB shows higher catabolic expression. Therefore, BatA and BatB are equivalent to mitochondrial and predominantly biosynthetic Bat1p and cytoplasmic and predominantly catabolic Bat2p in *S. cerevisiae* (50, 55, 56). BatA and BatB show redundancy in both biosynthesis and catabolism. BAT function is also distributed between two paralogs in *Lachancea kluyveri*, with one major biosynthetic BAT and both involved in aerobic metabolism (57). In contrast, *Kluyveromyces lactis* has just one BAT gene, which encodes a bifunctional enzyme for BCAA biosynthesis and degradation, and this is thought to be the ancestral type prior to the WGD and subfunctionalization of Bat1p and Bat2p in *S. cerevisiae* (50).

The dispensability of *batC*, *batD*, *batE*, and *batF* for BCAA biosynthesis and catabolism suggests evolution of novel roles. We showed that *batE* is regulated by leucine and *LeuB*, similar to other leucine biosynthesis genes, but expression levels are low and we did not observe a phenotype for the *batEΔ* mutant. However, *batE* expression is induced during hypoxia in the absence of glucose-to-ethanol fermentation, in association with elevated BCAA biosynthesis that occurs as a mechanism to generate NAD^+ and survive anaerobic stress (20, 58). BatE does not appear to contribute to BCAA metabolism under our normoxic growth conditions but may play a role during anaerobic stress. *batC* (*atnH*) and *batD* (*atnJ*) are members of the aspercryptin biosynthetic gene cluster, with presumed roles in transamination of the unusual BCAAs 2-aminocaprylic acid, 2-aminodecanoic acid, and 2-aminododecanoic acid (46, 47). Aspercryptins contain three BCAAs (isoleucine or valine, 2-aminocaprylic acid, and 2-aminododecanoic or 2-aminodecanoic acid). Expression of *batF* was undetectable under our growth conditions or growth conditions used for RNA-seq by others (59). *batF* is adjacent to the terriquinone A (*tdi*) biosynthetic gene cluster and may also be associated with secondary metabolism (45, 60–62).

Regulation of leucine biosynthesis is best understood in *S. cerevisiae* where both activation and repression are mediated by the $\text{Zn(II)}_2\text{Cys}_6$ transcription factor Leu3p (16). When leucine is abundant, it interacts with α -IPM synthetase, inhibiting its function, which decreases cellular α -IPM levels and leads to Leu3p acting as a repressor (16, 24). When leucine levels decrease, α -IPM synthetase is not inhibited, and α -IPM interacts with Leu3p, causing a conformational change and resulting in Leu3p switching to an activator (16, 30). We observed repression by exogenous leucine in wild-type cells of all six genes that function in leucine biosynthesis, *leuA*, *leuC*, *leuD*, *leuE*, *batA*, and *batB*, as well as *batE*, indicating this feedback mechanism operates in *A. nidulans*. Deletion of the *A. nidulans* *LEU3* ortholog *leuB* confers leaky leucine auxotrophy (19), which we have now shown is due to decreased expression of the leucine biosynthesis

genes. The *leuD*Δ mutant shows leaky leucine auxotrophy and increased expression of other leucine biosynthesis genes, which likely results from reduced cellular levels of the negative feedback mediator leucine and increased α-IPM inducer levels due to increased β-IPM levels increasing the reverse reaction rate carried out by the bidirectional α-IPM isomerase encoded by *luA*.

The absence of the leucine biosynthesis pathway in animals and the reduced virulence of leucine auxotrophs (4–6, 9, 33, 97) render leucine biosynthesis enzymes strong candidate targets for antifungals. Our studies of the genes in this pathway indicate that the feedback mechanisms and gene duplications present in the aspergilli must be considered in target selection to avoid increased LeuB-dependent expression of other leucine biosynthesis genes in response to an antifungal agent targeting this pathway. The strongest target would be α-IPM synthetase (LeuC), as reduced activity of this enzyme leads to decreased α-IPM and repression of leucine biosynthesis genes by LeuB (27). The benefit of targeting this step would be in cross regulation of nitrogen assimilation by reduced expression of *gdhA* and potentially reduced cellular glutamate and glutamine levels.

Overall, this study has completed the annotation of the genes required for leucine biosynthesis in *A. nidulans* and demonstrated regulation of the pathway genes by LeuB. We have found roles for *leuD* and *leuE* in leucine biosynthesis and for *batA* and *batB* in BCAA biosynthesis and catabolism. Roles for *batC* (*atnH*) and *batD* (*atnJ*) in aspercryptins production have now been reported (46, 47), but the roles of *batE* and *batF* remain to be determined.

MATERIALS AND METHODS

A. *nidulans* strains, media, and genetic analysis. *A. nidulans* strains and genotypes are listed in Table 3 using conventional nomenclature (98). *A. nidulans* growth conditions and media were as described previously (63, 64). *Aspergillus* nitrogen-free minimal medium (ANM), pH 6.5, containing 1% (wt/vol) glucose as the sole carbon source, was supplemented for auxotrophies and nitrogen sources (10 mM final concentration), unless otherwise stated. *A. nidulans* growth testing and genetic analysis were as described previously (64).

Standard molecular techniques. *Escherichia coli* NM522 cells [F' *proA*⁺*B*⁺ *lacI*^R Δ(*lacZ*)M15/Δ(*lac-proAB*) *glnV* *thi-1* Δ(*hsdS-mcrB*)5] (65) were employed for molecular cloning (66). Plasmid DNA was isolated using the Wizard Plus SV miniprep DNA purification kit (Promega). *A. nidulans* genomic DNA was isolated according to reference 67. PCR products and DNA fragments isolated from agarose gels were cleaned with the Wizard SV gel and PCR clean-up system (Promega). Restriction enzyme digestions (Promega, New England Biolabs), dephosphorylation with Arctic shrimp alkaline phosphatase (Promega), and ligations using T4 DNA ligase (Promega) followed the manufacturers' instructions. DNA was separated on 1 to 2% agarose gels by electrophoresis in 1× Tris-acetate-EDTA (TAE) buffer. PCRs used *Ex Taq* (TaKaRa), Phusion (Finnzymes), or AccuStart II Geltrack PCR supermix (Quanta Biosciences) DNA polymerases according to the manufacturers' instructions, with 1 ng plasmid or 100 ng *A. nidulans* genomic DNA templates. All reactions followed recommended denaturing and annealing conditions with 33 to 36 amplification cycles. Oligonucleotide PCR primers (Integrated DNA Technologies) are described in Table S1 in the supplemental material. DNA sequencing to confirm correct amplifications and cloning was performed at the Kansas State University DNA Sequencing and Genotyping Facility. Southern hybridizations used either Hybond N+ or Hybond XL membranes (GE Healthcare) and the DIG (digoxigenin) high prime DNA labeling and detection starter kit II (Roche) by following the manufacturer's instructions.

Strain construction. *A. nidulans* transformation was performed as described previously (27) using the *nkuA*Δ nonhomologous integration-defective mutant for targeted integration (68). Knockout constructs, generated by the *A. nidulans* whole-genome gene deletion constructs program (69), were sourced from the Fungal Genetics Stock Center, Manhattan, KS (70), and were transformed into MH11068 (*pyrG89 nkuA*Δ::Bar) and selected for uracil and uridine prototrophy to generate *leuD*Δ (AN0912Δ; RT411, Δ−7 to +1,431 bp), *leuE*Δ (AN2793Δ; RT413, Δ−6 to +1,233 bp), *batA*Δ (AN4323; RT415, Δ+65 to +1,722 bp), *batB*Δ (AN5957Δ; RT440, Δ+25 to +1,395 bp), *batC*Δ (AN7878Δ; RT475, Δ−10 to +1,222 bp), *batD*Δ (AN7876Δ; RT419, Δ−7 to +1,297 bp), *batE*Δ (AN0385Δ; RT417, Δ+27 bp to 1,302 bp), and *batF*Δ (AN8511; RT441, Δ−9 to +1,230 bp) strains. Selection media for *leuD*Δ and *leuE*Δ transformants were supplemented with 2 mM leucine, and BAT gene deletion transformants were supplemented with ILV (2 mM each). The *Aspergillus fumigatus pyrG* (*AfpyrG*) marker showed position effect in the *batC*Δ mutant, incompletely complementing the pyrimidine auxotrophy of the *pyrG89* mutation. Full complementation of *pyrG89* by *AfpyrG* was observed in the other deletion mutants generated in this study. Pyrimidine supplementation was used in all growth tests. All deletion mutants were confirmed by Southern blotting as a single homologous double-crossover integration at the correct locus by probing with the 982-bp KpnI-SspI fragment of *AfpyrG*⁺ (data not shown). Meiotic crossing was used to generate double, triple, and quadruple mutants. The presence of each deletion in the progeny of crosses was confirmed by diagnostic Southern blotting or diagnostic PCR. The *leuD*Δ mutant was repaired by introduction of a wild-type *leuD* PCR product (−960 to +3216) amplified from MH1, with direct selection for simultaneous resistance to 1 mg ml^{−1} 5-fluoroorotic acid (5-FOA) in the absence of exogenous leucine. The *leuD*Δ *leuE*Δ mutant was

TABLE 3 Strains used in this study

Strain	Origin	Genotype ^a
MH1	M. J. Hynes	<i>biA1</i>
MH10865	R. B. Todd	<i>yA1 pabaA1 pyrG89 argB::fmdS-lacZ areAΔ(5')::riboB</i>
MH11068	M. J. Hynes	<i>pyrG89 pyroA4 nkuAΔ::Bar</i>
MH12609	M. A. Davis	<i>yA1 pabaA1 leuBΔ::riboB pyroA4 nkuAΔ::Bar niiA4</i>
MH12181	Downes et al. (27)	<i>leuBΔ::riboB amdS::AfpYroA-gdhA(−753 bp)-lacZ pyroA4 niiA4</i>
RT411	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuDΔ::AfpYrG</i>
RT412	RT411 × MH10865	<i>yA1 pabaA1 pyrG89 leuDΔ::AfpYrG</i>
RT413	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar leuEΔ::AfpYrG</i>
RT414	RT413 × MH10865	<i>yA1 pabaA1 pyrG89 leuEΔ::AfpYrG</i>
RT415	Transformant of MH11068	<i>pyrG89 batAΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT416	RT415 × MH10865	<i>yA1 pyrG89 pabaA1 batAΔ::AfpYrG</i>
RT417	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar batEΔ::AfpYrG</i>
RT418	RT417 × MH10865	<i>yA1 pabaA1 pyrG89 batEΔ::AfpYrG</i>
RT419	Transformant of MH11068	<i>pyrG89 batDΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT440	Transformant of MH11068	<i>pyrG89 batBΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT441	Transformant of MH11068	<i>pyrG89 pyroA4 nkuAΔ::Bar batFΔ::AfpYrG</i>
RT444	RT411 × RT414	<i>pyrG89 pyroA4 leuEΔ::AfpYrG leuDΔ::AfpYrG</i>
RT452	MH12181 × MH11068	<i>pyrG89 leuBΔ::riboB amdS::AfpYroA-gdhA(−753 bp)-lacZ pyroA4</i>
RT453	MH12181 × MH11068	<i>pyrG89 leuBΔ::riboB amdS::AfpYroA-gdhA(−753 bp)-lacZ pyroA4 niiA4</i>
RT454	RT418 × RT419	<i>yA1 pabaA1 pyrG89 batDΔ::AfpYrG batEΔ::AfpYrG</i>
RT457	RT416 × RT440	<i>pyrG89 batBΔ::AfpYrG batAΔ::AfpYrG pyroA4</i>
RT458	RT412 × RT453	<i>yA1 pabaA1 pyrG89 amdS::AfpYroA-gdhA(−753 bp)-lacZ leuDΔ::AfpYrG</i>
RT460	RT412 × RT453	<i>yA1 pabaA1 pyrG89 leuBΔ::riboB amdS::AfpYroA-gdhA(−753 bp)-lacZ leuDΔ::AfpYrG</i>
RT462	RT412 × RT453	<i>leuBΔ::riboB amdS::AfpYroA-gdhA(−753 bp)-lacZ leuDΔ::AfpYrG niiA4</i>
RT466	RT441 × RT454	<i>pabaA1 pyrG89 batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT475	Transformant of MH11068	<i>pyrG89 batCΔ::AfpYrG pyroA4 nkuAΔ::Bar</i>
RT520	RT457 × RT466	<i>batBΔ::AfpYrG batAΔ::AfpYrG batEΔ::AfpYrG</i>
RT523	RT457 × RT466	<i>pabaA1 batBΔ::AfpYrG batAΔ::AfpYrG batFΔ::AfpYrG</i>
RT524	RT457 × RT466	<i>pabaA1 batBΔ::AfpYrG batAΔ::AfpYrG batEΔ::AfpYrG</i>
RT525	RT457 × RT466	<i>batBΔ::AfpYrG pyroA4 batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT526	RT457 × RT466	<i>batBΔ::AfpYrG batFΔ::AfpYrG</i>
RT531	RT457 × RT466	<i>batBΔ::AfpYrG batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT636	RT525 × RT524	<i>batBΔ::AfpYrG batEΔ::AfpYrG</i>
RT642	RT525 × RT523	<i>batBΔ::AfpYrG batAΔ::AfpYrG pyroA4 batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT645	RT415 × RT466	<i>pabaA1 batAΔ::AfpYrG pyroA4 batFΔ::AfpYrG</i>
RT647	RT415 × RT466	<i>pabaA1 batAΔ::AfpYrG batFΔ::AfpYrG batEΔ::AfpYrG</i>
RT648	RT415 × RT466	<i>batAΔ::AfpYrG pyroA4 batEΔ::AfpYrG</i>
RT793	RT453 × RT415	<i>pyrG89 batAΔ::AfpYrG leuBΔ::riboB pyroA4 nkuAΔ::Bar niiA4</i>
RT794	RT453 × RT440	<i>pyrG89 batBΔ::AfpYrG leuBΔ::riboB pyroA4 nkuAΔ::Bar niiA4</i>

^aAll strains carry *veA1*.

complemented with the plasmid pJS249, which carries *leuE* (−913 to +2877) PCR amplified from MH1 and cloned into pGEMTeasy by transformation with direct selection for leucine prototrophy. The *batAΔ batBΔ* double mutant was complemented with the wild-type *bata* (−717 to +2558) or *batB* (−725 to +2187) gene using plasmids (pJS244 and pJS255, respectively) containing PCR-amplified DNA from MH1 cloned into pGEMTeasy. Transformants were directly selected for growth in the absence of exogenous ILV.

β-Galactosidase assays. β-Galactosidase assays were performed as described previously (71) using soluble protein extracts. β-Galactosidase specific activity is defined as $A_{420} \times 10^3 \text{ min}^{-1} \text{ mg}^{-1}$ of soluble protein. Protein concentrations were determined using Bio-Rad assay reagent (Bio-Rad).

RNA preparation. Total RNA was isolated by grinding mycelia under liquid nitrogen and subsequent addition to RNA extraction buffer (7.0 M urea, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1.0% sodium dodecyl sulfate) followed by two phenol-chloroform-isoamyl alcohol extractions and one chloroform extraction (66). RNA was precipitated in 3 M ammonium acetate and 50% isopropanol, resuspended in diethyl pyrocarbonate- H_2O , and reprecipitated overnight in 4 M lithium chloride at −20°C. RNA quality was determined by visualization after electrophoretic separation in a 1.2% agarose gel containing 1.1% formaldehyde run in 1× morpholinepropanesulfonic acid (MOPS) buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA). RQ1 DNase (Promega) treatment of RNA followed the manufacturer's instructions.

RT-qPCR. For reverse transcriptase-quantitative PCR (RT-qPCR), cDNA was produced using the reverse transcriptase system (Promega) or qScript cDNA supermix (Quanta Biosciences). RT-qPCR used a MyiQ thermocycler (Bio-Rad) with iTAQ universal SYBR green supermix (Bio-Rad), and results were analyzed with iQ5 v2.1 (Bio-Rad). Fold change was calculated using the $\Delta\Delta C_T$ method with β-tubulin-encoding *benA* as the reference gene (72–74). Primers (IDT) were designed to specifically amplify cDNA by overlapping a splice junction. Primer sequences used for RT-qPCR, target regions, and efficiencies are listed in Table S2.

RNA-seq. PolyA⁺ RNA, isolated from three independent biological replicates of wild-type (MH1) mycelia grown for 16 h in supplemented liquid ANM with 10 mM ammonium, glutamine, or alanine, was fragmented to 180 bp and indexed using the TruSeq stranded total RNA sample preparation kit (Illumina). Multiplexed libraries were sequenced using 50-bp single-end reads on the Illumina Hi-Seq 2500 system (Kansas University Medical Center Genome Sequencing Facility, Kansas City, KS). RNA-seq analysis was conducted using Galaxy (www.galaxyproject.org) (75–77). Reads were processed with FASTQ Groomer (78) and FastQC and aligned to the *A. nidulans* FGSC_A4 genome (79, 80) using TopHat (v2.0.6) (81) default settings, with exceptions (minimum intron length, 10; maximum intron length, 4,000; maximum alignments, 40; minimum read length, 20). Strand-specific reads were separated using SAMtools view (v1.1) (82). Strand-specific transcripts were identified using AspGD annotations (s10_m03_r15) and Cufflinks (v2.1.1.7) (83, 84) default settings, with exceptions (max intron length, 4,000; bias correction, yes; multiread correction, yes). Identified transcripts from all growth conditions were combined into a single annotation using Cuffmerge guided by the reference annotation. Differential expression was determined using CuffDiff (84) and cummeRbund (v2.8.2) (85).

Bioinformatics and in silico analyses. DNA and protein sequences were downloaded from the *Aspergillus* Genome Database, AspGD (www.aspgd.org) [83], the *Saccharomyces* Genome Database, SGD (www.yeastgenome.org) [86], the Broad Institute genomes database (www.broadinstitute.org), the NCBI protein database (www.ncbi.nlm.nih.gov/protein/), and the EMBL-EBI Pfam database (<http://pfam.xfam.org>) [87]. Protein sequence database searches used BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Protein conserved domains were identified using the NCBI Conserved Domain Database (88). Pairwise protein sequence comparisons, and percent identity and similarity were calculated using EMBOSS Needle (EMBL-EBI) with default parameters. Sequences were analyzed in Geneious version 5.3.5, created by Biomatters (www.geneious.com). Multiple sequence alignments were made using ClustalW2 (89) or Clustal Omega (90) on the EMBL-EBI server (<http://www.ebi.ac.uk/Tools/msa>) and shaded using online BoxShade 3.2 (K. Hofmann and M. D. Baron) at ExPASy (https://embnet.vital-it.ch/software/BOX_form.html). Predicted subcellular localization of proteins was determined using Predotar 1.03 (<https://urgi.versailles.inra.fr/predotar/>) [42], TargetP v1.1 (<http://www.cbs.dtu.dk/services/TargetP/>) [40, 41], and DeepLoc-1.0 (<https://services.healthtech.dtu.dk/service.php?DeepLoc-1.0>) [43]. Colinearity of syntenic regions was illustrated using the GBrowse genome browser of FungiDB with genomes clustered based on whole-genome phylogenies (91–93, 99).

Phylogenetic analyses. The Pfam database (<http://pfam.xfam.org/>) [87] was used to identify orthologs in the isocitrate/isopropylmalate dehydrogenase family (PF00180) and the aminotransferase class IV family (PF01063). Protein sequences were aligned and phylogenies were constructed using MAFFT (94). The neighbor-joining method with 100 bootstraps was used to generate consensus unrooted phylogenetic trees of β -IPM dehydrogenases and BATs. Tree visualization and label editing used Interactive Tree Of Life (iTOL) (95).

Data availability. RNA-seq fastq files and bigwigs have been deposited in NCBI's Gene Expression Omnibus (96) and are accessible through GEO Series accession number (GSE145035).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

DATA SET S1, XLSX file, 0.04 MB.

FIG S1, PDF file, 0.6 MB.

FIG S2, PDF file, 2 MB.

FIG S3, PDF file, 0.3 MB.

FIG S4, PDF file, 2.8 MB.

FIG S5, PDF file, 0.9 MB.

FIG S6, PDF file, 0.8 MB.

TABLE S1, PDF file, 0.02 MB.

TABLE S2, PDF file, 0.03 MB.

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